

***IN VITRO AND IN VIVO INVESTIGATIONS INTO THE
INTERACTIONS BETWEEN THE ACYL GLUCURONIDE
METABOLITE OF DICLOFENAC AND SERUM
ALBUMIN***

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BY

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DECLARATION

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

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This research was carried out in the MRC Centre for Drug Safety Science,
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IN VITRO AND IN VIVO INVESTIGATIONS INTO THE INTERACTIONS BETWEEN THE ACYL GLUCURONIDE METABOLITE OF DICLOFENAC AND SERUM ALBUMIN

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Adverse drug reactions represent a major challenge to clinicians, healthcare systems, pharmaceutical companies and academia. With carboxylic acid drugs accounting for the most common class of drugs withdrawn from the market, the carboxylate pharmacophore has received much attention as a potential toxicophore. Direct glucuronidation of the carboxylate group, producing chemically unstable and protein reactive acyl glucuronide (AG) metabolites has received much attention as a bioactivation pathway responsible for generation of these off-target hypersensitivity and hepatotoxicity. It is the chemical instability and protein reactivity of AG metabolites that has led to their hypothesised ability to covalently modify proteins *in vivo* and subsequently stimulate inappropriate immune responses in susceptible patients. Despite this, whilst the reactivity of AGs has been shown *in vitro*, their reactivity has never been confirmed in any *in vivo* system, meaning their association with toxicity may be unjustified. The focus of this thesis was to investigate whether acyl glucuronides could identify covalent adducts to protein *in vivo*.

To address this aim, the thesis first investigates the chemistry of interaction between acyl glucuronides and protein during *in vitro* investigation. 2mM 1- β diclofenac-AG was found to degrade spontaneously via acyl migration following incubation with 0.1M phosphate buffer pH 7.4 at 37°C with a degradation half-life of 0.78 hours, confirming diclofenac as amongst the most reactive AGs. Further incubations confirmed the action of human serum albumin (HSA) as a mild esterase, and the presence of plasma esterases acting to hydrolyse AGs. The covalent binding of diclofenac-AG to HSA was confirmed using both an alkaline hydrolysis as well as direct mass-spectrometric analyses of modified proteins. Covalent modification of lysine residues was specifically identified, and was found to be concentration and time dependent. Further *in vitro* incubation experiments revealed for the first time that the 1- β isomer of AGs is responsible for the formation of transacylation adducts, and confirmed previous suggestion that acyl migration is required for the extensive glycation of HSA.

Following characterisation of the interaction of diclofenac-AG with HSA, investigations were undertaken in the rat to identify interactions of AGs with circulating rat serum albumin *in vivo*. *In vitro* incubations of diclofenac-AG revealed RSA contained fewer binding sites when compared to HSA. Further to this no covalent modification of RSA could be detected *in vivo* following intravenous administration of 60mg/kg diclofenac-AG. The rapid plasma clearance of diclofenac-AG ($67.81 \pm 12.83 \text{ ml min}^{-1} \text{ kg}^{-1}$) in the rat was shown to be 600 fold faster than that of diclofenac ($12.00 \pm 2.98 \text{ ml min}^{-1} \text{ kg}^{-1}$) following bolus intravenous administration. Use of a continuous intravenous infusion drug delivery system revealed an adaptive change in rats upon continuous infusion of diclofenac, resulting in enhanced plasma elimination of the drug, and induction of the

ROS scavenging enzymes catalase and superoxide dismutase-2, without detection of hepatotoxicity.

The final experiments in the thesis revealed for the first time the detection of glycation adducts to HSA extracted from volunteer patients receiving chronic diclofenac therapy. These were shown through the detection of glycation adducts in three out of six patients tested. Between 1 and 4 lysine residues were identified in patients, with modifications towards one or all of lysine residues 195, 199, 432 and 525. Transacylation adducts were detected towards lysine residues in all six patient samples analysed. Whilst identification of transacylation adducts reveals bioactivation of the carboxylic acid functional group, it is the identification of glycation adducts to albumin isolated from three of the six patients which reveals, for the first time, definitive evidence for AG reactivity *in vivo*. This reinforces concerns over the potential of AGs to act as haptens, and re-affirms the carboxylic acid structure as a site of bioactivation forming reactive metabolites.

PUBLICATIONS

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LIST OF ABBREVIATIONS

ADR, Adverse Drug Reaction

AG, Acyl Glucuronide

ALT, Alanine Aminotransaminase

AMAP, N-acetyl-*m*-aminophenol

APAP, Acetaminophen, Paracetamol

AUC, Area Under the Curve

BSEP, Bile Salt Export Pump

Co A, Coenzyme A

COX, Cyclooxygenase

CL_p, Plasma Clearance

CRM, Chemically Reactive Metabolite

CYP450, cytochrome P450 mixed function oxidase

GLDH, Glutamate dehydrogenase

H&E, Haematoxylin and Eosin

HCl, Hydrochloric Acid

HPLC or LC, High Pressure Liquid Chromatography, Liquid Chromatography

Hr, Hour

HSA, Human Serum Albumin

IHC, Immunohistochemistry

IL, Interleukin

LD₅₀, Median Lethal Dose

MHC, Major Histocompatibility Complex

MIDAS, Multiple Reaction Monitoring-Initiated Detection and Sequencing

MRM, Multiple Reaction Monitoring

MRP, Multidrug Resistance Protein

MS/MS, Tandem Mass Spectrometry

NAPQI, N-acetyl-p-benzoquinone imine

NCE, New Chemical Entity

NSAIDs, Non-Steroidal Anti-Inflammatory Drugs

RSA, Rat Serum Albumin

$t_{1/2}$, half-life

ULN, Upper Limit of Normal

QStar, QSTAR Pulsar i Hybrid Mass Spectrometer

QTrap, 5500 QTRAP Hybrid Triple-Quadrupole/Linear Ion Trap Mass Spectrometer

CHAPTER ONE

GENERAL INTRODUCTION

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1.1 ADVERSE DRUG REACTIONS

1.1.1 Introduction to adverse drug reactions

Adverse drug reactions (ADR) have been defined by Edwards and Aronson as: ‘an appreciably harmful or unpleasant reaction, resulting from an intervention related to the use of a medicinal product, which predicts hazard from future administration and warrants prevention or specific treatment, or alteration of the dosage regimen, or withdrawal of the product’ (Edwards and Aronson, 2000).

Approximately 6.5% of hospital admissions in the U.K. have been attributed to ADRs, incurring an estimated cost of approximately £500 million for the NHS (Pirmohamed et al., 2004). In addition, drug withdrawals or restrictions on drug usage impose a major financial burden on pharmaceutical companies. Therefore ADRs represent a major challenge for clinicians, regulatory authorities and the pharmaceutical industry.

A huge financial investment is required to assess the value of a candidate drug in clinical trials. Currently the second most frequent cause of attrition of novel compounds following their administration to man is concerns with respect to drug safety (Kola and Landis, 2004). Consequently, early identification of properties of novel compounds that correlate with a high risk of toxicity is required. If this can be achieved, it may be possible to select lead compounds for development that have low toxicity risk for development, so that large investments can be targeted towards molecules that have the greatest chance of success.

1.1.2 On-target adverse drug reactions

On-target ADRs (also known as Type A) usually present as an exacerbation of the known primary or secondary pharmacological response of the drug target. As a consequence of this, they usually show a clear dose-response relationship (Rawlins and Thompson, 1977), meaning that they are often simply managed clinically through a reduction of dose or through administration of combinational pharmacological intervention.

A classic example of an on-target ADR is bleeding associated with warfarin therapy. Warfarin inhibits the vitamin K cycle, reducing the carboxylation, and consequently the biological activity, of pro-coagulation factors including factors VII, IX and X. This reduces the risk of embolism, and has been shown to reduce risk of stroke in atrial fibrillation patients by 70% (Hart et al., 2003). However, a major limitation to warfarin therapy is the risk of serious haemorrhage, occurring with an incidence of between 1.3 to 4.2 per 100 patient years of drug exposure (1994; 1996; Aithal et al., 1999). Serious haemorrhage is encountered as a consequence of the exacerbation of the drug's pharmacological action, resulting from above desired inhibition of the vitamin K cycle. Warfarin is known to have a notably small therapeutic window, where the doses required for pharmacological effect and generation of haemorrhage are not markedly different. Extensive inter-individual variation in response to warfarin, partly due to differences in vitamin K dietary intake, makes haemorrhage not uncommon (Aithal et al., 1999).

Whilst undesirable, on-target adverse drug reactions are usually simply managed, often without the necessity of drug withdrawal. Frequently, dose reduction with close

monitoring of the patient is sufficient for reduction of toxicological risk, whilst maintaining drug therapy.

1.1.3 Off target ADRs

Unlike on-target ADRs, off target ADRs (also known as Type B) do not show a clear dose-response relationship, cannot be predicted from the known primary or secondary pharmacology of the compound, cannot be predicted using known animal models and are only apparent in a small subset of the population (Rawlins and Thompson, 1977; Kalgutkar and Soglia, 2005). Essentially they are unexpected reactions with signs and symptoms inconsistent with the known toxicity of the drug. Consequently, accurate associations between drugs and off-target ADRs cannot be made until sufficiently large populations are exposed (Park et al., 2000), often during late-stage clinical trials or following drug release to the market. Once a drug has reached these stages in its development massive financial investments in the compound have already been made. Clinically, off-target ADRs range from mild to severe, and may occasionally be life-threatening. However, it is their unpredictable nature that makes management of off-target ADRs difficult (Park et al., 2000). Often, upon detection of the reaction, withdrawal of the otherwise effective drug from the patient is required, especially for off-target ADRs with suspected immunological pathogenesis.

A good drug example of off-target ADRs is represented by hepatotoxicity associated with ximelagatran. Ximelagatran was the first of the direct thrombin inhibitor anticoagulants, providing a wider therapeutic window than the coumarins, reducing the risk of bleeding and consequently eliminating the necessity for tight patient monitoring (Mattsson et al., 2005). Its efficacy was proven in man and a good safety profile was observed in pre-clinical and short term clinical trials. Longer clinical trials

(>35 days), however revealed the potential of the molecule to elicit hepatotoxicity in a low frequency of individuals (Petersen et al., 2003). Plasma alanine transaminase (ALT) activities were raised to above 3x the upper limit of normal (ULN) in 11 patients at 3 weeks following cessation of drug exposure, while one patient was hospitalised due to plasma ALT activities up to 27x ULN (Agnelli et al., 2009). Subsequently, ximelagatran's development was ceased and the drug was withdrawn from all markets (Keisu and Andersson, 2010).

As with other off-target ADRs, no clear relationship between ximelagatran dose and incidence of toxicity could be elucidated following retrospective analysis of clinical trials (Keisu and Andersson, 2010). Despite re-analysis of pre-clinical safety testing, and retrospective analysis, to date, hepatotoxicity has not been established in any animal model, meaning mechanisms for its pathogenesis in man have not been established.

Until mechanisms of off-target ADRs are understood, which integrate the biochemistry of the compound with the intrinsic variation in biology or environment of the patient, early prediction of off-target ADRs administration to large numbers of humans will not be possible.

1.2 XENOBIOTIC METABOLISM

Humans are continually exposed to a wide range of xenobiotics, either via the diet, non-dietary environmental exposure or pharmaceutical treatment. Many xenobiotics have the potential to be harmful. Consequently, xenobiotic deactivation and subsequent clearance from both the cell and organism is important to avoid potential toxicological consequences (Park et al., 2005). One of the key mechanisms in xenobiotic deactivation and clearance is metabolism. The overall chemical modifications of a compound usually enhance its hydrophilicity and its affinity for export transporters, resulting in enhanced clearance (Meyer, 1996). Xenobiotic metabolism has been usefully categorised into two biochemical phases. Phase I metabolism frequently enhances the electrophilicity of a molecule through insertion of polar functional groups via one of several metabolic pathways. Molecules with enhanced electrophilicity are often more susceptible to conjugative (Phase II) metabolic reactions than parent molecules. In Phase II reactions, molecules are conjugated with polar residues, resulting in complexes that possess sufficient hydrophilicity to enable their rapid excretion (Gibson and Skett, 2001).

1.2.1 Phase I metabolism

The apparent biochemical purpose of Phase I metabolism is to enhance a molecule's electrophilicity, usually through the provision of functional groups susceptible to Phase II conjugation, and consequently the excretion of the molecule. In some cases, however, Phase I metabolites may have sufficient polarity for direct elimination without the necessity for conjugation.

Phase I metabolism involves several different metabolic reactions, including oxidation, reduction, hydrolysis, hydration and isomerisation, with the reactions almost invariably

requiring enzymatic catalysis by enzymes including cytochrome P450s, monoamine oxidases and esterases. Being collectively the most abundantly and widely expressed, and responsible for the metabolism of an enormous range of substrates, the isoforms of the cytochrome P450 superfamily are the most important Phase I metabolising enzymes (Gibson and Skett, 2001; Guengerich, 2001).

1.2.2 Phase II metabolism

Phase II metabolism involves the conjugation of endogenous moieties to both xenobiotics and other endogenous metabolism products (Caldwell, 1982). This frequently results in the conjugate's increased hydrophilicity and affinity for export transporters, enhancing renal and biliary elimination. Several different conjugation reactions are considered in Phase II metabolism, including glucuronidation, sulfation, methylation, acetylation, as well as the conjugation of glutathione and amino acids (Gibson and Skett, 2001). These conjugations are site specific, targeted to electrophilic sites of molecules. Occasionally these sites may possess intrinsic chemical reactivity, which, if not deactivated may have harmful consequences, as is found in the quinone-imine metabolite of paracetamol. Consequently, conjugation at these sites has historically thought to abate a potential toxicological risk (Caldwell, 1982). This traditional view of phase II metabolites as metabolic end products lacking pharmacological activity, however, has been challenged in recent years, with pharmacological activity reported for the glucuronide metabolites of morphine-6 β -glucuronide (Paul et al., 1989), codeine-6-glucuronide (Vree et al., 2000) and dabigatran-AG (Stangier et al., 2007).

1.2.3 Glucuronidation

Phase II metabolism by glucuronidation is the conjugation of glucuronic acid to xenobiotics or endogenous molecules, catalysed by UDP-glucuronosyltransferase (UGT) enzymes of the endoplasmic reticulum, usually targeted to hydroxyl (alcohol and phenol), carboxyl, sulphhydryl and amine groups of xenobiotics (Caldwell, 1982; Tukey and Strassburg, 2000). Expression of 19 human UGT isoforms belonging to two distinct families (UGT1 and UGT2) (Ritter, 2000; Tukey and Strassburg, 2000; Mackenzie et al., 2005) is thought to allow the accommodation of this wide range of substrates. Like P450s, mammalian UGTs display somewhat broad and often overlapping substrate specificities (Picard et al., 2005; Alonen et al., 2008), stereoselectivity (Mano et al., 2007a) and genetic polymorphisms (Daly et al., 2007).

Conjugation of substrates by glucuronidation is usually considered to be a low affinity, high capacity, reaction. Due to the abundance of glucuronic acid, this pathway plays a major role in drug metabolism, with glucuronidation a listed metabolic pathway of 8% of the top 200 prescribed drugs in the US in 2002 (Williams et al., 2004). Glucuronidation is also estimated to account for approximately 10% of the major drug elimination pathways (Gardiner and Begg, 2006). Products of glucuronidation are conventionally categorised into four broad types dependent on the atom to which the glucuronic acid is linked, namely O-glucuronides, C-glucuronides, N-glucuronides and S-glucuronides (Figure 1. 1). Of these conjugation reactions, O-glucuronidation is most frequently encountered.

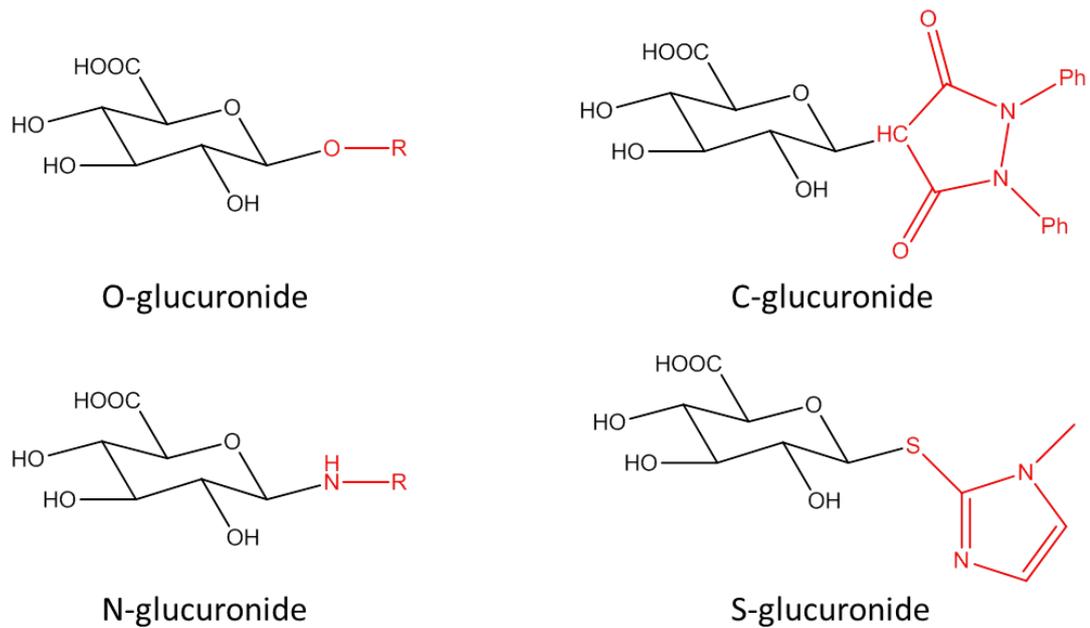


Figure 1. 1: Representative subtypes of glucuronides. O-glucuronides include alcohol, phenol, carboxyl and N-carbamoyl conjugates. Certain drug substrates can form more than one subtype of glucuronide in a mammalian species, e.g. an acyl glucuronide and either a phenolic glucuronide (Dickinson et al., 1993; Picard et al., 2005) or an N-glucuronide (Alonen et al., 2008). A glucuronide can itself be a substrate for glucuronidation, sulphonation or oxidation by 450s (Kumar et al., 2002).

The principal site of glucuronidation is the liver. However, expression of UGTs has also been reported in the kidney, GI tract and lung, as well as in the mammary gland and prostate, where glucuronidation of steroids is important (Ohno and Nakajin, 2009). UGTs are usually found expressed as membrane bound enzymes, located in the endoplasmic reticulum (Tukey and Strassburg, 2000).

1.3 ROLE OF DRUG BIOACTIVATION IN ADVERSE DRUG REACTIONS

The usual consequence of drug metabolism – with the important exception of pro-drug activation - is the conversion of active substrates into pharmacologically inactive products, primed for rapid elimination from biological systems (Park et al., 2005). Occasionally, however, metabolism may lead to the formation of intermediates or end products with intrinsic chemical reactivity, instability, or activity at secondary pharmacological targets.

1.3.1 Bioactivation of xenobiotics to chemically reactive metabolites (chemical activation)

Bioactivation through metabolism may also result in the formation of compounds with intrinsic chemical reactivity. Usually these chemically reactive metabolites (CRMs) are rapidly bio-inactivated, generally through phase II metabolism. Occasionally, however, exposure or intrinsic reactivity of CRMs may overwhelm detoxification pathways. In this situation CRMs may elicit a toxicological effect (Park et al., 2005). This may be either through covalent binding to endogenous macromolecules, possibly resulting in cell death, immune sensitisation or carcinogenesis. Alternatively, the chemical instability of CRMs may result in their redox cycling, resulting in cellular stress through oxidative stress.

1.3.2 Potential of CRMs to elicit intrinsic cellular stress through covalent modification of endogenous macromolecules

Strong associations have been drawn between the formation of CRMs, their covalent modification of endogenous macromolecules, and subsequent potential for toxicity and cell death (Brodie et al., 1971; Gillette et al., 1974; Park et al., 2005; Zhou et al., 2005). This relationship, however, is somewhat complex, with covalent modification of

macromolecules not universally resulting in toxicological output (Evans et al., 2004). An example of this is comparison of the model hepatotoxin acetaminophen (APAP) with its isomer N-acetyl-*m*-aminophenol (AMAP). Both molecules are metabolised to reactive benzoquinone and benzoquinoneimine intermediates *in vivo*, and have been shown to form covalent adducts to hepatic proteins in mice to a similar extent (Rashed and Nelson, 1989; Rashed et al., 1990). However, whilst APAP was found to elicit hepatotoxicity in mouse models, AMAP did not (Nelson, 1980). Perturbation of function of macromolecules involved in molecular pathways critical for cellular survival through covalent adduct formation is thought to have a role in the mechanism and aetiology of some ADRs (Kalgutkar and Soglia, 2005; Park et al., 2005), as shown by Figure 1. 2. Examples of this are highlighted by Table 1. 1, including APAP's modification of mitochondrial proteins (Nazareth et al., 1991; Landin et al., 1996) and covalent modification of DNA by polycyclic aromatic hydrocarbons (PAH) resulting in carcinogenesis (Brookes and Lawley, 1964; Mastrangelo et al., 1996). Alternatively, whilst not perturbing cellular function, modification of endogenous macromolecules is thought to have a key role in the generation of several drug allergies including halothane (Kenna et al., 1988a; Kenna et al., 1988b; Kenna et al., 1992) and penicillins (Brander et al., 1995; Padovan et al., 1997). Consequently, identification of drugs capable of forming covalent adducts *in vivo* leads to anxiety as to their management, and future progression in development (Park et al., 1994; Evans et al., 2004; Kalgutkar and Soglia, 2005).

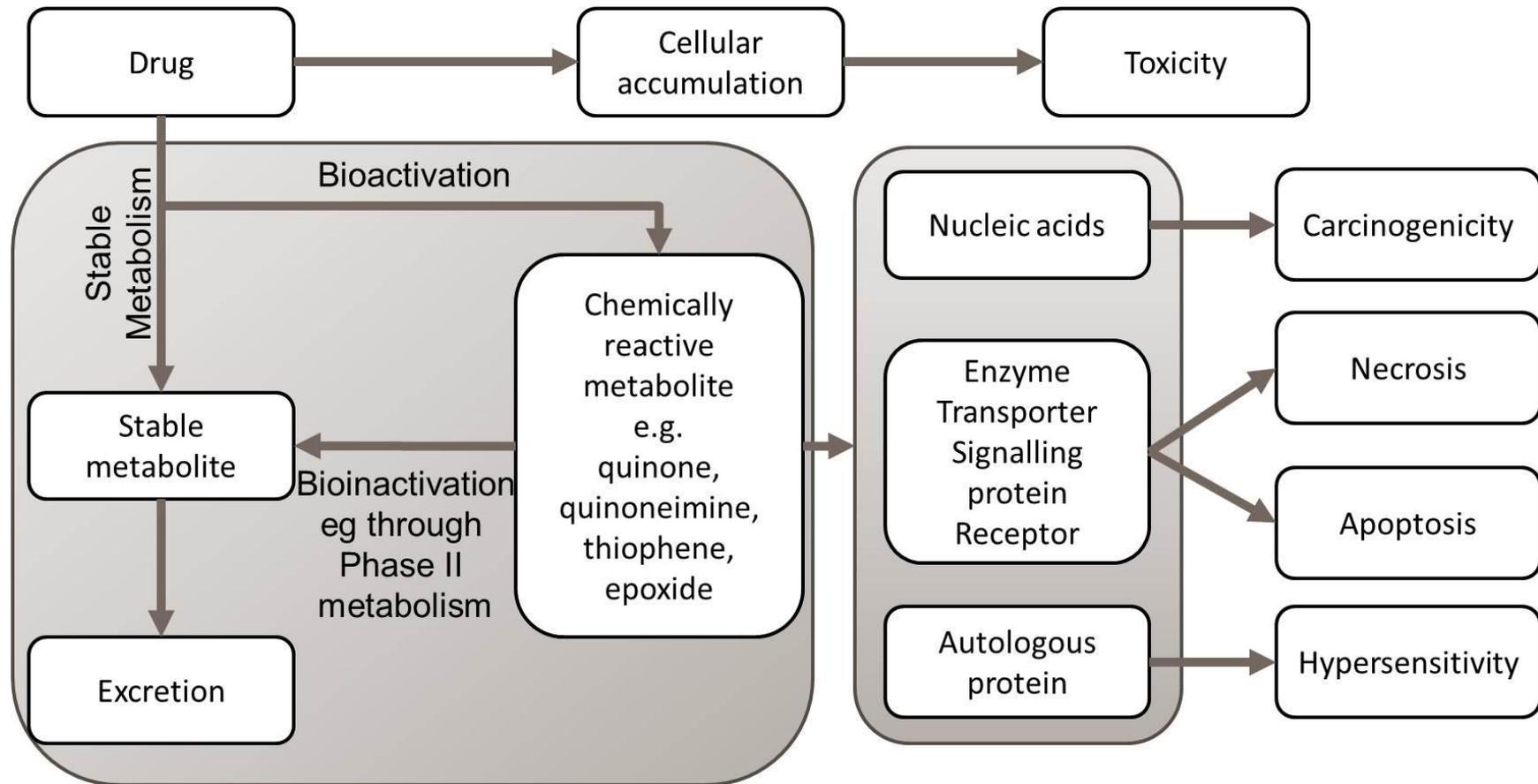


Figure 1. 2: Physiological consequences of drug bioactivation. Adapted from (Park et al., 2005)

Table 1. 1: Drugs associated with eliciting toxicity through their metabolism into chemically reactive metabolites, and their associated protein targets

Drug	Bioactivating enzyme	Chemically reactive metabolite	Protein target if identified	Toxicity associated	References
Paracetamol	CYP1A2, CYP2E1, CYP3A4	<i>N</i> -acetyl- <i>p</i> -benzoquinone imine	Microsomal glutathione synthetase, Nuclear lamin A, Selenium-binding protein, <i>N</i> -10-formyltetrahydrofolate dehydrogenase, Mitochondrial glutamate dehydrogenase, Mitochondrial aldehyde dehydrogenase	Hepatotoxicity	(Nazareth et al., 1991; Bartolone et al., 1992; Pumford et al., 1992; Patten et al., 1993; Bulera et al., 1995; Landin et al., 1996; Pumford et al., 1997a; Pumford et al., 1997b)
Halothane	CYP2E1	Trifluoroacetyl chloride	Glutathione-S-transferase b, CYP2E1, CYP2B, Protein disulphide isomerase, UDP-glucose glycoprotein glucosyltransferase, Carboxylesterase, Erp72, BiP/GRP78, Erp99.	Hepatitis	(Kenna et al., 1988b; Satoh et al., 1989; Martin et al., 1993; Pumford et al., 1993a; Eliasson and Kenna, 1996; Amouzadeh et al., 1997; Pumford et al., 1997a)
Diclofenac	UGT 2B7, CYP2C8, CYP3A4, CYP2C9	Acyl Glucuronide 1', 4'-Quinoneimine 2', 5'-Quinoneimine Coenzyme A thioester	Dipeptidyl peptidase IV	Hepatotoxicity	(Stierlin and Faigle, 1979; Hargus et al., 1995; Shen et al., 1999; Poon et al., 2001; Kenny et al., 2004)
Tienilic Acid	CYP2C9	Thiophene-S-oxide	CYP2C9	Hepatotoxicity	(Zimmerman et al., 1984; Koenigs et al., 1999)
Troglitazone	CYP3A4	<i>p</i> -benzoquinoneimine quinone-epoxide	Microsomal proteins BSEP	Hepatotoxicity	(Kawai et al., 1997; Watkins and Whitcomb, 1998; Funk et al., 2001; Kassahun et al., 2001; Yamamoto et al., 2002; He et al., 2004)

1.4 CARBOXYLIC ACID FUNCTIONAL GROUPS IN PHARMACEUTICAL DRUGS

Carboxylic acid groups are important molecular tools for medicinal chemists, incorporated into pharmaceutical agents for improved metabolism and disposition profiles for lead compounds. Most carboxylic acid drugs are monocarboxylates but there are examples of dicarboxylates (MacFadyen et al., 1993; Wen et al., 2007). In addition to being unmasked by facile hydrolysis of ester moieties (MacFadyen et al., 1993), carboxyl groups can be formed on carboxylate (Sumner et al., 1975; Balani et al., 1997; Hermening et al., 2000) and non-carboxylate (Dalgaard and Larsen, 1999; Paulson et al., 2000a; Paulson et al., 2000b; Paulson et al., 2000c; Mutlib et al., 2002) drugs by oxidative metabolism, lactone hydrolysis (Prueksaritanont et al., 2002; Ramadan et al., 2006) or a combination of oxidative and hydrolytic reactions (Pekol et al., 2005). In certain cases the pathways of formation are complex and unexpected (Umehara et al., 2004), exacerbating the difficulties of assessing the overall biological impacts of pharmaceutical carboxylates. For a number of classes of drugs, including classes currently in development, the carboxylic acid group may be the pharmacophore, or a component of the pharmacophore, having a direct role in the mechanism of action of the compound (Istvan and Deisenhofer, 2001; Rowlinson et al., 2003). Consequently, over 450 pharmaceuticals marketed worldwide contain a carboxylic acid functional group (Smith, 2010).

For some compounds, the carboxylic acid group may have a direct role in the mechanism of action of the compound. In the simplest cases, carboxylic acid drugs and/or their metabolites match the functional group chemistry of natural substrates or ligands. A prime example of this is in the carboxylic non-steroidal anti-inflammatory drugs (NSAIDs), where the carboxylic acid structure is important for the mimicking of

the arachidonic acid metabolite (Rowlinson et al., 2003); resulting in the competitive antagonism of arachidonic acid binding to cyclooxygenase (COX-2 and/or COX-1) isozymes (Rowlinson et al., 2003; Khairullina et al., 2007), inhibition of prostaglandin synthesis, and consequently, anti-inflammatory effects (Waterbury et al., 2006). The propensity of carboxylic acids to form hydrogen bonds with polypeptide side chains and the ability of carboxylate anions to engage in electrostatic interactions (ion pairing) are also important in forming non-covalent interactions with target receptors and active sites of enzymes (Istvan and Deisenhofer, 2001).

Carboxylate groups are also commonly incorporated into medicinal compounds as a method to provide improved aqueous solubility. Good aqueous solubility is often a desired property for a pharmaceutical compound, as this enhances a molecule's passive absorption from the GI tract to the bloodstream, without requiring affinity for transporters. However, if the carboxylic acid is matched with a complementary basic centre in the molecule, and the resulting isoelectric point is close to pH 7.4, the compound's zwitterion species will have increased membrane penetration at physiological pH and potentially increased bioavailability (Cavet et al., 1997). Due to their polar nature, represented by their ionisation in aqueous conditions and ability to form hydrogen bonds with water through the (=O) acting as a hydrogen bond acceptor and the (-OH) acting as a hydrogen bond donor, carboxylic acid structures are often desired to enhance hydrophilicity of lead compounds. Alternatively, due to their polarity and electronegativity, carboxylic acid groups may be inserted into aromatic ring side-chains to protect against P450 metabolism, pulling electrons away from sites of susceptible oxidation (Smith, 2010).

However, the increased polarity and resulting hydrophilicity introduced to compounds through carboxylic acids can also result in their notoriously poor ability to passively diffuse across plasma membranes, particularly when it is in negatively charged states (Austin et al., 1995). Consequently, without the action of uptake transporters, distribution of carboxylate compounds to intracellular targets is poor. This poor diffusion across plasma membranes represents a particular challenge for drugs targeted towards the central nervous system (CNS) where passive absorption across the blood brain barrier is required for efficacy (Pajouhesh and Lenz, 2005). This resulting low volume of distribution (V_{DSS}) of carboxylic acid drugs (Smith and Obach, 2005; Smith, 2010) is further reduced through their extensive non-covalent binding to plasma proteins (often >99% fraction bound) (Kratochwil et al., 2002). In order to overcome these problems with distribution, carboxylic acid drugs are frequently administered at high daily doses in order to allow drug disposition to its mechanistic site of action at concentrations sufficient to elicit pharmacological effect, meaning that circulating doses of the drug generally are high. With carboxylate drugs associated with liability towards generating off-target liabilities as a consequence of bioactivation at this site (see sections 1.4.1, 1.4.3, 1.6 and 1.8.1) this is a situation which is desirably avoided (Sakatis et al., 2012).

1.4.1 Association of carboxylic acid drugs with ADRs

Clinically, patients are exposed to a wide range of carboxylic acids, for varying therapeutic indications, including NSAIDs, fibrates, loop diuretics, iron chelators, antiasthmatics, antibiotics, anticonvulsants, antiretrovirals, angiotensin II receptor antagonist, thromboxane synthase inhibitors, direct thrombin inhibitors and PPAR α/β agonists. The most important of which are NSAIDs, of which 6% of the adult

population in the US reported using a prescription NSAID in a month, and 24% using non-prescription ibuprofen (Paulose-Ram et al., 2003). This large range of important therapeutic drug classes means large numbers of the population are exposed to carboxylic acid pharmaceuticals. The majority of patients receive effective therapy, with few safety concerns.

Despite the majority of carboxylic acids acting as safe therapeutics to the majority of patients who are exposed to them, on rare occasions, carboxylic acids individually, or as a sub-class, such as the carboxylic NSAIDs, have been associated with eliciting immune mediated hepatotoxic and hypersensitivity adverse drug reactions. This association led to 17 out of 121 (14%) drugs withdrawn between 1960-1999 containing a carboxylic acid functional group (Fung et al., 2001). Further to this, some older carboxylic acid drugs remaining on the market, such as diclofenac, do so with black box warnings or reported cases of eliciting these ADRs (Skonberg et al., 2008).

1.4.2 Immediate hypersensitivity reactions

NSAIDs have been associated with a wide range of differing hypersensitivity adverse drug reactions. These ADRs may be immediate, with disease pathogenesis occurring within 1-7 hours of first administration of the compound. Immediate hypersensitivity reactions are generally less serious than delayed hypersensitivity reactions, although occasionally these immediate ADRs may progress into more serious generalised anaphylactic reactions (Stevenson et al., 2001; Berkes, 2003). Therefore it is generation of delayed hypersensitivity adverse drug reactions that is the most common cause for carboxylic acid drug withdrawal.

1.4.3 Delayed hypersensitivity reactions

Delayed hypersensitivity ADRs are generally classified as developing over 24 hours following drug exposure, but usually develop between 1-8 weeks following initiation of drug therapy (Gunawan and Kaplowitz, 2004; Kaplowitz, 2001). The most common delayed ADRs associated with carboxylic acid drugs are cutaneous (including severe bullous cutaneous reactions, fixed drug eruptions and maculopapular drug eruptions). These ADRs led to the withdrawal of zomepirac (Levy and Vasilomanolakis, 1984). Further to this, several carboxylic acid drugs were also associated with the generation of tissue specific hypersensitivity reactions including hepatotoxicity, leading to the withdrawal of drugs including benoxaprofen, bromfenac and fenclozic acid, although these reactions were less common (Bakke et al., 1995; Fung et al., 2001; Sanchez-Borges et al., 2005).

Drug induced delayed hypersensitivity ADRs are off-target, and consequently only affect a small fraction of the population (Strom et al., 1987; Banks et al., 1995), with confident prediction of 'at-risk' individuals not currently possible. Adaptive immune responses are thought to play a key role in the pathogenesis of these ADRs (Posadas and Pichler, 2007). Evidence for this is strengthened with observations that following withdrawal of the compound to alleviate ADRs, re-exposure may lead to rapid re-stimulation of the ADR (Helfgott et al., 1990). The short timeframe between re-exposure and redevelopment provides further suggestion of adaptive immune involvement, with reactions suspected to be cytotoxic T-cell mediated (Posadas and Pichler, 2007).

1.5 ROLE OF ANTIGEN PRESENTATION IN DELAYED HYPERSENSITIVITY REACTIONS

1.5.1 Pro-hapten and hapten hypotheses

Most pharmaceutical compounds on the market are small molecules. Consequently, they are assumed to be of insufficient size to be recognised as foreign and presented to the immune system. It has been hypothesised that parent compounds or their CRMs must be presented to the immune system in an altered state with higher molecular weight for recognition as foreign entities (Padovan et al., 1997).

The hapten concept describes how low molecular weight organic compounds (<1000 Da), undetectable by the immune system due to their size, may be presented through the formation of covalent adducts with endogenous macromolecules (Park et al., 1987; Padovan et al., 1997; Park et al., 2001; Posadas and Pichler, 2007). This suggests that parent molecules must have protein reactivity. Most pharmaceuticals, however, are devoid of chemical reactivity but their bioactivation into CRMs capable of forming covalent adducts with endogenous macromolecules may lead to their presentation (Kenna et al., 1988b; Park et al., 2001; Naisbitt et al., 2002; Aithal et al., 2004; Posadas and Pichler, 2007). Molecules able to directly modify endogenous macromolecules without processing are known as haptens, whereas molecules requiring bioactivation into CRMs are known as pro-haptens.

Haptenation may occur on autologous proteins, or alternatively, may occur directly onto the major histocompatibility complex (MHC) of T-cells. Those antigens formed on endogenous macromolecules, either intracellularly or to extracellular cell surfaces, may require processing by antigen presenting cells before presentation to T-cells for stimulation of sensitization and immune responses (Posadas and Pichler, 2007).

1.5.2 Antigen presentation as a response to the cellular site of endogenous modification

Following adduct formation with endogenous protein, the resulting drug-protein complex may either activate MHC molecules directly or require processing for this. Antigen presenting cells cleave the hapten-protein adduct into peptide fragments, onto some of which the hapten may be bound. The haptened peptide is able to bind weakly to the MHC in the cell (Naisbitt et al., 2007). Where the haptened peptide locates in the cell has an effect on which class of MHC molecule it is presented by. Haptens which bind to extracellular proteins usually require capture in the endosome, followed by subsequent lysozyme degradation, and are usually presented by MHC class II molecules, leading to CD4⁺ T-cell responses. Intracellular haptens, on the other hand, appear to be presented specifically by MHC class I molecules. Following processing of the protein adducts, the antigen presenting cell locates to the lymphatic organs, where antigens are then presented to the T-cells through the MHC molecules, resulting in CD8⁺ T-cell responses (Pichler, 2003).

1.5.3 Pharmacological interaction (PI) hypothesis

Whilst immune stimulation through the covalent binding of drug species to endogenous macromolecules is assumed to play a large role in a wide range of ADRs, Pichler (Pichler, 2002) has proposed that in some cases of drug hypersensitivity covalent binding may not be critical. Instead, compounds form weak, reversible, non-covalent interactions with MHCs and T-cell receptors. Interaction with the MHC may result in antigen presentation to T-cells expressing appropriate T-cell receptors, subsequently resulting in the initiation of immune stimulation.

1.5.4 Danger hypothesis

To elicit an efficient immune response, the innate immune system needs to be activated. The innate immune response provides defence against a wide range of pathogens but its defence is non-specific. However, the innate immune system includes the antigen presenting cells, which, as described above, present hapten-peptide adducts to T-cells, initiating the more specific adaptive immune response (Medzhitov and Janeway, 2000). The activation of the antigen presenting cells has also been postulated to cause the expression of co-stimulatory molecules, which aid in activating resting T-cells (Kamradt and Mitchison, 2001). It is also likely that an underlying infection may result in the activation of the innate immune system as described above, resulting in cytokine and co-stimulatory molecule release. This effect, when occurring concomitantly with the administration of an immunogenic drug, may also result in increased T-cell activation, producing an immune response to the drug which otherwise might not have been initiated (Heller, 2000). This may help to explain why individuals suffering with an underlying infection often show an increased risk of developing immune mediated adverse drug reactions (Pichler, 2003).

Another factor which is thought to be involved in drug hypersensitivity reactions is Matzinger's danger hypothesis. The danger hypothesis proposes that an inflammatory response is not initiated through just the recognition of a non-self antigen, but that other signals are required to indicate that the foreign antigen is dangerous (Matzinger, 1994a). Curtsinger (Curtsinger et al., 1999a) has subsequently hypothesised that three signals are required for the eliciting of an immune response. The first signal is the presentation of the antigen to the T-cell through the MHC complex of an antigen presenting cell. The second signal is from co-stimulatory molecules and pro-

inflammatory cytokines, which cause antigen-presenting cells to release other stimulatory molecules. The third signal is from polarising cytokines, which act on T-cells and lead to immune responses. Whilst signal 1 is the recognition of the foreign antigen, receipt of only this signal results in tolerance to the antigen (Matzinger, 1994b), further receipt of signal 2 results in T-cell activation and signal 3 acts as an adjuvant, resulting in the propagation in the immune response (Cai et al., 1996; Curtsinger et al., 1999b; Pirmohamed et al., 2002) (Figure 1. 3).

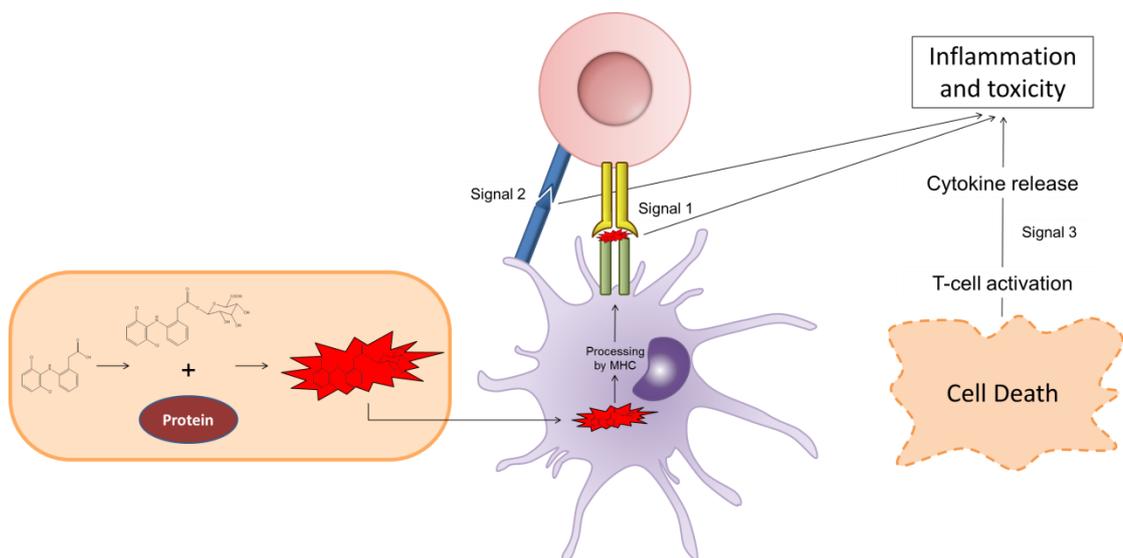


Figure 1. 3: Potential mechanism for carboxylic acid drugs to elicit inappropriate immune responses according to the hapten and danger hypotheses.

1.6 DRUG METABOLISM TO ACYL GLUCURONIDE METABOLITES

Direct glucuronidation, resulting in the formation of AG metabolites is quantitatively the most important route of biotransformation for carboxylic acid drugs (Skonberg et al., 2008). Acyl glucuronidation of both mono- and di- carboxylic acids have been reported (Wen et al., 2007), with di- carboxylic acids capable of forming either two distinct mono-AG (Devissaguet et al., 1990; Wen et al., 2006), as well as di-AG conjugates (Wen et al., 2006).

In addition to acyl glucuronidation, many carboxylic acid drugs also undergo oxidative (Stierlin and Faigle, 1979; Balani et al., 1997; Hermening et al., 2000; Wang et al., 2006; Albrecht et al., 2008), and other conjugative metabolism (Dickinson et al., 1993; Sun et al., 1996; Dahms et al., 1997; Picard et al., 2005; Alonen et al., 2008). Further to this, oxidative metabolites of carboxylate pharmaceuticals may also undergo acyl glucuronidation (Hermening et al., 2000; Zhou et al., 2001; Kenny et al., 2004; Wang et al., 2006; Albrecht et al., 2008). Oxidative metabolism of non-carboxylate drugs may also reveal carboxylic acid functional groups, which may subsequently be glucuronidated into AG metabolites as is shown for celecoxib (Paulson et al., 2000a; Paulson et al., 2000b; Paulson et al., 2000c).

Direct glucuronidation of carboxylic acids, forming AG metabolites can represent a large fraction of their metabolic clearance (Zhou et al., 2001; Wang et al., 2006), with the dicarboxylic acid fibrate gemcabene cleared almost exclusively by acyl glucuronidation followed by renal elimination (Yuan et al., 2009). In rare instances, acyl glucuronidation may represent the sole pathway of biotransformation of a carboxylic acid, as appears to be the case for telmisartan (Stangier et al., 2000) and ifetroban (Dockens et al., 2000), although glucuronidation is not a universal metabolic

pathway of carboxylate drugs, as shown by the lack of a glucuronide metabolite for captopril, and identification of the acyl glucuronidation of two of four structurally similar di-acid ACE inhibitors (Devissaguet et al., 1990; Drummer et al., 1990; Tan et al., 2009).

Glucuronidation of numerous pharmaceuticals is catalysed by the UGT isoform UGT2B7, including carboxylic acids such as diclofenac (King et al., 2001), gemfibrozil (Mano et al., 2007b) and mycophenolic acid (Picard et al., 2005), for all of which it is the predominant catalyst. However, other human UGT isoforms contribute to the clearance of carboxylic acid drugs and carboxylate metabolites, with UGT2B7 not always the major catalyst (Wen et al., 2007; Alonen et al., 2008), as exemplified by the glucuronidation of bilirubin in man catalysed by the UGT1A1 isoform (Kadacol et al., 2000). Turnover of various carboxylic acids to their acyl glucuronides is exemplified by Table 1. 2.

Table 1. 2: Carboxylic acid drugs that undergo glucuronidation in human liver microsomes or following clinical administration of the parent drug (Regan et al., 2010)

Drug	Drug class	Fraction glucuronidated
Zomepirac	NSAID	90
Isoxepac	NSAID	90
Ketoprofen	NSAID	74
Carprofen	NSAID	75
Diclofenac	NSAID	75
Keterloac	NSAID	73
Suprofen	NSAID	62
Clofibrac Acid	Fibrate	61
S-Naproxen	NSAID	57
Aclofenac	NSAID	56
Nalidixic Acid	Antibacterial	54
Tiaprofenic Acid	NSAID	51
Fenoprofen	NSAID	49
Benoxaprofen	NSAID	47
Pirprofen	NSAID	45
Probenacid	Uricosuric agent	44
Diflusal	NSAID	40
Fenofibrac Acid	Fibrate	39
Oxaprozin	NSAID	39
Valproic Acid	Anticonvulsant	33
Tocainide	Antiarrhythmic	31
Etodolac	NSAID	25
Salicylic Acid	Keratolytic agent	10
Lumiracoxib	NSAID	2.5

Generally AGs and other glucuronides are eliminated in urine and bile. For example the acyl and phenolic glucuronides of the NSAID diflusal are both excreted in urine and bile in both humans (Verbeek et al., 1988) and rats (Brunelle and Verbeek, 1997). Biliary excretion of AGs in humans has only been investigated on few occasions but it is clearly a highly variable process, representing a minor route of elimination for

certain AGs (Verbeeck et al., 1988; Balani et al., 1997) and a major route for others (Hofmann et al., 2005; Wang et al., 2006).

1.6.1 Toxicological concerns regarding acyl glucuronide formation

Phase II conjugative metabolism is usually seen as a detoxification process. This is through the pharmacological deactivation of the drug, and increased rate of clearance via enhanced hydrophilicity of the molecule and increased affinity for export transporters (Caldwell, 1982; Miners and Mackenzie, 1991; Mulder, 1992; Spahn-Langguth and Benet, 1992). AG metabolites, however, have been shown to be chemically unstable and protein reactive *in vitro* (Munafo et al., 1990; Volland et al., 1991; Spahn-Langguth and Benet, 1992; Ding et al., 1993; Ding et al., 1995; Qiu et al., 1998; Ebner et al., 1999; Iwaki et al., 1999; Mortensen et al., 2001; Walker et al., 2007), resulting in much interest in these metabolites as a potential toxicological concern (Faed, 1984; Spahn-Langguth and Benet, 1992; Shipkova et al., 2003). The protein reactivity of AG metabolites has led to suggestion of their ability to act as CRMs capable of forming haptens *in vivo*, and consequently having a mechanistic role in the pathogenesis of idiosyncratic delayed hypersensitivity reactions associated with their parent drugs (Spahn-Langguth and Benet, 1992). These general assumptions have been incorporated in the Food and Drug Administration's Metabolites In Safety Testing (MIST) guidance, which states:

“Phase II conjugation reactions generally render a compound more water soluble and pharmacologically inactive, thereby eliminating the need for further elimination. However, if the compound forms a toxic conjugate such as an acyl glucuronide, additional safety assessment may be required (Faed, 1984)” (FDA, 2008).

This assertive labelling of AG metabolites as toxic, however, is controversial. Whilst their reactivity *in vitro* is well defined, when compared with other CRMs, reactivity of AG metabolites is low. This low reactivity of AGs does allow them to be widely distributed through the blood stream (Volland et al., 1990; Benet et al., 1993; Mayer et al., 1993; Zia-Amirhosseini et al., 1994; Stangier et al., 2000; Zhou et al., 2001; Wang et al., 2006), and transported into tissues (Shackleford et al., 2006), but despite this wide distribution, no definitive identification of AG metabolites reactivity towards endogenous macromolecules has ever been shown using *in vivo* models or in man. The only reported chemical reaction of drug AG administered intravenously to experimental animals is rapid and extensive hydrolysis (Watt et al., 1991; Iwaki et al., 1995). Consequently, evidence associating AG metabolites with toxicity may not be justified. Nonetheless, the speculative association of AG formation and clinical toxicity has resulted in persistent concern in the pharmaceutical industry regarding management of compounds forming reactive AG metabolites, even when the glucuronide is a minor product (Lundahl et al., 2009).

1.6.2 Acyl glucuronide chemical instability and protein reactivity

Chemical instability of AGs following *in vitro* incubation under aqueous conditions is almost a universal chemical characteristic of these phase II metabolites. The actual chemical instability of AG metabolites is greatly variable, with the degradation half-life for the disappearance of the 1-beta isomer ranging from 0.26 hours for tolmetin-AG to 79 hours for valproic acid-AG (Ebner et al., 1999). This marked variation of the intrinsic reactivity of differing acyl glucuronides has been attributed to the electronic and steric properties of the aglycones (Benet et al., 1993). Currently, research is being undertaken into assessing structure activity relationships of aglycone structure to

allow understanding of the attributes of chemical structure attributing to this variation in acyl glucuronide reactivity. The general rate of acyl glucuronide reactivity is understood to be acetic acid > propanoic acid > benzoic acid derivatives (Benet et al., 1993; Wang et al., 2004). This order is thought to be attributable to the level of substitution of the carbon alpha to the carboxylic acid in the aglycone structure. With good correlations ($r^2 = 0.995$) being drawn between the intrinsic chemical instability of AG metabolites and their reactivity towards protein following *in vitro* incubation (Benet et al., 1993), compounds extensively metabolised to reactive AGs have been suggested to provide greater risk for generation of ADRs. Comparisons of AG instability with the risk of ADR generation and fate of both previously and currently marketed carboxylic acid drugs, however, are not always clear.

1.7 ACYL GLUCURONIDE REACTIVITY WITH PROTEINS *IN VIVO*

1.7.1 Evidence from studies in humans

Circumstantial evidence for the ability of AG metabolites to act as haptens *in vivo* has been provided through the identification of modification of plasma proteins in patients administered either zomepirac, ibuprofen or tolmetin, as measured using an alkaline hydrolysis technique, correlating with plasma exposure (AUC) of the AG metabolite (Smith et al., 1986; Hyneck et al., 1988; Castillo et al., 1995). Further identification that AG metabolites are responsible for these covalent adducts was obtained through the co-administration of probenecid and zomepirac to volunteers. Probenecid increased plasma zomepirac-AG exposure by competing for renal clearance, consequently increasing zomepirac's modification of plasma protein (Smith et al., 1986).

1.7.2 Animal models

Further indications that the protein reactivity of AG metabolites is operative *in vivo* have been achieved through investigations using rats and guinea pigs as model species.

As with studies in humans, covalent modifications of plasma proteins following administration of several carboxylic acid drugs were detected, with binding correlating with AG exposure (Sallustio and Foster, 1995; Liu et al., 1996; Dong et al., 2005). Further suggestion for the role of AG metabolites to covalently bind with endogenous macromolecules was achieved through the detection of increased covalent binding through disruption of chemical and surgical disruption of elimination pathways, with chemical induction of renal failure in rats through the administration of uranyl nitrate enhancing plasma AG exposure following administration of salicylic acid, along with subsequent enhanced irreversible modification of plasma proteins (Liu et al., 1996).

Interruption of biliary excretion through surgical ligation of the bile duct in rats was also shown to increase the plasma exposure of zomepirac-AG, causing enhanced modification of plasma proteins (Wang and Dickinson, 2000). Chemical inhibition of plasma esterases (using phenylmethylsulfonylfluoride PMSF) has also been used to increase plasma AG exposure, through reduction of AG hydrolysis *in vivo*. In guinea pigs, bile duct ligation was required in order to produce detectable levels of zomepirac-AG in the plasma. Concomitant administration of PMSF to guinea pigs administered an intravenous dose of zomepirac resulted in enhanced plasma AG exposure. This further increase in AG plasma exposure was again found to again show good correlation with irreversible binding, and to date is the most convincing *in vivo* evidence of the ability of AG metabolites to covalently modify plasma proteins (Smith et al., 1990).

(Bailey and Dickinson, 1996), using four test carboxylate drugs (zomepirac, diflunisal, clofibrac acid and valproic acid) in rats, observed a generally rationalizable relationship between the intrinsic chemical reactivity of the drug's acyl glucuronides *in vitro* and the extent of protein adduction in liver and plasma. However, there was an important exception: the protein adduction by valproic acid in the liver was disproportionately high. This enhanced covalent binding was attributed to the localised reactions of acyl CoA (thioester) conjugates, in addition to that from AGs. Western blot analysis with anti-drug antibodies was used for partial identification of hepatic protein adducts, but this only allowed estimation of the molecular weights of proteins that were modified.

1.7.3 Detection of hepatic adducts in experimental animal models

Whilst some carboxylic acid drugs are associated with generalised delayed hypersensitivity reactions, others, including benoxaprofen, diclofenac and ibufenac are more frequently associated with delayed off-target hepatotoxicity reactions (Driver et al., 1982; Rake and Jacobs, 1983; Banks et al., 1995; Fung et al., 2001; Boelsterli, 2002a; Goldkind and Laine, 2006). Strong suggestions of immune involvement in the pathogenesis of these ADRs have also led to the investigation of hepatic protein adducts in animal models.

Administration of diclofenac, zomepirac, ibuprofen and diclofenac to the rat or mouse has revealed selective modification of hepatic proteins. Hepatic proteins modified were found to have molecular weights including 110, 140 and 200 kDa (Bailey and Dickinson, 1996; Wade et al., 1997). Work by (Hargus et al., 1995) went on to identify the 110 kDa protein using sequence analysis and immunoblotting as the enzyme dipeptidylpeptidase IV (DPPIV and also known as CD26) following diclofenac administration. Work with zomepirac also identified DPPIV as a protein target (Wang et al., 2001; Wang et al., 2002). Further adducts of 50 and 70 kDa were identified by (Pumford et al., 1993b; Wade et al., 1997).

Immunohistochemical analysis has allowed sub-cellular location of hepatic protein modification by carboxylic acid drugs in rats. Covalent adducts in the rat were found localised to the canalicular membrane (Hargus et al., 1994; Seitz et al., 1998; Aithal et al., 2004). TR⁻ rats, which do not express functioning MRP2 export proteins on the canalicular membrane, show minimal biliary excretion of diclofenac-AG and do not show canalicular protein adducts following treatment with diclofenac (Seitz et al., 1998). Due to the extensive biliary export of acyl glucuronides, in part due to the

abundance of export transporters (Sallustio et al., 1996; Sallustio et al., 2000), exposures of proteins along the canalicular membrane to AGs are likely to be high. This association has also resulted in further implication of acyl glucuronide metabolites in eliciting these canalicular localised adducts.

1.8 OTHER FACTORS INFLUENCING TOXICOLOGICAL RISK POSED BY ACYL GLUCURONIDES IN HUMANS

The implication of acyl glucuronidation as a potential toxicological process is centred on the potential of AGs to act as haptens, forming covalent protein adducts. This has been well defined and characterised following *in vitro* incubation (Benet et al., 1993; Ding et al., 1993; Ding et al., 1995; Qiu et al., 1998), and is the basis of much of the concern within the pharmaceutical industry as to the management of novel compounds extensively metabolised to reactive acyl glucuronides. Despite this persistent concern, no evidence as yet has definitively shown AG metabolites of drugs are directly responsible for covalent adduct formation *in vivo*. Potentially this may mean the anxiety over AGs, at least in the respect of direct protein adduction, may be unjustified. Further to this, many AG-forming drugs remain on the market providing safe and effective therapy for patients, as well as considerable profit for the pharmaceutical industry (Paulose-Ram et al., 2003; Barton et al., 2006).

1.8.1 Metabolism of carboxylic acid drugs to CRMs other than acyl glucuronides

Commonly, xenobiotic carboxylic acids, including drugs, may also undergo conjugation reactions catalysed by acyl-Coenzyme A synthetases forming acyl-Coenzyme A thioesters (Olsen et al., 2002; Grillo et al., 2003; Olsen et al., 2005; Olsen et al., 2007). Formation of coenzyme A thioesters is thought to represent a low abundance metabolic pathway, however, the resulting metabolites are considered as highly reactive. This is in comparison to AG metabolites, which are considered to represent high exposure but lower reactivity (Boelsterli, 2002b). The synthetic coenzyme A thioester of 2-phenylpropionic acid has been shown to undergo a transacylation

reaction with glutathione, and to react with serum albumin *in vitro* (Li et al., 2002). Naproxen coenzyme A thioester acylates several amino acid side chains of HSA *in vitro* (Olsen et al., 2003b). Whilst coenzyme A thioesters are becoming an area of interest as a potential toxicological concern, this interest has only begun relatively recently (Boelsterli, 2002b). However, whereas drug AGs are frequently found in plasma, coenzyme A conjugates are thought to be confined to their cellular site of formation. Thus the existence of coenzyme A conjugates of xenobiotics *in vivo* is known only from the presence of stable metabolic derivatives (Olsen et al., 2003a; Grillo and Hua, 2008; Grillo et al., 2008).

Further to reactive biosynthetic esters, individual carboxylate compounds may have sites of reactivity generated through oxidative metabolism. An example of this mechanism is the formation of a quinone-imine structure following oxidative metabolism of diclofenac to the 5-hydroxy metabolite (Poon et al., 2001). These reactive metabolites also have the ability to form covalent adducts with proteins (Shen et al., 1999), as is the case with NAPQI. Therefore a drug might be metabolised to several chemically reactive and potentially toxic intermediates, with an AG being just one of them.

1.8.2 Limitations of alkaline hydrolysis as a method to investigate acyl glucuronide reactivity *in vivo*

Alkaline hydrolysis with subsequent HPLC analysis (Hyneck et al., 1988; Smith et al., 1990; Benet et al., 1993; Munafo et al., 1993; Bischer et al., 1995) and antibody techniques (Hargus et al., 1994; Bailey and Dickinson, 1996; Aithal et al., 2004) have been the usual techniques for the identification of carboxylic acid compound modification of proteins *in vivo*. A major limitation of this approach, however, is that

these techniques are unable to identify or differentiate between the reactive metabolites responsible for the modifications detected. Consequently, with carboxylic acids potentially metabolised to several different products with the potential for covalent modification of macromolecules *in vivo* (Li et al., 2002; Olsen et al., 2002; Kenny et al., 2004; Sidenius et al., 2004), adducts detected may not necessarily be AG-metabolite mediated.

Whilst covalent modifications to plasma proteins in rodent and human studies correlate to plasma AG exposure (Hyneck et al., 1988; Benet et al., 1993; Castillo et al., 1995; Liu et al., 1996), it is also possible that metabolism into other metabolites not tested may also show equal or improved correlation. Consequently, until the identification of glycation adducts of proteins in samples taken *in vivo* is achieved using MS/MS techniques, questions will remain as to the true potential of AG metabolites to act as haptens.

1.9 DICLOFENAC AS A TEST CARBOXYLATE DRUG

Diclofenac, a phenylacetic acid NSAID, is currently used principally in the treatment of rheumatoid arthritis (Boelsterli, 2003). The mechanism of action *in vivo* is expected to be through non-selective inhibition of the cyclooxygenase (COX-1 and COX-2) isoenzymes, inhibiting the metabolism of arachidonic acid to prostaglandins (Waterbury et al., 2006). Approximately 15% of patients taking repeated doses of diclofenac develop raised liver enzyme activity, with a threefold rise in transaminase levels reported in 5% (Banks et al., 1995). Usually these minor rises are not a clinical cause for concern, and treatment may be continued with monitoring of the patient. Diclofenac has also been associated with more serious off-target hepatotoxic reactions, with 6.3 out of 100,000 diclofenac users requiring hospitalisation (de Abajo et al., 2004). These off-target ADRs usually manifest with either a hepatocellular or cholestatic pattern (Banks et al., 1995; Aithal and Day, 2007; Aithal, 2011). Further to this, diclofenac has also been associated with case reports of generalised hypersensitivity ADRs (Alkhawajah et al., 1993; Romano et al., 1998).

In humans, diclofenac is metabolised to several metabolites (Stierlin and Faigle, 1979). Diclofenac is oxidised by CYP2C9 to the 4'-OH metabolite, and by CYP2C8, 3A4 or 2C19 to the 5'-OH metabolite (Tang et al., 1999a; Tang et al., 1999b). Spontaneous oxidation of the 5'-OH metabolite yields a chemically reactive quinone-imine (Shen et al., 1999; Sparidans et al., 2008). Both 4'-OH-diclofenac and 5'-OH-diclofenac yield electrophilic quinone-imine derivatives that react with GSH *in vivo* (Poon et al., 2001; Kenny et al., 2004). Additionally, diclofenac may be conjugated with coenzyme A, resulting in the formation of a reactive CoA thioester conjugate (Grillo et al., 2003), as well as directly glucuronidated to an acyl glucuronide metabolite (Stierlin and Faigle,

1979). The S-acyl glutathione adduct of diclofenac produced in human hepatocytes might be formed via transacylation of GSH by a CoA thioester intermediate and/or the known AG conjugate (Grillo et al., 2003).

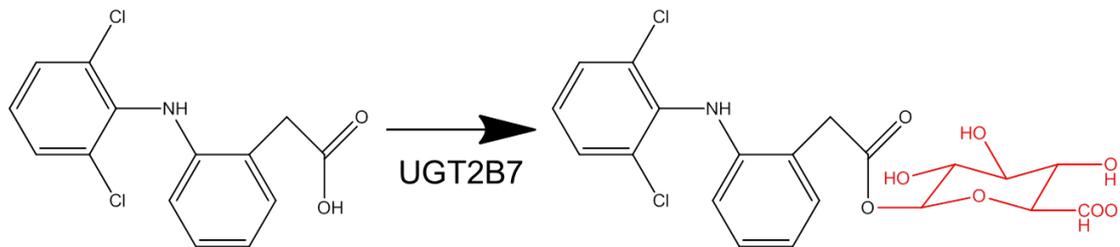


Figure 1. 4: Metabolism of diclofenac to its acyl glucuronide metabolite in human liver. Diclofenac is glucuronidated principally by UGT2B7, to a lesser extent by UGT1A9, and to minor extents UGT1A6 and UGT2B15 (King et al., 2001).

Hepatic microsomal metabolism experiments estimated acyl glucuronidation of diclofenac to be approximately 75% of the drug's clearance in man (Kumar et al., 2002). In phosphate buffer, pH7.4, the acyl glucuronide of diclofenac is chemically unstable, with a half-life of 0.51 hours (Ebner et al., 1999). Nevertheless, it has been detected in the plasma of mice administered diclofenac (Sparidans et al., 2008). Diclofenac-AG has been shown to form covalent adducts with HSA *in vitro* (Kenny et al., 2004) and with protein in rat liver microsomes (Kretz-Rommel and Boelsterli, 1994). Identification of antibodies to diclofenac metabolite-modified liver protein adducts in the sera of 7/7 patients diagnosed with diclofenac-induced hepatotoxicity, and 12 out of 20 without hepatotoxicity and none of four healthy controls (Aithal et al., 2004) provides evidence that reactive diclofenac metabolites form covalent adducts in man. However, as with all other AG-forming compounds, no definitive evidence of AG metabolites being responsible for these adducts has ever been shown. Due to these properties, diclofenac represents a suitable model compound for investigating idiosyncratic reactions to carboxylic acid drugs, and more specifically the

potential of AG reactive metabolites to form covalent adducts with proteins *in vivo* (Boelsterli, 2003; Aithal, 2004).

1.10 AIMS OF THE THESIS

AG drug metabolites remain a major challenge for the pharmaceutical industry as to their management. Extensive metabolism of a novel chemical entity into a carboxylic acid drug results in much anxiety as to its management and future progression. The reason for this is the assumed potential of these reactive metabolites to form haptens capable of eliciting inappropriate autoimmune responses historically associated with carboxylic acid drugs. Whilst the protein reactivity of AG metabolites has been shown following *in vitro* incubation, and associations have been drawn from *in vivo* studies, direct and definitive associations for carboxylic acid drugs to form covalent adducts *in vivo* or in patients have never been reported. Consequently the anxiety of AG metabolites to act as haptens, and have a causal role in eliciting the inappropriate immune responses which are characteristic in the pathogenesis of carboxylic acid mediated hypersensitivity and hepatotoxicity may be unjustified. Until the mechanisms of carboxylic acid drug induced delayed hypersensitivity reactions are fully elucidated, and metabolites responsible are identified, prediction of the risk of novel chemical entities will remain challenging, and potentially inaccurate. The work undertaken in this thesis is designed to provide a further insight into the potential of AG metabolites to act as haptens through the attempt to define their ability to form covalent adducts in man, using the AG metabolite of diclofenac as a test compound. This will be undertaken through the following experimental aims.

- a. To define the chemical instability and protein reactivity of diclofenac-AG as well as the chemistry of interaction between the AG metabolite and protein through analysis of their protein binding and stabilities in phosphate buffer, HSA solution and plasma solutions.** Acyl glucuronides have been shown to be chemically unstable and protein reactive molecules, subsequently leading to their association with idiosyncratic ADRs. Chemical instability of AGs in aqueous conditions has been associated with their rate of covalent adduct formation, subsequently leading to the current working hypothesis within drug development that generation of a chemically unstable acyl glucuronide metabolite is a toxicological concern. Through this chapter, the *in vitro* chemical instability and protein reactivity of the acyl glucuronide of the model drug diclofenac will be explored. Diclofenac-AG will be incubated in phosphate buffer to assess its chemical instability. Incubations with HSA solution will investigate its protein reactivity. Degradation studies will be used to provide increased information on the chemical interactions between AG metabolites and protein.
- b. Investigate the plasma clearance of diclofenac-AG from the rat following intravenous administration.** The physiological role of glucuronidation of carboxylic acids is to i) deactivate the pharmacophore ii) increase rate of clearance, through enhancing polarity (and therefore hydrophilicity) and affinity for export transporters. Despite AGs being protein reactive *in vitro*, their potential rapid clearance *in vivo* may prevent any accumulation of metabolite concentrations sufficient to form detectable adducts. To

investigate this, the rate of clearance of diclofenac-AG following intravenous administration to the rat was measured and compared to that of the aglycone

- c. Investigate the use of continuous infusion to enhance plasma diclofenac-AG exposure to detectable levels, and as a method to investigate potential mechanisms for diclofenac-induced production of danger signals.** Clinically, diclofenac may be administered chronically for many years. Subsequently acute exposure *in vivo* experiments may not be adequate in replicating the clinical situation. Further to this, rats are known to exhibit higher rates of xenobiotic clearance than man. Continuous infusion technologies, resulting in higher drug exposure may better represent the clinical situation. Plasma pharmacokinetics of diclofenac were measured. Investigation into potential hepatotoxicity of the drug was also undertaken, to investigate whether continuous drug exposure may elicit cell death, potentially revealing a mechanism for production of danger signals.
- d. Investigate if diclofenac is metabolised into its AG metabolite in patients receiving therapeutic doses, and if detectable serum albumin proteins can be detected in these patients using tandem mass spectrometry.** AG metabolites have been associated with forming covalent adducts to endogenous protein resulting in initiation of inappropriate immune responses in susceptible individuals. Despite this hypothesis, no definitive identification of AG metabolite adduct formation has ever been definitively shown *in vivo*. Through these experiments, using plasma samples isolated from patients receiving therapeutic doses of the drug will be assessed for covalent adducts using the

most sensitive mass spectrometry equipment available, in an attempt to reveal whether AG metabolites actually can form covalent adducts *in vivo*.

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CHAPTER TWO

CHEMICAL ASSESSMENT OF THE INTERACTIONS BETWEEN ACYL GLUCURONIDE DRUG METABOLITES AND PROTEIN USING HUMAN SERUM ALBUMIN AS A MODEL PROTEIN

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2.1 INTRODUCTION

Chemical instability and protein reactivity of AGs are well-established characteristics of these metabolites from *in vitro* incubation experiments (Stachulski et al., 2006; Regan et al., 2010). The perceived potential of AGs to manifest the same chemistry *in vivo*, and consequently form potentially immunogenic covalent adducts with endogenous proteins is central to the anxiety that AG metabolites of carboxylate drugs will elicit ADRs (Williams et al., 1992; Worrall and Dickinson, 1995; Bailey and Dickinson, 2003).

Spontaneous chemical instability of AGs in aqueous solution at pHs > 7 is almost a universal characteristic of these Phase II metabolites. As summarised by Table 2. 1, AG metabolites of drugs exhibit extensive variation in their chemical stabilities, with $t_{1/2}$ of degradation at pH 7.4 and 37°C ranging from 0.26 hours for tolmetin-AG to 79 hours for the AG of valproate. Strong correlations have been drawn between the chemical instability of AGs and their protein reactivity ($r^2 = 0.995$ for nine AGs of carboxylate NSAIDs) (Benet et al., 1993). These observations and associations between the chemical instability of AGs in phosphate buffer and toxicological risk of the parent carboxylate drug (Sawamura et al., 2010) have resulted in much interest in structural factors affecting the chemical stability of AGs (Nicholls et al., 1996; Johnson et al., 2007; Johnson et al., 2010), and incorporation of AG stability studies into pre-clinical development of carboxylate drugs (Ebner et al., 1999; Wang et al., 2004; Walker et al., 2007; Potter et al., 2011).

The intrinsic chemical reactivity of an AG derives fundamentally from the electrophilicity of the ester carbonyl carbon (Baba and Yoshioka, 2009). Consequently, these compounds degrade spontaneously under aqueous conditions and more rapidly when in the presence of simple nucleophiles, such as the hydroxide ion and short-

chain alcohols (Dickinson and King, 1991; Silvestro et al., 2011), by two distinct chemical pathways, namely hydrolysis or acyl migration (Stachulski et al., 2006). The relative rates of these pathways can vary considerably between AGs, as determined by the structure, including the stereochemistry of the aglycone (Johnson et al., 2007) and nature of the matrix (Johnson et al., 2010).

The ester carbonyl carbon of the AG can undergo nucleophilic attack by $-OH$ ions in biological media (Hyneck et al., 1988a), or be targeted by intracellular and extracellular enzymatic (hydrolase) attack (Smith et al., 1990b; Dubois-Presle et al., 1995; Bailey and Dickinson, 2003; Kenny et al., 2005; Iwamura et al., 2012). This results in displacement of glucuronic acid and regeneration of the parent aglycone, and in vivo is responsible for conjugation-deconjugation cycles of AG metabolites (Brunelle and Verbeeck, 1997). Degradation of 1- β AG metabolites by intramolecular acyl migration is a consequence of nucleophilic attack on the carbonyl group by the adjacent hydroxyl group. This causes electron displacement, resulting in the formation of an ortho-acid ester intermediate, before stabilisation with the aglycone moiety moved along the sugar ring as outlined by Figure 2.1. Subsequently, this pathway allows the aglycone to move around the sugar ring from the C-1 position to the C2, 3 and 4 positions. Following initial migration from the C-1 position, migration between the C2, 3 and 4 positions is reversible, however direct back migration to the C-1 position is not thought to be possible. When the acyl residue is at C2, 3 and 4 positions, the hydroxyl group on C-1 may reversibly move from the β to α orientation after a transient open ring intermediate is formed. When the 1- β AG has rearranged to the 2 α isomer, back acyl migration to the C-1 position is possible, forming the highly unstable 1 α AG isomer (Corcoran et al., 2001; Baba and Yoshioka, 2009). The α -1-O-acyl isomer in turn

undergoes acyl migration to a mixture of α/β -2-O-acyl isomers. Consequently, through acyl migration and epimerisation, the 1 β AG metabolite can degrade into eight isomers *in vitro*, and through hydrolysis, may revert to the parent aglycone as outlined by Figure 2.1, Figure 2.2 and (Bailey and Dickinson, 2003; Stachulski et al., 2006).

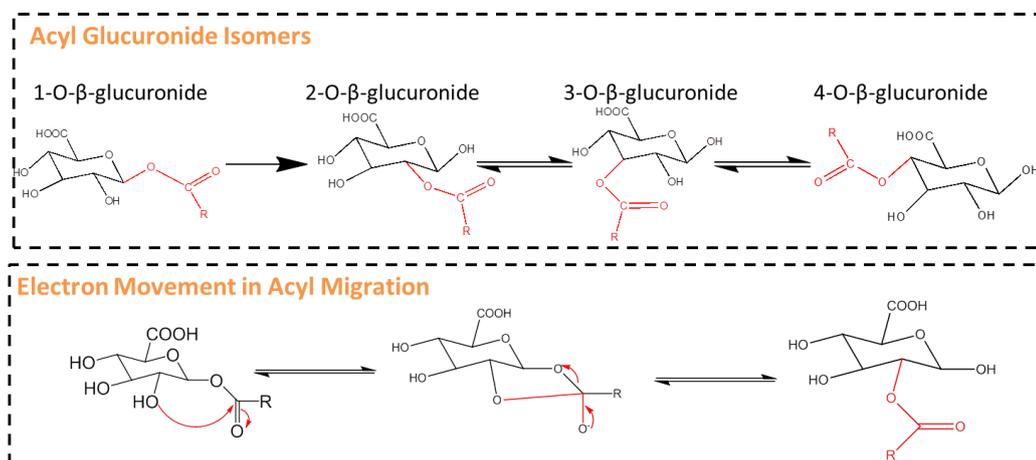


Figure 2. 1: Positional isomers of acyl glucuronides and the electronic mechanism of acyl migration via ortho-ester transition states (Berry et al., 2009; Regan et al., 2010)

Covalent modification of protein during *in vitro* incubation is essentially a generic reaction of AGs (Benet et al., 1993). The rate and extent that AGs modify protein is likely to be distinct to each AG. Factors influencing AG modification of protein include the chemistry of the aglycone, pH of the incubation mixture, concentration of protein and AG and the time course of incubation (Spahn-Langguth and Benet, 1992)

The chemistry of the covalent interactions between AG metabolites and protein has been ascertained in part through the use of chemical modifications of the protein (site blocking) and its adducts (imine stabilisation) (Wells et al., 1987; Smith et al., 1990a) and radiochemical analysis of modified proteins following *in vitro* incubation with AGs (Ruelius et al., 1986). This work has underpinned the identification of two pathways of AG adduct formation, namely acylation (intermolecular transacylation) and glycation (Ruelius et al., 1986; Wells et al., 1987; Smith et al., 1990a; Williams et al., 1992; Kretz-

Rommel and Boelsterli, 1994; Williams and Dickinson, 1994; Iwaki et al., 1999). However, it was not until the use of tandem mass spectrometric analysis that the covalent binding of AG to HSA was fully characterised (Ding et al., 1993; Ding et al., 1995; Qiu et al., 1998). Chemical pathways of adduct formation map closely to degradation pathways of the AG molecule, apparently forming covalent adducts only by these two pathways.

As with the degradation of AGs by hydrolysis, AGs are also susceptible to nucleophilic attack by amino, guanidine, and hydroxyl groups found on proteins, resulting in the displacement of glucuronic acid. This results in the acyl residue of the aglycone being covalently bound to the protein via amide and ester linkages (Ding et al., 1995; Qiu et al., 1998). Formation of glycation adducts, however, from first principles of hemiacetal/aldehyde (pyranose/aldose) chemistry (Zhu et al., 2001; Brown et al., 2011) requires prior migration of the acyl group to the 2, 3 and 4 ring positions (Smith et al., 1990a). Following this rearrangement, a transient opening of the sugar (pyranose) ring can occur at C-1, during which the positional isomers can move between the β and α conformations. The aldehyde function of the opened sugar ring (acyclic aldose structure) is susceptible to nucleophilic attack by amino groups found on proteins. This aldehyde condenses with these groups, resulting in the reversible formation of imine adducts (Schiff bases) (Smith et al., 1990a). The covalent reactions of aldohexoses and other sugars with proteins, at least *in vitro*, are governed generally by the proportion of the molecule present in its acyclic form (Srovy, 1994). However, this molecular parameter is apparently less influential in the reactions of AG with protein than the reversible non-covalent interactions preceding adduction (Smith and Wang, 1992). The C-3 and C-4 Schiff bases may subsequently be stabilised through an

Amadori rearrangement, yielding 1-amino-keto adducts (Acharya and Sussman, 1984; Neglia et al., 1985; Smith et al., 1990a). Based upon the rearrangements of simple sugar adducts of lysine residues, the rate of the hydroxyimine-ketoamine tautomerization can vary considerably between molecular sites; probably depending upon the proximity of nucleophilic amino acid side-chains that facilitate the isomerization (Acharya et al., 1991; Nacharaju and Acharya, 1992). The formation of certain ketoamine (Amadori) adducts, such as those of glucose on N-terminal amino acids (Neglia et al., 1985), is nearly irreversible. Adducts on ϵ -amino groups of lysines appear to be somewhat labile (Acharya and Sussman, 1984). Buffer conditions considerably influence this reversibility *in vitro*: it is significantly higher in a TRIS buffer, pH 7.5, than in a phosphate buffer of the same pH (TRIS can act as a nitrogen nucleophile via its amino group and as an oxygen nucleophile via its hydroxyl groups). It has been suggested that under physiological conditions ketoamine adducts on ϵ -amino side-chains are in equilibrium with the imine tautomer, the equilibrium being biased toward the ketoamine (Acharya and Sussman, 1984). Importantly, the phosphate buffer used for the present incubations of diclofenac-AG and HSA will have favoured retention of the Amadori rearrangement products. The distinguishing feature of these glycation adducts is that the glucuronic acid moiety is retained in the covalent adduct (Figure 2. 2).

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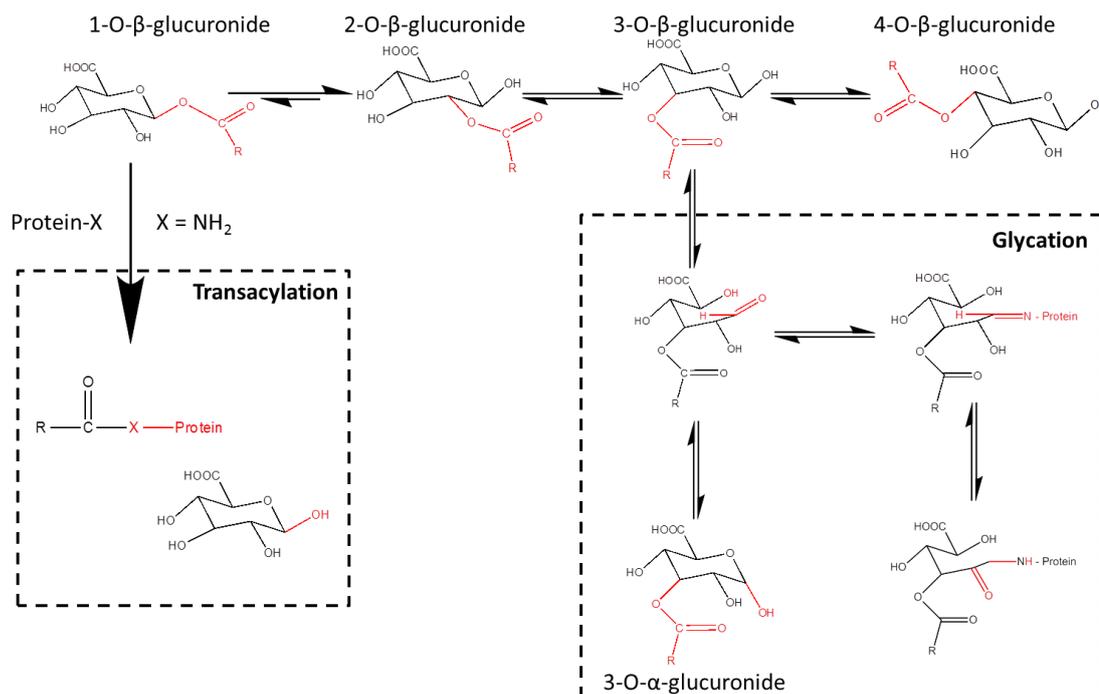


Figure 2. 2: Chemical pathways of formation of protein adducts from an acyl glucuronide, adapted from (Bailey and Dickinson, 2003). In principle, all the open-chain forms of C-2, C-3 and C-4 positional isomers of an AG can undergo glycation reactions with proteins. All the ring-open structures undergo mutarotation, giving α -anomers at C1 on the sugar ring which themselves might undergo reactions with proteins. Within the constraints of steric hindrance, C2→C3 and C3→C4 acyl migrations, and even back migrations, might occur after formation of the Schiff base (imine) adducts. Therefore, potentially, the haptens formed by glycation are both highly heterogeneous and dynamic. However, only Schiff bases of the O-3 and O-4 esters can undergo the Amadori rearrangement

AGs have been shown to modify HSA in a time-dependent manner through the use of alkaline hydrolysis to liberate bound drug from the protein adducts, and subsequent LC-UV quantification of the drug. Generally, modification occurs to a point where C_{max} is reached. Once this point is reached, covalent binding subsequently plateaus or slowly decreases. The latter observation suggests that AG adduction of protein may not be fully irreversible, and subsequently, AG and aglycone residues may be spontaneously hydrolysed away from albumin. This pattern has been shown for the AGs of oxaprozin (Wells et al., 1987), zomepirac (Smith et al., 1986), tolmetin (Munafò

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et al., 1990), diflunisal (Watt and Dickinson, 1990), ketoprofen (Presle et al., 1996) and ibuprofen (Castillo et al., 1995).

Alkaline and mild-acid hydrolysis of AGs and HSA (Smith et al., 1990a) combined with selective inhibitors of reversible protein binding allowed some early suggestions as to the sites on the HSA molecule covalently modified by AGs (Williams and Dickinson, 1994). Whilst not yet allowing quantitative assessment of the covalent protein binding, MS/MS allows identification of the chemical route of modification and identification of the amino acids modified. Consequently, whilst AGs had previously been suggested to form glycation adducts (Smith et al., 1990a), it was not until tandem mass spectrometric identification that these adducts were confirmed (Ding et al., 1993). Subsequently tandem mass spectrometric analyses of HSA adducts of the AGs of benoxaprofen and tolmetin (Ding et al., 1993; Ding et al., 1995; Qiu et al., 1998) and synthetic activated esters of tolmetin and zomepirac (Zia-Amirhosseini et al., 1995) have been reported.

HSA is a protein containing three homologous helical domains (named I, II and III), each divided into subdomains (A and B) (Ghuman et al., 2005). Within these binding domains, as a consequence of the protein's folding in its tertiary structure, sites of the protein have been identified as binding pocket, largely located in hydrophobic pockets the first of which (the first named Sudlow Site I or warfarin binding site and the second as Sudlow Site II, benzodiazepine or diazepam binding site) (Sudlow et al., 1975; Sudlow et al., 1976).

Covalent adducts formed by AGs were formed preferentially in these binding pockets of the HSA molecule, at the Sudlow I and II sites (located on HSA subdomains IIA and IIIA, respectively) (Sudlow et al., 1975; Sudlow et al., 1976; Ghuman et al., 2005).

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Modifications were found to several amino acids, including Lys-199, Lys-195 and Lys-525 through the glycation pathway, as well as Lys-199, Ser-312 and Arg-521 through the transacylation pathway (Ding et al., 1995; Zia-Amirhosseini et al., 1995; Qiu et al., 1998).

The principle aim of this *in vitro* work was to validate diclofenac-AG as a suitable tool to test the ability of AGs to form covalent adducts with HSA *in vivo*. Due to the extensive literature of the interaction of AGs with HSA (Smith et al., 1986; Munafo et al., 1990; Watt and Dickinson, 1990; Ding et al., 1993; Mayer et al., 1993; Dubois et al., 1994; Castillo et al., 1995; Ding et al., 1995; McGurk et al., 1996; Sallustio et al., 1997; Qiu et al., 1998) allowing comparison to our data, and its high concentration in human plasma (Anderson and Anderson, 2002) from which it can be readily extracted (Greenough et al., 2004; Jenkins et al., 2009) resulting in its applicability to *in vivo* assessment of covalent modification to be undertaken in later chapters, HSA was seen as a good model to investigate the interactions of diclofenac-AG with protein.

For diclofenac-AG to represent a suitable tool for the investigation of the AG to form covalent adducts *in vivo*, it should be chemically unstable in solution at physiological pH, ideally, like the AGs of R-benoxaprofen and tolmetin with a degradation $t_{1/2}$ of \leq 2hrs (Table 2. 1), and it should also form persistent covalent adducts with the protein. If a novel chemical entity was extensively metabolised to an unstable and protein-reactive AG, anxiety would arise over its progression. Further aims of this work were to obtain additional insights into the chemistry of the interactions between AGs and protein, and specifically to obtain reference data that would indicate how the AG might react *in vivo*.

Table 2. 1: Clinical doses of selected carboxylic acid drugs and the degradation half-lives of their AG metabolites in phosphate buffer, pH 7.4, at 37°C. Table adapted from (Regan et al., 2010). ^aTaken from (Ebner et al., 1999). Note, only the protein (HSA) adducts of benoxaprofen AG (Qiu et al., 1998) and tolmetin AG (Ding et al., 1993; Ding et al., 1995) had been fully characterised (in vitro) before the present investigations.

Drug	Clinical dose	AG Degradation Rate $t_{1/2}$ (h)
Tolmetin	1.2g-1.8g daily	0.26
Probenecid	Up to 4g daily	0.3
Zomepirac (ZP)	100mg	0.45
Diclofenac (DCF)	75–150mg daily	0.51 ^a or 0.79 (see Table 2. 4)
Naproxen	0.5-1.25g daily	R-Naproxen 0.92 S-Naproxen 1.8
Ibuprofen		1.1
Indomethacin	50–200 mg daily in divided doses	1.4
Benoxaprofen	600mg/dose	R-Benoxaprofen 2.0 S-Benoxaprofen 4.1
Ibuprofen	900mg – 2.4g daily	3.3
Bilirubin	n/a	4.4
Furosemide	20-120mg daily	5.3
Clofibrate	2g daily	7.3
Mefenamic acid	1.5g daily	16.5
Telmisartin	20-80mg daily	26
Gemfibrozil	0.9-1.2g daily	44
Valproic Acid	1.5g-2.250g daily	79

2.2 MATERIALS AND METHODS

2.2.1 Materials

Acetonitrile (LC-MS grade), ammonium acetate (LC-MS grade), concentrated phosphoric acid (99%), diethyl ether (HPLC grade), ethanol (LC-MS grade), ethyl acetate (HPLC grade), isopropanol (LC-MS grade), methanol (LC-MS grade) and trifluoroacetic acid (LC-MS grade) were purchased from Fisher Scientific, Leicestershire. Bio Rad Bradford reagent was purchased from Bio Rad, Hertfordshire, UK. Modified trypsin was purchased from Promega, Hampshire, UK. Acetic acid (LC-MS grade), ammonium hydrogen carbonate, diclofenac sodium salt, dithiothreitol, formic acid (LC-MS grade), HSA (approx. 99% pure, essentially globin free and fatty acid free) iodoacetamide, potassium hydroxide pellets and zomepirac sodium salt were purchased from Sigma-Aldrich, Dorset, UK.

0.1M phosphate buffer pH 7.4 was made using 0.3117% monosodium phosphate monohydrate, 2.0747% disodium phosphate, heptahydrate w/v in distilled water.

All other reagents were purchased from Sigma-Aldrich (Dorset, UK) unless otherwise stated.

2.2.2 Chemical synthesis of 1- β diclofenac acyl glucuronide

Synthetic 1- β diclofenac-AG was synthesised in a three stage process and characterised as described by (Bowkett et al., 2007). This synthesis was kindly performed by Xiaoli Meng at the University of Liverpool and Stuart Bennet at AstraZeneca, Alderley Park, UK and is described in Supplementary section 1.1. Purity of synthetic 1- β diclofenac-AG was assessed using a combination of LC-UV and tandem mass-spectrometric assessment. For acceptable use in experiments, purity was above 98%. LC-UV conditions as described in section 2.2.4 were used to assess diclofenac purity.

2.2.3 Degradation of synthetic 1 β -diclofenac acyl glucuronide in phosphate buffer, human serum albumin solution and isolated human plasma

Chemically synthesised 1- β diclofenac-AG at a concentration of either 400 μ M or 2mM was incubated in triplicate for 16 hours in either 0.1M phosphate buffer, pH7.4, HSA solution (40 μ M in phosphate buffer) or isolated plasma pooled from three healthy male volunteers of ages between 21 and 25 years at 37°C. The phosphate buffer, HSA solutions and plasma were incubated at 37°C for 20 minutes prior to addition of diclofenac-AG.

For phosphate buffer incubations, at desired time points, 99 μ l of the incubation mixture were removed and immediately acidified through the addition of 1 μ l of concentrated phosphoric acid. Acidified solutions were then immediately analysed by HPLC-UV (Kenny et al., 2004).

For incubations with HSA and human plasma, 100 μ l of the incubation mixture were removed. Degradation of the AG was stopped and protein was precipitated through the immediate sequential addition of two volumes of ice-cold acetonitrile containing 2% acetic acid (v/v) and one volume of ice-cold ethanol. Samples were vortex mixed, and protein immediately separated by centrifugation at 14,000rpm at 4°C. The supernatant was removed, evaporated to dryness under a constant stream of nitrogen at 37°C, reconstituted in 100 μ l of 0.1M phosphate buffer containing 1% phosphoric acid (v/v), and immediately analysed by HPLC-UV.

2.2.4 HPLC-UV analysis of diclofenac acyl glucuronide degradation

1- β diclofenac-AG, its α -anomer, its 2-, 3- and 4- positional isomers and the parent aglycone (Figure 2. 3) were chromatographically resolved using a Zorbax Eclipse XDB-C8 column (150 x 4.6mm, 5 μ m; Agilent Technologies, Santa Clara, California, USA) fitted with a SecurityGuard guard column (Phenomenex, Macclesfield, Cheshire, UK). The Dionex Summit HPLC system comprised of an ASI-100 automated sample injector, a P580 pump and a UVD170S UV detector (Dionex Ltd., Macclesfield, Cheshire, UK) operated using Chromeleon software (Dionex Ltd).

Analytes in aliquots of the incubation solutions after processing (20 μ l for phosphate buffer incubations, and 40 μ l for incubations in HSA solution and human plasma) were eluted at room temperature with a linear gradient of methanol containing 10% acetonitrile (v/v) in 10mM ammonium acetate, pH 4.5: 15-67.5% over 75minutes. Following this, the column was purged with methanol-acetonitrile (9:1, v/v) for 5 minutes, and then re-equilibrated for a further 10 minutes. The eluent flow rate was maintained at 0.9ml/minute. Absorbance of eluted compounds was monitored at 254 nm. Analyte peak areas were calculated using Chromeleon, and represented as a percentage with respect to the combined peak areas of the AG isomers and aglycone. No peaks were detected on the UV chromatogram of blank HSA or human plasma samples which would have interfered with AG or aglycone analyte peaks. Peaks with a signal-to-noise ratio of less than three were considered below the limit of detection and discounted from analysis.

Samples containing AG and aglycone were assayed at 254nm, the standard wavelength for detection of aromatic systems, as glucuronic acid is not reported to have any absorbance at this wavelength (Shirao et al., 1994). Although *O*-glucuronidation can

alter the absorbance of some phenolic compounds (Singh et al., 2010), the isomolar absorbency of the aglycone and AGs of the NSAIDs diflusalin (Dickinson and King, 1991) and ketoprofen (Terrier et al., 1999) has been reported previously. To confirm this observation applies to diclofenac, 100 μ l aliquots of the 2 mM solution of the 1- β AG isomer and a 90 minute degraded sample, both spiked with 10 μ l of 10 mM zomepirac, were assayed at 254nm using the same HPLC-UV conditions outlined above. Less than 5% variance in the peak area ratio of analyte and internal standard were detected between the 1- β diclofenac-AG and its degradation products, confirming isomolar absorbance of the AG isomers and diclofenac at 254nm.

The identities of the chromatographic peaks of the 1- β diclofenac-AG isomer and the parent aglycone were confirmed using synthetic standards run on the assay. Retention times of 1- β diclofenac-AG and diclofenac were confirmed using LC-MS. The four resolved AG isomers showed qualitatively identical positive-ion electrospray mass spectra which were all indistinguishable from the 1- β AG's spectrum. AG spectra were all dominated by neutral loss of dehydroglucuronic acid.

Following identification of the 1- β diclofenac-AG and diclofenac peaks, the remaining positional isomers were assigned from their chronological appearance during incubation in phosphate buffer, pH 7.4, at 37°C (Figure 2. 6A). This method has been employed in previous studies of AG rearrangement (Watt and Dickinson, 1990). The observed order of isomer elution (4-, 1- α , 1- β , 3-, 2-O-AG) is the most frequently reported order for AG isomer elution from reversed phase columns when the isomers have been defined by NMR spectroscopy (Corcoran et al., 2001; Akira et al., 2002; Stachulski et al., 2006). Whilst complete or near-complete separation of the 2-, 3-, and 4- isomers was achieved, separation of anomers was only achieved for the 1- α and 1- β

anomers. The minor chromatographic peak directly in front of the 1- β anomer was designated the 1- α anomer in conformity with the standard verified order of elution of these epimers (Corcoran et al., 2001; Akira et al., 2002). Chromatographic separation of the 1- β and 1- α anomers is not invariably achieved (Ebner et al., 1999). No attempt was made to achieve chromatographic separation between the anomers of the 2-, 3- or 4- isomers. Anomeric separation of these isomers is usually incomplete, and is associated with distorted peak shapes due to rapid inter-conversion of the anomers (Stachulski et al., 2006).

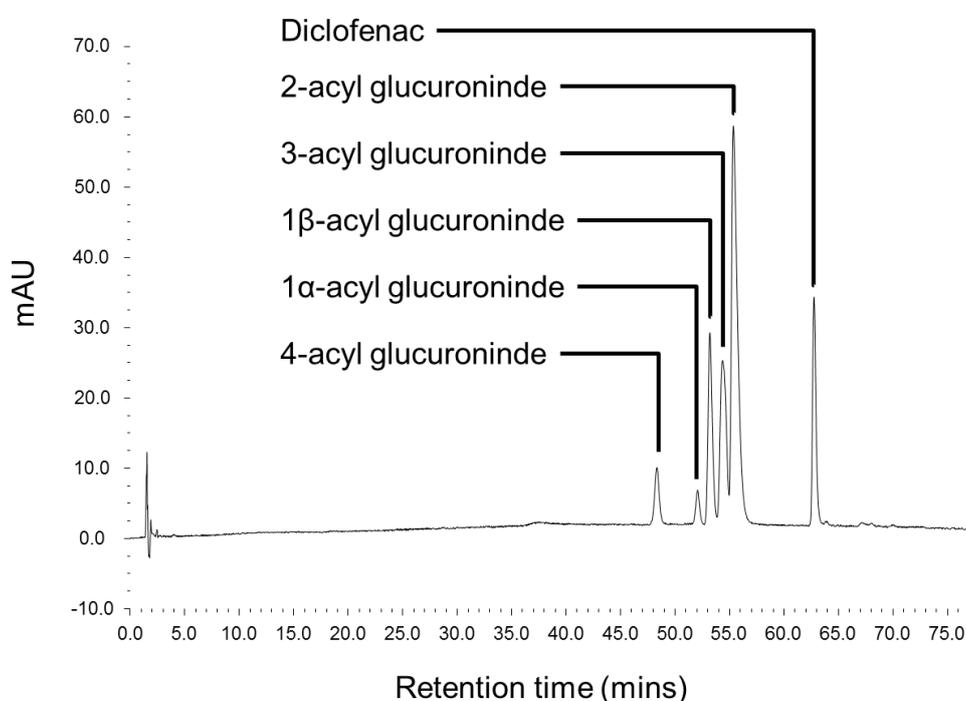


Figure 2. 3: Representative LC-UV trace showing chromatographic separation of 1- β diclofenac-AG, its α anomer, the 2-, 3- and 4- positional isomers and its parent aglycone at 254nm. The mixture was produced by incubating 1- β diclofenac-AG in 40 μ M HSA solution, pH 7.4, at 37°C for 90 min. The AG positional isomers were identified from their chronological appearance during the incubation. The α -anomer was identified from its chromatographic position, by comparison with isomer/anomer sets of other AG that have been characterised ab initio by NMR spectroscopy (Corcoran et al., 2001; Akira et al., 2002).

2.2.5 Covalent binding of 1- β diclofenac acyl glucuronide to human serum albumin determined using an alkaline hydrolysis technique

750 μ M and 150 μ M concentrations of diclofenac-AG were incubated with a 15 μ M HSA solution in phosphate buffer, pH 7.4, in triplicate at 37°C. Covalent binding was assessed using an alkaline hydrolysis technique based upon published methods (Zia-Amirhosseini et al., 1994; Zia-Amirhosseini et al., 1995). Aliquots (500 μ l) were removed at intervals between 5min and 16 hours. The protein was precipitated through the sequential addition of 1.5ml of ice-cold acetonitrile followed by 500 μ l ice-cold isopropanol. The protein solution was separated by centrifugation at 2500 rpm. Following protein precipitation, non-covalently bound drug was washed from the protein pellet through vortex-mixing with seven 5 ml aliquots of methanol-diethyl ether (3:1, v/v). The 6th and 7th organic extracts were retained, evaporated to dryness under a constant stream of nitrogen at 37°C and reconstituted in 250 μ l of 75% 500mM ammonium acetate pH 4.5, 25% acetonitrile (v/v) for LC-MS/MS analysis. No drug was detected in these wash extracts by LC-MS/MS analysis. The fully extracted protein pellet was dried under a constant stream of nitrogen at 37°C. 1 ml of 0.25M potassium hydroxide was added to the protein pellet, and subsequently incubated at 80°C in a water bath for 90 minutes to liberate covalently bound diclofenac. An aliquot of the solution (20 μ l) was then removed for protein assay (Bradford, 1976), giving a recovery of 63.29 \pm 10.14%. The remaining alkaline hydrolysate was neutralised through addition of 45 μ l of 43% phosphoric acid (v/v in aqueous solution). 55 μ l of 100 μ M zomepirac (in 50% acetonitrile) was added to the neutralised hydrolysate as an internal standard before addition of 5ml ethyl acetate. Following mixing and centrifugation at 2500 rpm for 5 minutes, the upper phase was removed and

evaporated to dryness under a constant stream of nitrogen at 37°C. The hydrolysate was reconstituted in 250µl of 35% 8.5mM ammonium acetate containing 0.0075% formic acid (v/v), 65% methanol (v/v), for LC-MS/MS quantification of liberated diclofenac.

2.2.6 LC-MS/MS assay of diclofenac liberated from HSA adducts of diclofenac acyl glucuronide using an alkaline hydrolysis technique

25µl of reconstituted sample following hydrolysis was injected onto the HPLC column. Analyte separation was performed at room temperature using a Zorbax Eclipse XDB-C8 column (150 x 4.6mm, 5µm; Agilent Technologies, Santa Clara, California) fitted with a SecurityGuard guard column (Phenomenex, Macclesfield, Cheshire, UK), connected to a PerkinElmer series 200 HPLC system (PerkinElmer, Norwalk, Connecticut, USA) and an API 2000 triple quadrupole mass spectrometer (AB Sciex, Warrington, UK). Analytes were eluted at room temperature with a linear gradient of methanol in 8.5mM ammonium acetate containing 0.0075% formic acid (v/v), 65-85% over 7 minutes. Following this, the column was purged with methanol for 1 minute before re-equilibration for 3 minutes. The eluent flow rate was maintained at 1ml/minute, achieving separation of diclofenac and zomepirac as represented by Figure 2.4. MS operating parameters for the multiple reaction monitoring (MRM) assay of diclofenac with zomepirac as internal standard are shown in Table 2. 2. The lower limit of detection of diclofenac spiked into buffered HSA solution before alkaline hydrolysis, as defined by a signal-to-noise ratio >3, was 100µM. The corresponding lower limit of quantification, as defined by accuracy of between 80-120%, and precision (coefficient of variation) < 20%, was 100µM (accuracy 90.7% and precision of 17.3%).

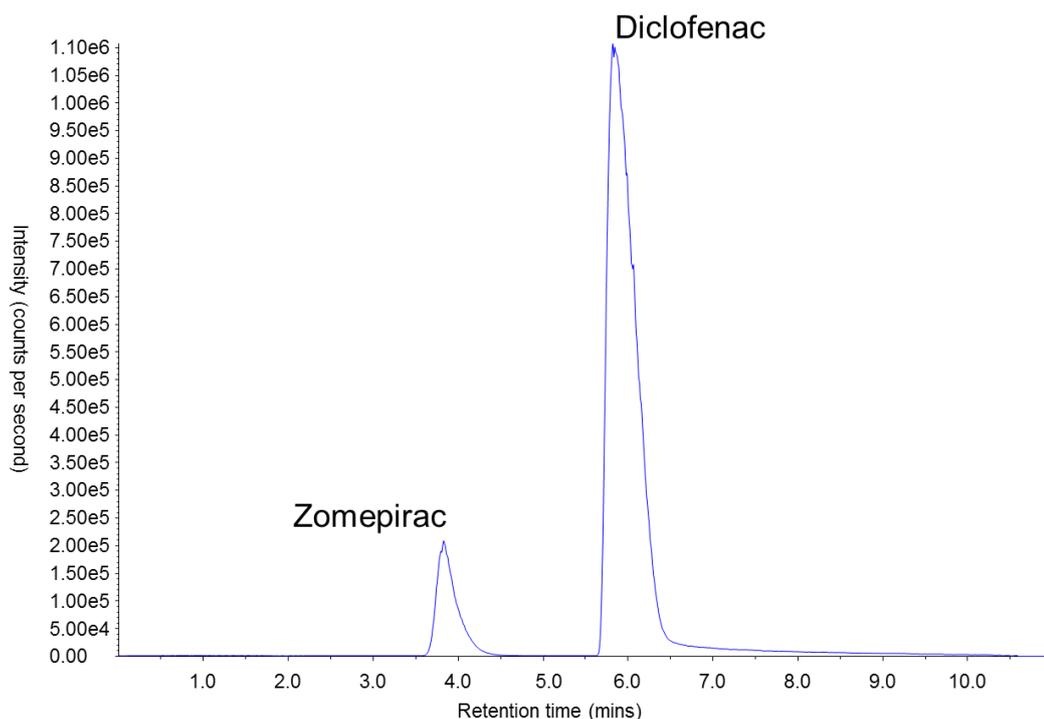


Figure 2. 4: Total ion current chromatogram representing chromatographic separation of diclofenac and zomepirac (internal standard) in the alkaline hydrolysis assay of covalent binding of diclofenac-AG to HSA *in vitro*. The compounds were analysed by LC-MS/MS

Table 2. 2: API 2000 operating parameters for the MRM assay of diclofenac with zomepirac as an internal standard.

Parameters	Diclofenac	Zomepirac
Fragmentation transition	296.066→214.9	292.038→139.000
Declustering potential (V)	11.00	16.00
Focussing Potential (V)	360.00	360.00
Entrance Potential (V)	6.00	10.50
Collision energy (eV)	25.000	27.00
Collision cell entrance potential (V)	12.000	12.000
Collision cell exit potential (V)	10.00	6.00
Dwell time (ms)	150	150

2.2.7 Covalent modification of human serum albumin by diclofenac acyl glucuronide during *in vitro* incubation

Concentration-dependent modification of HSA by diclofenac-AG was investigated *in vitro*. Increasing concentrations of 1- β diclofenac-AG (400nM, 4 μ M, 40 μ M, 400 μ M and 2mM) were incubated with 40 μ M HSA in 0.1M phosphate buffer, pH 7.4, at 37°C for 16 hours. To investigate time-dependent modifications of HSA, 2mM diclofenac-AG was incubated with 40 μ M HSA for time points between 30 minutes to 16 hours. To investigate the role of the 1- β isomer in the chemical pathways of AG covalent binding, a final concentration-effect experiment was performed. Diclofenac-AG was pre-degraded for 3 hours in 0.1M phosphate buffer, pH 7.4, at 37°C. Separate experiments have shown that this incubation results in degradation of the 1- β isomer to 5.52 (\pm 1.37)% presence in the incubation mixture, with acyl migration to the 2-, 3- and 4-isomers and minimal hydrolysis to parent diclofenac (Figure 2. 6A). Degraded diclofenac-AG was incubated with 40 μ M HSA at a final concentration of 2mM for 16 hours. A solution of 2mM undegraded 1- β diclofenac-AG was incubated with 40 μ M HSA alongside the degraded AG incubation for 16 hours for direct comparison.

At desired time points 100 μ l aliquots of the reaction mixture were removed. The reaction was stopped and protein precipitated through immediate vortex mixing with 900 μ l ice-cold methanol and subsequent centrifugation at 24,000g at 4°C for 15 minutes. The supernatant was removed, and the pellet washed three times with 60 μ l ice-cold methanol. The precipitated protein was dissolved in 50 μ l of 0.1M phosphate buffer pH 7.4, reduced with dithiothreitol (10mM) for 15 minutes at room temperature and alkylated with iodoacetamide (55mM) for a further 15 minutes at room temperature to cap cysteine residues, preventing the formation of disulphide bridges

of peptides before mass spectrometric analysis. The protein was then precipitated and washed with ice-cold methanol. The protein pellet was re-dissolved in ammonium hydrogencarbonate solution (50 μ M, 30 μ l), assayed for protein content using the Bradford assay (Bradford, 1976), and 30 μ l aliquots of 3.2mg/ml protein solution were digested with 5 μ g trypsin overnight. The digests were desalted using 0.6 μ l bed C18 - Zip-Tip pipette tips (Millipore, Cork, Republic of Ireland) as per the manufacturer's instructions, eluted with 10 μ l 50% acetonitrile, 0.1% trifluoroacetic acid in deionised water and dried by centrifugation under vacuum (SpeedVac, Eppendorf UK Ltd, Cambridge) prior to LC-MS/MS analysis.

2.2.8 Mass-spectrometric analysis of diclofenac acyl glucuronide modified tryptic peptides of human serum albumin

Modified tryptic peptides were detected on a 5500 QTRAP hybrid triple-quadrupole/linear ion trap instrument fitted with a Nanospray II source (AB Sciex, Foster City, California, USA). MRM transitions specific for peptides modified by diclofenac-AG by either the transacylation or glycation pathway were selected as follows: the m/z values of all singly charged HSA peptides with a missed trypsin cleavage at the modified lysine residue and mass additions of either 277 amu (for the transacylated peptide) or 453 amu (for the glycated peptide) were calculated. These m/z values were paired with the m/z values of the dominant fragment ions of diclofenac, namely m/z 215 and m/z 250 (Figure 2. 5) to complete the MRM transitions. HSA peptides identified as modified following incubation with diclofenac-AG (2mM diclofenac-AG, 40 μ M HSA) following exhaustive analysis using a QStar i hybrid mass spectrometer (Applied Biosystems, Warrington UK) (see chapter 3) were selected for MRM transitions, combined with other peptides identified as modified by

a range of drugs in our laboratory. Transitions for which modified HSA peptides were detected following *in vitro* incubation are outlined in Supplementary Table 1 and 2. Relative MRM peak heights for each of the modified peptides were computed by MultiQuant software version 2.0 (AB Sciex) to produce an ‘epitope profile’ that is characteristic of each type of modifying species, i.e. transacylating and glycating. However, due to the unavailability of standards of the modified peptides allowing relative ionisation efficiencies of modified peptides to be ascertained, these ratios must be regarded as approximations.

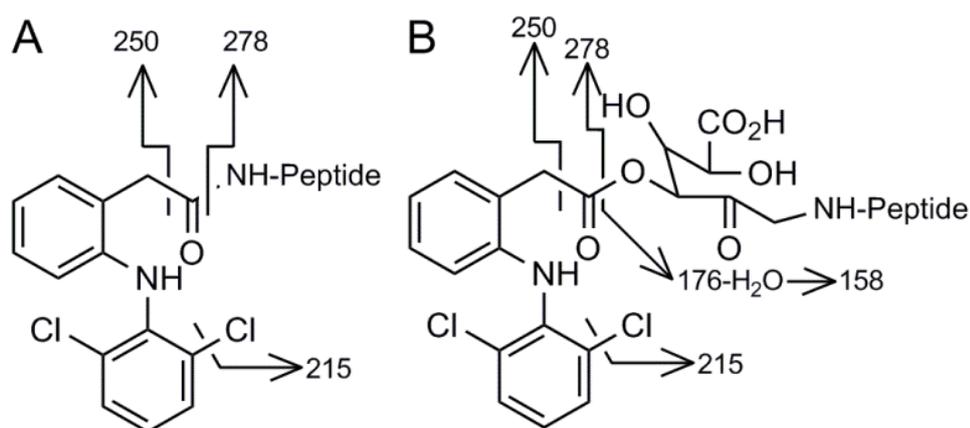


Figure 2. 5: Mass-spectrometric fragmentations of diclofenac and diclofenac-AG residues when covalently bound to protein. (A) represents transacylation adducts (B) represents glycation adducts. (The 2-ketoamine form of the 3-*O*-ester conjugate is shown; see **Figure 2. 2**). Mass values corresponding to the $^{12}\text{C}^{35}\text{Cl}_2$ isobars of adduct fragments are shown. See (Galmier et al., 2005) for the product-ion spectrum of diclofenac obtained by electrospray ionisation.

The total ion count for each sample of tryptic peptides was normalised to that of the HSA conjugate produced by incubation of 2mM diclofenac-AG with 40 μ M HSA for 16 hours. This allows the magnitudes of MRM signal to be adjusted for differences between amounts of sample loaded on to the LC column.

Aliquots of the modified tryptic peptides were injected onto a C₁₈ PepMap column (75 μ m x 15 cm; Dionex) fitted with a 5 mm C18 nano-precolum using an Ultimate

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3000 HPLC system. A gradient of 2% acetonitrile, 0.1% formic acid (v/v), to 50% acetonitrile, 0.1% formic acid (v/v), over 60 minutes at a flow rate of 300 nl/minute was used to separate the peptides, and they were delivered to the mass spectrometer through a 10 μ m i.d. PicoTip ionspray emitter (New Objective, Woburn, MA). The ionspray potential was set to 2200-3500 V, the nebuliser gas to 19 and the interface heater to 150°C. MRM transitions were acquired at unit resolution in both Q1 and Q3 to maximise specificity. Collision energies were optimised for each MRM and dwell times were 20ms. MRM survey scans were used to trigger up to three enhanced product-ion scans of modified peptides according to the multiple reaction monitoring-initiated detection and sequencing (MIDAS) technique (Unwin et al., 2005; Unwin et al., 2009), with Q1 set to unit resolution and with dynamic fill of the trap. The complete or partial amino acid sequence of the peptide and the location of the adduct were deduced from these product-ion spectra.

2.3 RESULTS

2.3.1 Investigations into the chemical instability of synthetic 1 β diclofenac acyl glucuronide in 0.1M phosphate buffer pH7.4, human serum albumin solution and isolated human plasma at 37°C

Synthetic 1- β diclofenac-AG was found to degrade spontaneously in 0.1M phosphate buffer, pH7.4, and it also degraded in 40 μ M HSA solution and human plasma at 37°C (Figure 2. 6). Degradation of the 1- β isomer appeared to follow first-order kinetics. This allowed the rate of degradation to be analysed using non-linear regression analysis. A curve was fitted to the lines following the equation $C=C_0\exp^{-k_{deg}(time)}$, where C represents the peak area ratio of the 1- β isomer in the degradation mixture and k_{deg} represents the degradation rate constant (Figure 2. 7), allowing the half-life of degradation ($t_{1/2}$) to be calculated. All regression analyses were fitted to an r^2 value of above 0.99 (Table 2. 4).

During incubation in 0.1M phosphate buffer, pH 7.4, a 2 mM solution of 1- β diclofenac-AG was found to degrade with a half-life of 0.78 hours (Figure 2. 6A). Degradation was primarily through the acyl migration pathway, resulting in sequential appearance of the 2-, 3- and 4- positional isomers. The time taken for maximal appearance of these isomers was 100 (\pm 17.32), 260 (\pm 34.6) and 960 (\pm 0) minutes, respectively. Together, over the 16 hour time course, these three positional isomers accounted for 87.75 (\pm 2.1) % of combined AG and aglycone exposure. Little back migration to the 1- α isomer or hydrolysis to the parent aglycone was detected throughout the experiment, accounting for only 2.21 (\pm 0.39) and 1.65 (\pm 0.28)% exposure in the incubation mixture over the 16 hour incubation respectively.

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Little change in the kinetic profile of the degradation of 2 mM diclofenac-AG was seen when HSA was added to the incubation mixture at 40 μ M (Figure 2. 6B). A slight increase (21.8%) in the degradation $t_{1/2}$ of the 1- β isomer was observed, however, this is likely to be attributable to assay variation rather than a real difference. Only a minor change in the % exposure of the degradation products was observed throughout the time course for any of the AG isomers or the parent aglycone, with less than 2% variation seen for the 1- α , 1- β , 3- isomers or diclofenac, and less than 5% variation for the 2- and 4- isomers.

A five-fold reduction in the concentration of 1- β diclofenac-AG incubated with 40 μ M HSA to 400 μ M, however, caused an increased level of hydrolysis (Figure 2. 6C). This effect resulted in the % exposure of diclofenac over the 16 hour time course to increase from 2.33 (\pm 0.61)% for the 2 mM incubation to 24.23 (\pm 1.28)%. Accompanying the increased extent of hydrolysis, acyl migration was reduced, resulting in a combined reduction in exposure to the 2-, 3- and 4- isomers by 19.1%. Little change in the maximal presence of the 2-isomer in the incubation mixture was seen, but the shorter time to reach this concentration (by 60 minutes when compared to the 2 mM AG incubation in HSA solution) suggested the rate of acyl migration from C-1 to C-2 may also be enhanced. However, evidence for an increased rate of further acyl migration to the 3- and 4- isomers is not clear. The rate of appearance of diclofenac shows a similar profile to the loss of the 1- β isomer, where little further appearance of the aglycone was detected after 180-300 minutes, when exposure to the 1- β isomer is reduced to below 10% presence in the incubation mixture. With exposure to the other positional isomers remaining considerable following this

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timepoint, this observation may suggest that hydrolysis catalysed, or at least accelerated by HSA is selective to the 1- β isomer.

Incubation of 2 mM 1- β diclofenac-AG with human plasma resulted in a faster rate of degradation of the conjugate, with its degradation $t_{1/2}$ reduced to 7 minutes. In human plasma, the principal mechanism of 1- β diclofenac-AG degradation was through hydrolysis, with exposure to diclofenac accounting for 54.56 (\pm 8.59) % of combined AG and aglycone exposure over the 16 hour time course of the assay. A reduction in the time taken for maximal exposure of the 2- isomer to 15 minutes, 85, 105 and 45 minutes shorter than incubation in phosphate buffer, or incubation in HSA solution at 2mM or 400 μ M, respectively, combined with a 57% increase in the maximal concentration of this isomer, suggests an increased rate of acyl migration from C-1 to C-2. The continuing rise of exposure to diclofenac following loss of the 1- β AG suggests in human plasma AG hydrolysis is not selective to that isomer.

Reducing the concentration of the 1- β AG isomer in the incubation mixture with human plasma to 400 μ M resulted in a further shortening of the degradation $t_{1/2}$ of the conjugate to 5.41 minutes. Hydrolysis was further enhanced, with 82.24 (\pm 1.26) % of AG/aglycone exposure during the 16 hour time course attributable to diclofenac. Reduction of the concentration of diclofenac-AG also appeared to further increase the rate of acyl migration, as maximal concentrations of the 2- and 3- isomers remained similar to those in the other incubations, but the time taken to reach these concentrations was shortened to 10 and 30 minutes, respectively. The % exposure of the combined 2-, 3- and 4- positional isomers was reduced to 16.68 (\pm 2.14) % exposure over the 16 hour incubation. From the incubation at 400 μ M, it is clear that hydrolysis of diclofenac-AG is not isomer specific, as the concentration of diclofenac

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increased consistently throughout the incubation until almost all the AG ($98.21 \pm 0.28\%$) was hydrolysed by the end of the experiment.

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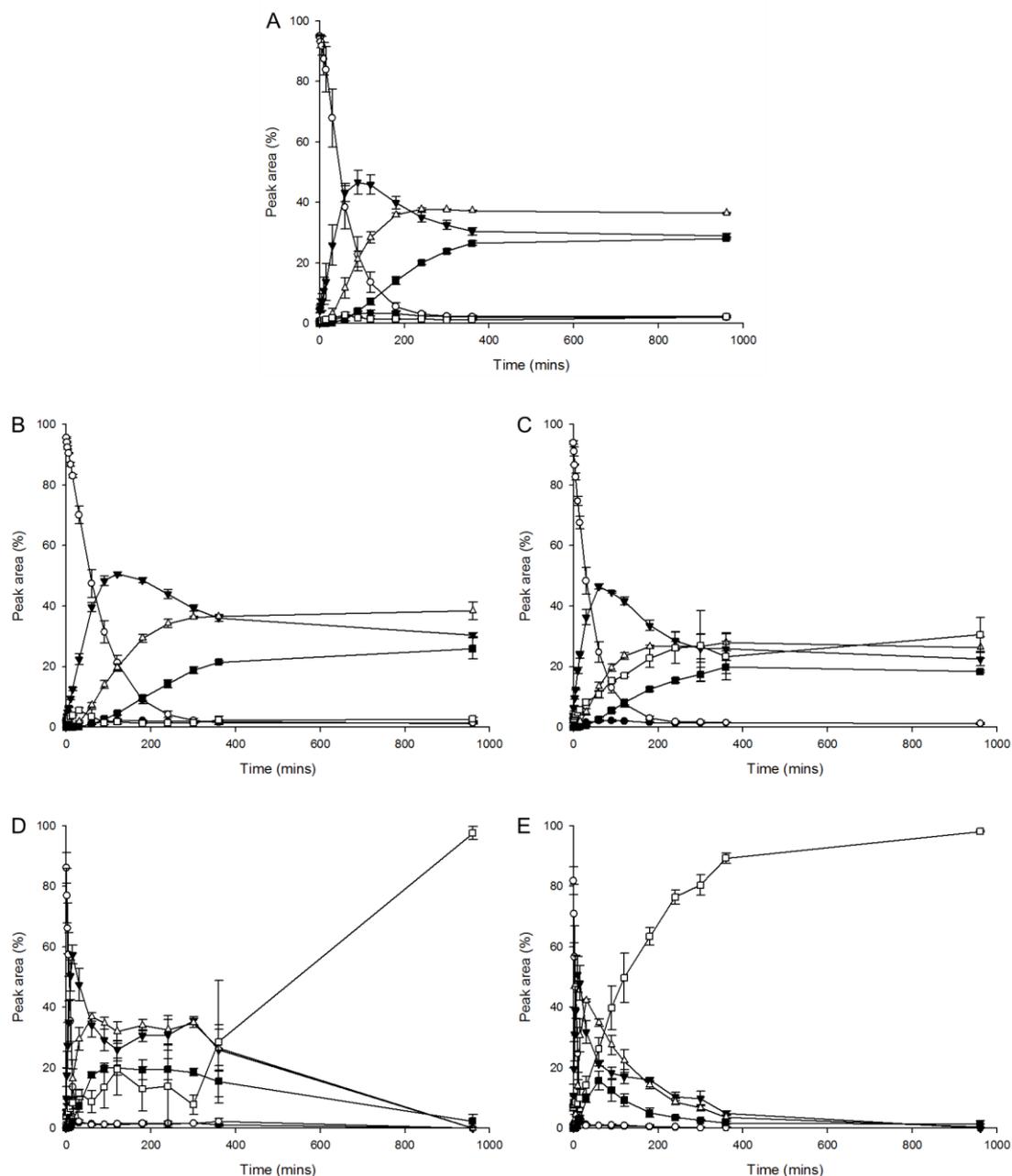


Figure 2. 6: Degradation of 1- β diclofenac-AG to its positional isomers, α anomer and parent aglycone during incubation in 0.1M phosphate buffer, pH 7.4, HSA solution or human plasma at 37°C. Synthetic 1- β diclofenac-AG was incubated for 16 hours in 0.1M phosphate buffer, at pH 7.4, 2mM (A), in buffered 40 μ M HSA solution at 2 mM (B) or 400 μ M (C), or pooled human plasma at 2 mM (D) or 400 μ M (E). Incubations were run in triplicate. Data are presented as means (\pm standard deviation; n=3). Diclofenac-AG isomers are represented as follows, 1- α is represented by filled circles, 1- β is represented by open circles, 2- is represented by filled triangles, 3- is represented by open triangles, 4- is represented by filled squares and diclofenac is represented by open squares.

Table 2. 3: Representation of % exposure of AG isomers and aglycone following incubation of the 1-β isomer in 0.1M phosphate buffer (pH 7.4), buffered HSA solution or pooled human plasma at 37°C over the 16 hour time course, calculated from AUCs (Figure 2. 6). Data presented as mean (± standard deviation; n=3).

	1-alpha	1-beta	2	3	4	Diclofenac
2mM DAG in phosphate buffer	2.21 (± 0.39)	8.39 (± 1.02)	32.07 (± 1.30)	33.64 (± 0.37)	22.04 (± 0.43)	1.65 (± 0.28)
2mM DAG in 40μM HSA	1.60 (± 0.09)	9.08 (± 0.89)	36.03 (± 0.28)	32.46 (± 1.04)	18.50 (± 1.00)	2.33 (± 0.61)
400μM DAG in 40μM HSA	1.38 (± 0.11)	5.74 (± 0.59)	27.47 (± 0.38)	25.13 (± 0.51)	16.05 (± 0.69)	24.23 (± 1.28)
2mM DAG in human plasma	0.65 (± 0.10)	1.75 (± 0.34)	15.14 (± 1.83)	14.55 (± 1.22)	13.34 (± 5.91)	54.56 (± 8.59)
400μM DAG in human plasma	0.23 (± 0.01)	0.86 (± 0.19)	6.38 (± 0.45)	7.16 (± 1.26)	3.14 (± 0.43)	82.24 (± 1.26)

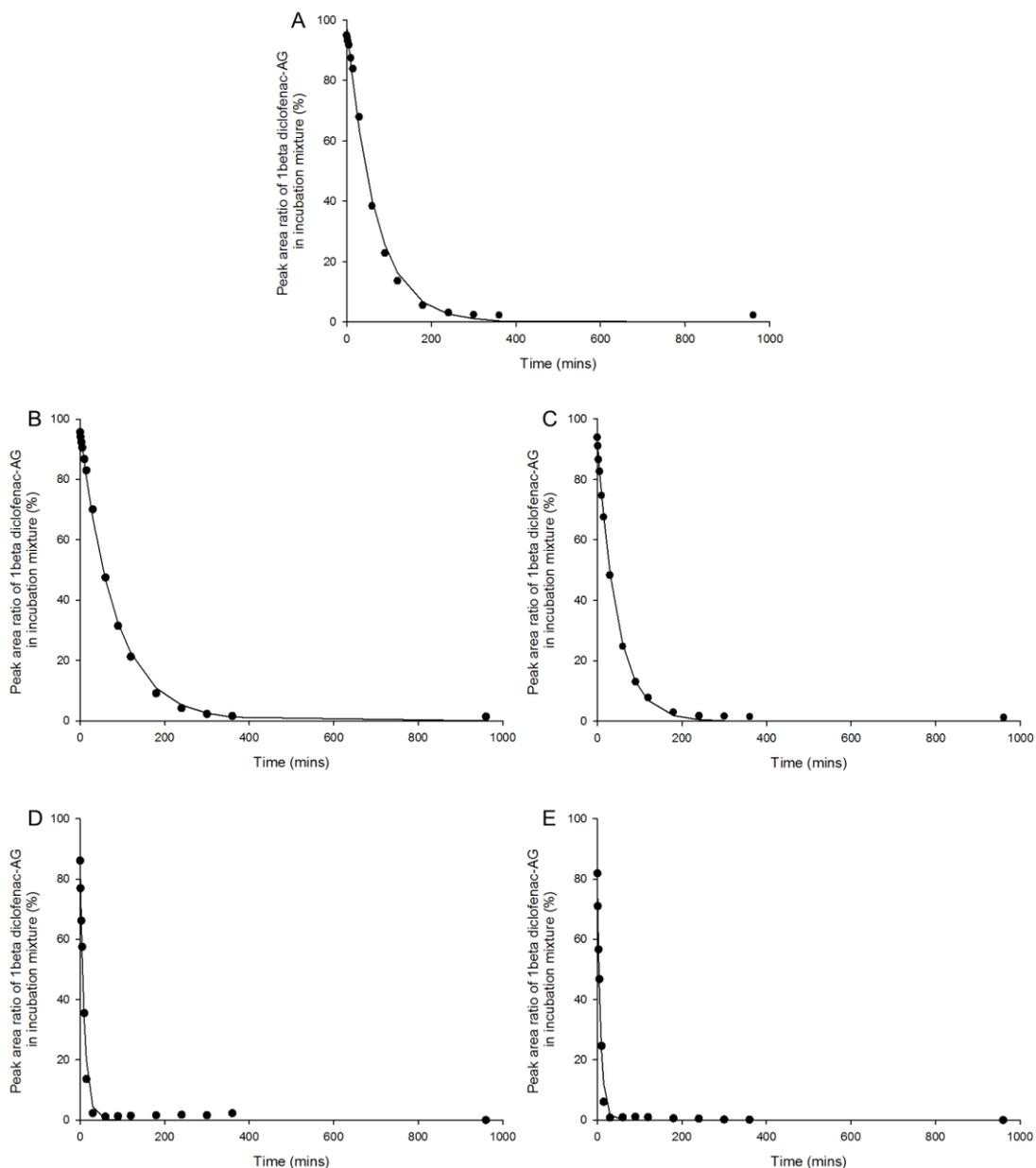


Figure 2. 7: 1st order regression curves fitted to the degradation of diclofenac-AG during *in vitro* incubations. 1st order regression curves fitted to mean 1-β diclofenac-AG degradation during incubation at 37°C in 0.1M phosphate buffer, pH 7.4, at 2mM (A), in 40 μM buffered HSA solution at 2mM (B) or 400μM (C), or in isolated human plasma at a concentration of 2mM (D) or 400μM (E). Regression curves are fitted to the equation $C = C_0 \exp^{-k_{deg}(time)}$. Mean 1-β diclofenac-AG degradation data are represented by black circles. Fitted regression curves are represented by the black line. Degradation rate constants are recorded in Table 2. 4.

Table 2. 4: Half-lives of degradation and parameters for the fitted degradation curves shown in Figure 2. 7. Data fitted to the first-order degradation rate equation $C=C_0\exp^{(-k_{deg})(time)}$.

	t ½ (hour)	t ½ (min)	K_{degradation}	R²
Phosphate buffer	0.78	46.52	0.0149	0.9953
2mM DAG in 40µM HSA	0.95	56.82	0.0122	0.9987
400µM DAG in 40µM HSA	0.53	31.94	0.0217	0.9994
2mM DAG in human plasma	0.12	7.02	0.0987	0.9933
400µM DAG in human plasma	0.09	5.41	0.1282	0.9953

2.3.2 Assessment of the covalent modification of human serum albumin by diclofenac acyl glucuronide during *in vitro* incubation using an alkaline hydrolysis method

Historically, alkaline hydrolysis has been the most commonly used technique in the analysis of acyl glucuronide modification of proteins (Smith et al., 1986; Hyneck et al., 1988b; Munafo et al., 1990; Dubois et al., 1993b; Presle et al., 1996). Consequently it was decided to apply this technique in the assessment of the covalent modification of HSA by diclofenac-AG.

To assess diclofenac-AG modification of HSA, a 750 µM and a 150 µM solution of synthetic diclofenac-AG was incubated with a 15 µM solution of HSA at pH 7.4. The molar ratios of AG : HSA were maintained consistent with the experiments investigating AG degradation in HSA solutions at 50 : 1 and 10 : 1.

Both the 750 µM and 150 µM incubations showed similar profiles for covalent modification of HSA during *in vitro* incubation at 37°C (Figure 2. 8). Over the first 6

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hours of incubation, an increase in modification of HSA was detected. Following this 6 hour time point, due to the variation in the assay, it is unclear whether additional bound diclofenac-AG and/or the aglycone is liberated from the adduct, or if binding has simply ceased, and no change in AG covalent binding between the 6 and 16 hour time points is evident.

To provide an assessment of the maximum % of diclofenac-AG in the incubation mixture covalently bound to HSA, the C_{max} of binding for each concentration (data not shown) was taken, and normalised to the amount of protein in the incubation mixture for both the 750 μ M and 150 μ M incubations (Table 2. 5). Total binding over the time course was represented by the area under the binding curve (AUC), and is recorded in Table 2. 5.

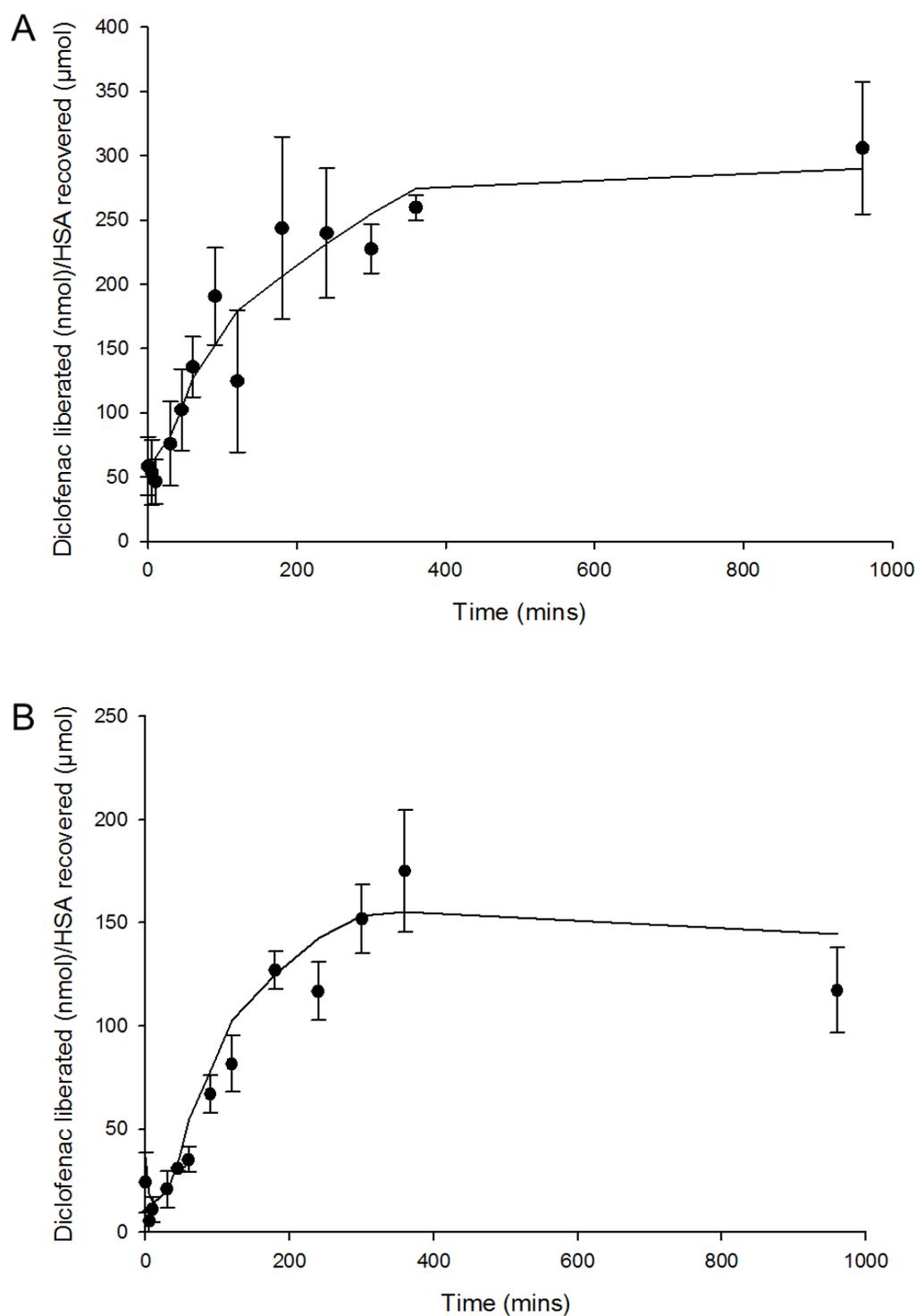


Figure 2. 8: Time course of the covalent modification of HSA during incubation with 1-β diclofenac-AG analysed by alkaline hydrolysis. A 750 μM (A) or 150 μM (B) concentration of diclofenac-AG was incubated with 15 μM HSA at pH7.4 and 37°C for 16 hours. Covalent binding was analysed using an alkaline hydrolysis method. Data presented as mean (± standard deviation; n=3).

Table 2. 5: Area-under-the-curve data of diclofenac-AG modification of HSA and maximum % of diclofenac-AG covalently bound to HSA during incubation of a 750 μ M of 150 μ M concentration of diclofenac-AG for 16 hours at 37°C. Data presented as mean (\pm standard deviation; n=3)

Concentration of diclofenac-AG incubated with 15 μ M HSA	AUC (ng hr ml ⁻¹)	Maximum % of diclofenac-AG covalently bound to HSA
750 μ M	238373.37 (\pm 17430.2)	0.62 (\pm 0.10)
150 μ M	124130.37 (\pm 15621.62)	1.78 (\pm 0.28)

2.3.3 Concentration-dependent modification of HSA during incubation with diclofenac acyl glucuronide

To investigate the covalent modification of HSA by diclofenac-AG, a concentration-effect experiment was used; incubating diclofenac-AG with 40 μ M HSA at concentrations ranging from 400nM to 2mM for 16 hours at pH 7.4 and 37°C. Normalised ion count data allowed some suggestions as to routes of adduct formation. Examples of mass spectra showing modification through the transacylation and glycation chemical pathway are shown in Figure 2. 9.

Detection of glycation adducts and absence of transacylation adducts at lower 400nM and 4 μ M concentrations suggested that glycation was the preferential chemical pathway of adduct formation over 16 hour incubations (Table 2. 6).

Modification of HSA by diclofenac-AG was found to be lysine specific (Table 2. 6). This is as would be expected, as most of the MRM transitions used for analysis were directed towards modification of lysine residues. Modification of only K190 by the glycation pathway was detected following incubation of diclofenac-AG at the lowest incubation concentration of 400nM. Incubation of diclofenac-AG at concentrations above 400nM resulted in the detected modification of several other lysine residues,

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and identification of transacylation adducts at concentrations above 40 μ M. At all of these concentrations, greater normalised ion counts are detected for modification of K190 than other amino acid, excepting for the detection of glycation adducts following incubation of diclofenac-AG at 40 μ M. At this concentration, normalised ion counts for modified K199 were greater. Nevertheless, as described above, care does have to be taken in the interpretation of normalised ion count.

A ribbon model of HSA was used to map the sites of covalent modification following incubation with 2mM diclofenac-AG (Figure 2. 10). The greatest number of modified lysine residues following incubation with 2mM diclofenac-AG – five of the 8 residues – were found on subdomains IIA and IIIA, and the cleft linking the two subdomains Table 2. 6. These subdomains contain hydrophobic pockets, Sudlow sites I and II, where compounds, including NSAIDs and their AGs (Iwakawa et al., 1990; Williams and Dickinson, 1994; Mizuma et al., 1999), often have strong non-covalent interactions (Ghuman et al., 2005). Only one site of AG modification was on subdomain IIIB, namely K525. A single modification was found on subdomain IIB at K137.

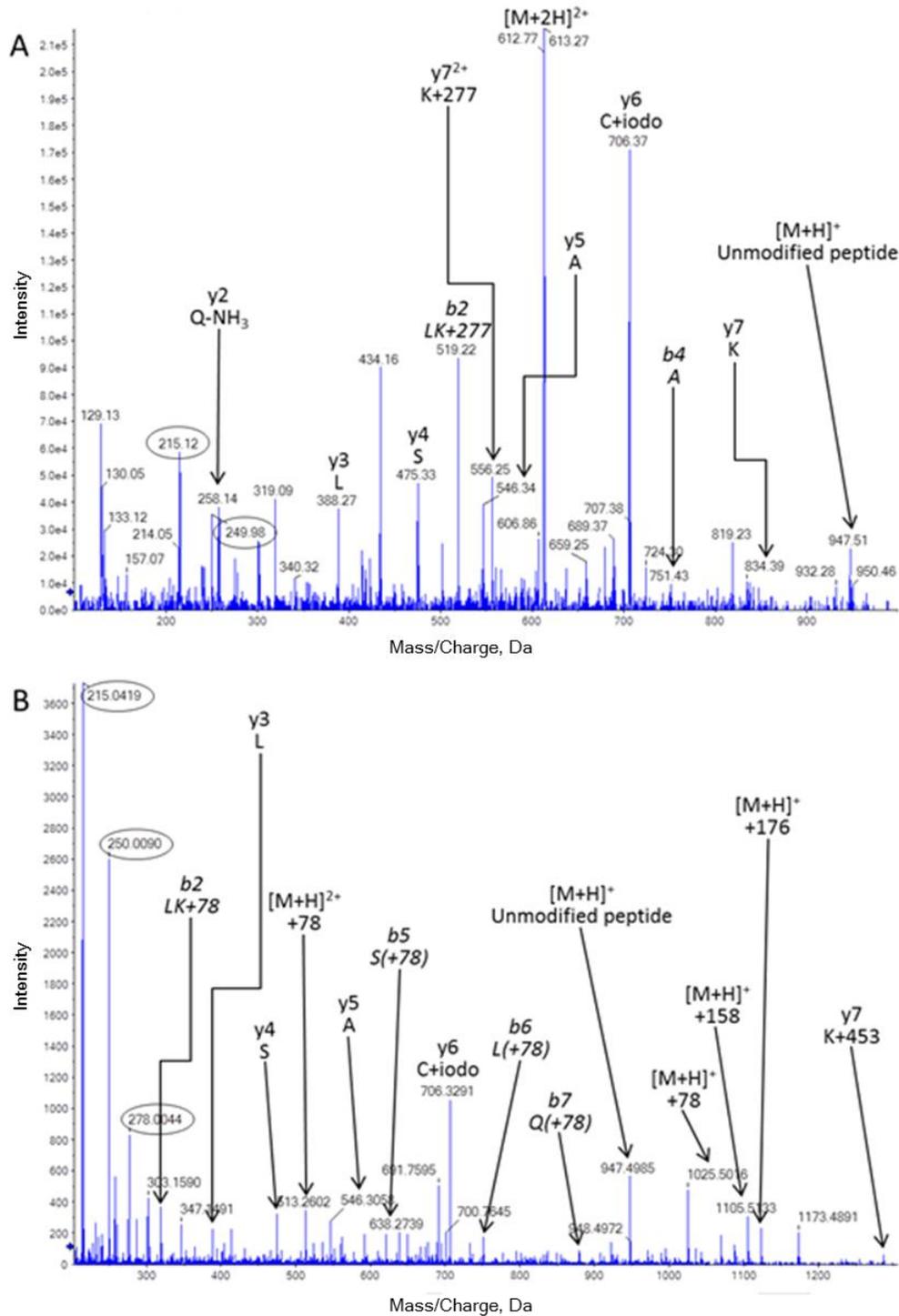


Figure 2. 9: Representative MS/MS spectra showing modification of the HSA tryptic peptide $^{198}\text{LKASLQK}^{205}$ at K199 by diclofenac-AG through transacylation (A) or glycation (B). A tryptic digest of modified, reduced and alkylated HSA was analysed by LC-MS/MS using a QTRAP 5500. Ions derived from the fragmentation of the hapten itself are circled (see Figure 2. 5). C+iodo= cysteine residue alkylated with iodoacetamide. Note, the modified and unmodified peptides were not resolved completely by LC. b ions are derived from the amino terminus of the peptide, and y ions are derived from the C-terminus.

Table 2. 6: Identification of lysine residues modified by diclofenac-AG and the chemical pathways of modification following incubation of the AG with HSA for 16 hours at 37°C. 40 μ M HSA solution was incubated with 1- β diclofenac-AG at pH 7.4. The amino acids modified and the chemical pathways of modification were identified by tandem mass spectrometry (Figure 2. 5 and Figure 2. 9). The adducted amino acid is marked with an asterisk. Glycation adducts are represented as G, transacylation adducts are represented as T.

Lysine	Tryptic peptide sequence	Concentration diclofenac-AG in incubation mixture				
		400nM	4 μ M	40 μ M	400 μ M	2mM
K137	K*YLEIAR				GT	GT
K190	LDELRDEGK*ASSAK	G	G	GT	GT	GT
K195	ASSAK*QR			GT	GT	GT
K199	LK*CASLQK		G	GT	GT	GT
K351	LAK*TYETTLEK			G	G	G
K432	NLGK*VGSK		G	G	GT	GT
K525	K*QTALVELVK			G	GT	GT
K541	ATK*EQLK			GT	GT	GT

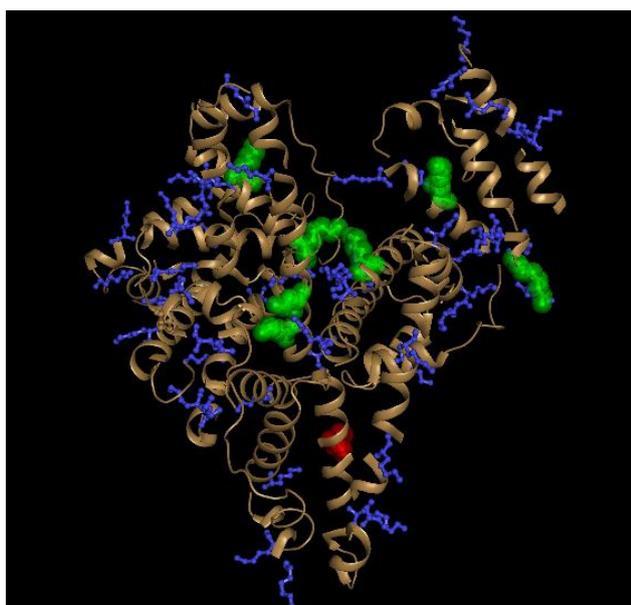


Figure 2. 10: A ribbon-model representation of the sites of modification of HSA during incubation with 2 mM diclofenac-AG. The eight lysine residues of HSA modified by 1- β diclofenac-AG were identified by tandem mass-spectrometric analysis of tryptic peptides (see Table 2. 6). The 51 unmodified lysine residues are represented by purple sticks, lysine residues modified by both transacylation and glycation pathways are represented by green spheres (see Figure 2. 2). K351 was modified by only the glycation pathway and is represented by red spheres. No lysine residues were modified by only the transacylation pathway. Image was produced using PyMol Molecular Graphics System (www.pymol.org)

2.3.4 Time-course of modification of HSA during incubation with diclofenac acyl glucuronide.

To allow further insight into the covalent interactions between diclofenac-AG and HSA a time-course experiment was run, incubating 2mM diclofenac-AG with 40 μ M HSA. Tryptic peptides were again analysed using mass spectrometry, allowing the earliest time point of detectable modification of each peptide to be estimated and the chemical route of formation to be ascertained. Normalised ion counts allowed semi-quantitative analysis of modification of the chemical pathways of adduct formation at individual lysine residues.

Following incubation with 2mM diclofenac-AG, all amino acids identified as modified at 16 hours were modified at the first 0.5 hour time point. Again, modification was found to be lysine selective. Normalised ion count data were used to provide a semi-quantitative analysis of the chemical route of modification of individual lysine residues. Over the first two hours, normalised ion counts of transacylation adducts were higher than those of glycation adducts for six of the eight modified lysine residues (HSA contains 59 lysine residues). Following this 2 hour time point, little further increase in transacylation adducts was detected, whereas ion counts for glycation adducts were greatly enhanced. These trends are illustrated by Figure 2. 11, showing normalised ion counts for two representative modified amino acid residues, namely K190 and K525. Whilst the time courses of modification of K199 and K351 did not follow the same profile, they differed substantially only in the fact that counts for transacylation adducts were higher than those of glycation adducts of K525 during the first three hours. Again, little increase in the detection of transacylation adducts was observed following two hours, whereas the intensity of glycation adducts increased.

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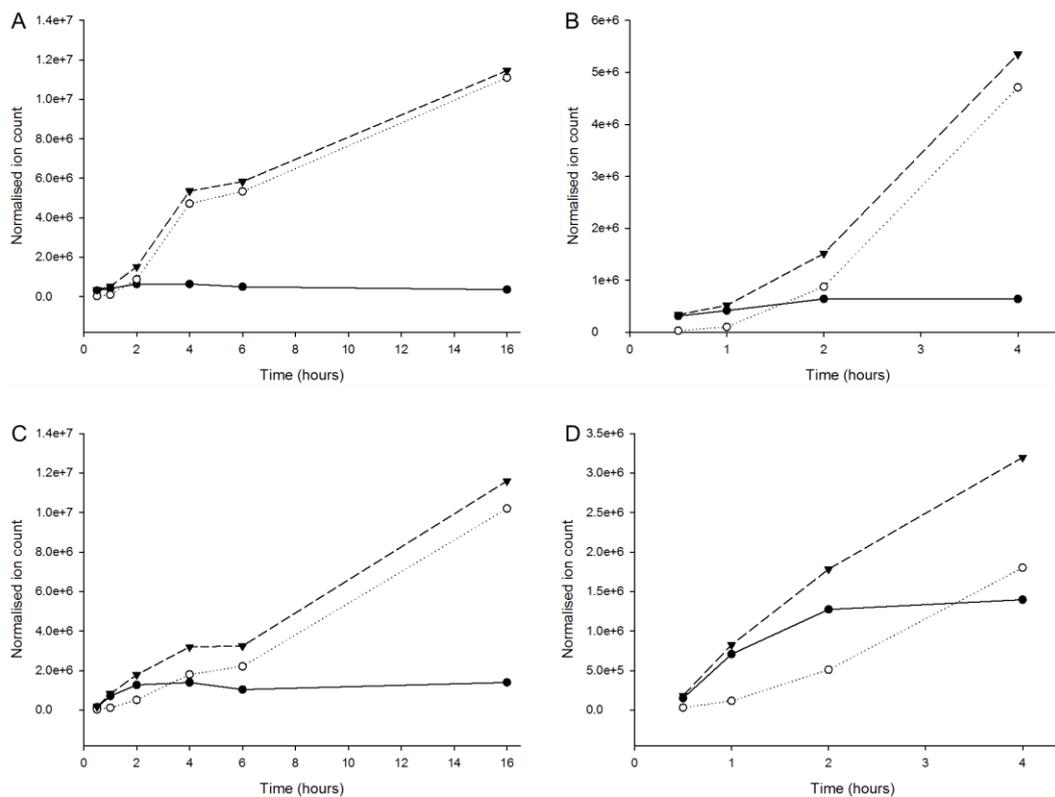


Figure 2. 11: Time courses of modification of K190 (A + B) and K525 (C + D) during incubation of HSA (40 μM) with diclofenac-AG (2 mM) at pH 7.4 and 37°C. Incubations over 16 hours (A + C) and the same incubations over the first 4 hours (B+D) are depicted. The total ion count for each sample of tryptic peptides analysed by LC-MS/MS was normalised to that of the HSA conjugate produced by incubation of 2 mM diclofenac-AG with 40μM HSA or 16 hours. Hollow circles represent glycation adducts, filled circles represent transacylation adducts and filled triangles represent the sum of transacylation and glycation adducts.

2.3.5 Covalent modification of HSA during incubation with 1- β diclofenac acyl glucuronide or a mixture of diclofenac acyl glucuronide isomers produced through pre-degradation of 1- β diclofenac acyl glucuronide

Following on from the observation that formation of covalent HSA adducts by the transacylation pathway did not increase after the first two hours of incubation with 1- β diclofenac-AG, with glycation apparently predominant thereafter, it was hypothesised that the transacylation adducts were formed preferentially by the 1- β diclofenac-AG isomer, whilst the esters generated by acyl migration were less productive. The reason for this is that at the two hour time point, following incubation of the same 2 mM concentration of diclofenac-AG with HSA, the 1- β isomer had degraded to only 21.2 (± 2.53)% abundance, with abundance of the 2-, 3- and 4- isomers representing 50.59 (± 0.63), 19.69 (± 1.20), 4.56 (± 0.58)%, respectively.

To test this hypothesis, 1- β diclofenac-AG was pre-incubated at 37°C in phosphate buffer, pH 7.4, for three hours before incubation with 40 μ M HSA for 16 hours at increasing concentrations of diclofenac-AG to HSA. Previous experiments showed pre-incubation of diclofenac-AG in phosphate buffer for three hours would degrade the 1- β AG isomer to approximately 9.07 \pm 1.39 % presence in the incubation mixture, with resulting formation of the 2-, 3- and 4- isomers to the extents of 48.6 (± 0.85), 29.4 (± 1.28) and 9.57 (± 0.85)% respectively. In this incubation only negligible degradation of the AG to the aglycone was detected, and the same observation was made when the 1- β AG was incubated at 2mM with HSA (Figure 2. 6). Also, no back migration to the 1- β isomer, requiring improbable sequential $\beta \rightarrow \alpha$ anomerization, C2 \rightarrow C1 migration and $\alpha \rightarrow \beta$ anomerization (Corcoran et al., 2001), was detected.

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Analysis of modification at individual lysine residues using normalised ion counts revealed evidence of the loss of transacylation adducts following removal of the 1- β isomer from the reaction mixture (Figure 2. 12). Complete loss of transacylation adducts was detected at K195 and 199 following incubation with 40 μ M pre-degraded AG. Further to this, following incubation at 400 μ M, loss of the 1- β isomer resulted in complete absence of transacylation adducts at K199, a 27.9% reduction at K195 and a reduction of between 53.6 and 62.1 % at other modified lysines. This trend continued at the 2mM AG concentration, with a complete loss in transacylation adducts at K199, a 30.2% loss at K195 and between a 63.1 and 81.5% loss of transacylation adducts at other modified peptides. Whilst differential ionisation of peptides means that normalised ion counts should be analysed sceptically, this comparison of the loss of transacylation adducts at amino acid residues negates the concern over differential ionisation, as it is a direct comparison of the same peptide, resulting in equal ionisation.

Removal of the 1- β AG isomer, as would be expected from the first principles of AG chemistry (Figure 2. 2), and in the absence of substantial AG hydrolysis (Figure 2. 6), had little effect on the abundance of glycation adducts detected.

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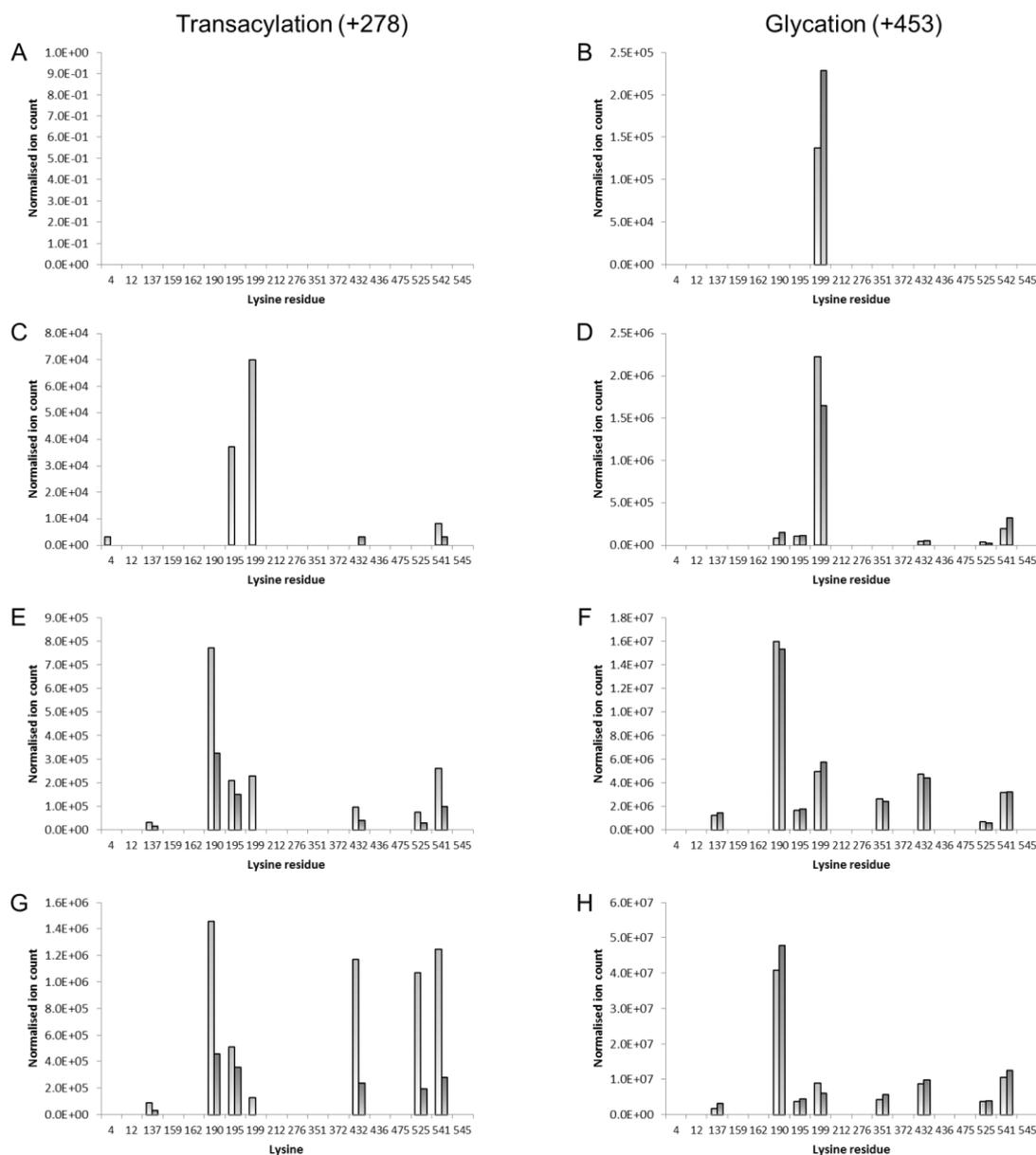


Figure 2. 12: Effect of removing the 1-β diclofenac-AG by pre-incubation on the modification of individual HSA lysine residues. Light bars represent incubation with 1-β diclofenac-AG. Dark bars represent incubation with diclofenac-AG pre-degraded in phosphate buffer, pH 7.4, at 37°C for three hours to remove the 1β isomer from the incubation mixture. (A+B) 4μM, (C+D) 40 μM, (E+F) 400 μM, (G+H) 2mM. (A+C+E+G) HSA modified by transacylation; (B+D+F+H) HSA modified by glycation. All the incubations with HSA (40μM) were performed at pH 7.4 and 37 OC for 16 hours. The total ion count for each sample of tryptic peptides analysed by LC-MS/MS was normalised to that of the HSA conjugate produced by incubation of 2mM diclofenac-AG with 40μM HSA for 16 hours.

2.4 DISCUSSION

The broad aims of this work were to define the chemical stability and protein reactivity of diclofenac-AG, and specifically the chemistry of its instability in different biochemical and biological matrices and its covalent interaction with protein, using HSA as a representative, well characterised, soluble protein that has numerous nucleophilic side chains (Kristiansson et al., 2003). Anticipating related studies in diclofenac patients (Chapter 5), HSA has the practical merits of being accessible and abundant (Johannesson et al., 2001; Jenkins et al., 2009; Meng et al., 2011; Whitaker et al., 2011) and is a physiologically relevant target for covalent modification because drug AGs frequently circulate in blood plasma (Volland et al., 1990; Benet et al., 1993; Mayer et al., 1993; Zia-Amirhosseini et al., 1994; Stangier et al., 2000; Zhou et al., 2001; Wang et al., 2006).

The degradation half-life of the 1- β AG isomer in phosphate buffer, pH 7.4, has been shown to be a good identifier for the protein reactivity of the molecule (Spahn-Langguth and Benet, 1992; Benet et al., 1993), as well as a useful marker of potential clinical toxicity of the parent drug (Sawamura et al., 2010). The degradation half-life of diclofenac-AG estimated in current investigations of 0.78 hours, shows the molecule to be amongst the most unstable AG metabolites studied (Table 2. 1), consequently suggesting that, if developed as a new chemical entity today, diclofenac might be considered to be at risk for generation of idiosyncratic reactions in man.

This degradation half-life of 1- β diclofenac-AG in phosphate buffer is slightly longer than the previously reported value of 0.51 hours (Ebner et al., 1999), but similar to that reported by Sawamura *et al* of 0.7 hours (Sawamura et al., 2010). Due to this expected rapid degradation of diclofenac-AG at pH 7.4, a greater number of sampling

time points (10 time points) were included over the first two hours of incubation, allowing more accurate tracking of the disappearance of the 1- β isomer from the degradation mixture (rather than the four sampling points used by (Ebner et al., 1999)). In addition to allowing better tracking of the loss of the 1- β isomer from the degradation mixture, may allow increased accuracy in fitting of regression curves to the data, resulting in improved precision in estimations of rate-constants of degradation and degradation half-lives.

Acyl migration was revealed to be the predominant pathway of diclofenac-AG degradation during incubation in 0.1M phosphate buffer, pH 7.4. Most AGs undergo preferential acyl migration, rather than hydrolysis, under these conditions (Watt and Dickinson, 1990; Iwaki et al., 1999; Corcoran et al., 2001; Akira et al., 2002; Berry et al., 2009; Johnson et al., 2010; Karlsson et al., 2010). Following the loss of the 1- β isomer from the incubation mixture, a sequential appearance of the positional isomers was detected chromatographically. The concentrations of these positional isomers appeared to reach consistent values towards the later time points of the incubation. This step-wise pattern of acyl migration has been reported for several other AGs (Spahn-Langguth and Benet, 1992; Ebner et al., 1999; Iwaki et al., 1999). It is generally accepted that the reason for this pattern of acyl migration is that the aglycone is only able to move around the ring one carbon at a time via ortho-ester transition states (Figure 2. 1) (Bradow et al., 1989; Berry et al., 2009). All of these intramolecular migrations are considered to be reversible, though the C2 \rightarrow C1 back migration is thought to require a preliminary $\beta\rightarrow\alpha$ anomerization (Corcoran et al., 2001).

Little hydrolysis of the AG occurred during incubation in phosphate buffer. This conflicts with the previous report of the kinetics of degradation of diclofenac-AG in

phosphate buffer, where, following 8 hours incubation, 20% of diclofenac-AG had hydrolysed to its parent aglycone (Ebner et al., 1999). Reasons for this discrepancy between these data are unclear. Whilst pH and temperature are well established to influence the rate of AG degradation (Spahn-Langguth and Benet, 1992; Stachulski et al., 2006), both experiments ensured these factors remained constant. Whilst the concentration of diclofenac-AG was greater in the experiment presented here (2 mM), rather than the 300 μ M incubations used by (Ebner et al., 1999), AG concentration is not known to influence the rate of degradation significantly (Watt and Dickinson, 1990; Berry et al., 2009); in conformity with the predominant degradation pathway being an intramolecular rearrangement. Differences in the source of conjugate used here and by (Ebner et al., 1999) may provide an explanation as the diclofenac-AG used by Ebner was isolated from bile of rats administered diclofenac, where slight contamination by biliary proteins or lipids undetected through their LC-MS analysis might have reduced the AG's stability.

Addition of 40 μ M HSA solution to the incubation of 2mM diclofenac-AG in 0.1M phosphate buffer, pH 7.4 had little effect on the kinetics of the AG's degradation. A slight increase in the half-life of degradation was observed, however this was only by 10 minutes, and variation in the precision of the assay cannot be discounted as a reason for this increased half-life. It has been suggested that HSA can retard acyl migration *in vitro*, at least beyond the C-2 isomer in the case of naproxen-AG (Iwaki et al., 1999). Serum albumin (human or rat) stabilises, additionally, the AG metabolites of diflunisal (Watt and Dickinson, 1990), tolmetin (Munafo et al., 1990), salicylic acid (Dickinson et al., 1994) and furosemide (Mizuma et al., 1999). Reduction of conjugate degradation in these experiments was far more profound than any effect of HSA on

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the rearrangement of 2mM diclofenac-AG. Also, HSA seems to accelerate both the intramolecular rearrangement and hydrolysis of 1- β gemfibrozil-AG (Sallustio et al., 1997). With the concentration of AG in this experiment also far exceeding the maximum exposure of AG relative to HSA in any of the earlier experiments, it is possible that sites on HSA responsible for hydrolysing the AG may be saturated; resulting in diclofenac-AG degradation remaining principally a consequence of acyl migration. Drug AGs are highly reversibly bound by HSA (Dubois et al., 1994; Williams and Dickinson, 1994; Sallustio et al., 1997; Mizuma et al., 1999), principally because of strong interactions between the aglycone residue and the protein (Dubois et al., 1994); generally, the glucuronic acid residue lowers the drug's binding affinity (Iwakawa et al., 1990).

Hydrolysis remained a minor pathway of diclofenac-AG degradation when the conjugate was incubated at 2mM with HSA. Only on incubation of a lower concentration of diclofenac-AG (400 μ M) was pronounced hydrolysis of the AG metabolite observed. The esterase-like activity of HSA (Ma et al., 2005), accelerating hydrolysis of AG *in vitro* is well known, with greater hydrolysis of the AGs of furosemide (Mizuma et al., 1999), naproxen (Iwaki et al., 1999), probenecid (Akira et al., 2002), gemfibrozil (Sallustio et al., 1997), and diflunisal (Watt and Dickinson, 1990) in HSA solution than in phosphate buffer alone. This hydrolytic activity of HSA, appropriately for an enzymic activity, can be highly dependent on the AG's structure, with variations in the esterase-like activity of HSA between the R- and S- enantiomers of carprofen (Georges et al., 2000) and ketoprofen (Dubois et al., 1994).

Few experiments have looked in depth at the effect of AG concentration on the esterase-like hydrolytic action of HSA. Concentration-activity studies using

ketoprofen-AG reported the action of HSA follows Michaelis-Menton kinetics, and consequently in accordance with this, increased AG concentration should result in increased rate of hydrolysis (Dubois et al., 1994). Conversely to this, little difference was observed between the hydrolysis of diflunisal-AG during incubation with HSA at 117 μ M and 23 μ M (Watt and Dickinson, 1990). Only on incubation at the lower concentration of 400 μ M was hydrolysis of diclofenac-AG evident, with only negligible hydrolysis detected at 2mM. Potentially, incubation of 5-fold the molar concentration of diclofenac-AG compared to HSA is likely to result in saturation of active sites of the HSA molecule, and consequently most of the AG metabolite is free to degrade following kinetics similar to incubation in the absence of HSA. Reducing the concentration of diclofenac-AG five-fold means a greater proportion is able to bind to hydrolytic sites of HSA, and less AG is free to degrade by acyl migration, resulting in a greater proportion of the AG hydrolysed at this concentration (Dubois-Presle et al., 1995). Hydrolysis is increased if HSA is both globulin- and fatty acid-free rather than just fatty-acid free (Watt and Dickinson, 1990). This is thought to be a consequence of globulin's hindrance of AG access to sites of the HSA molecule capable of hydrolysing the metabolite. It should be noted that even commercially produced HSA preparations contain very large numbers of minor/trace co-purified peptides and full-length proteins, and some of the proteins are associated with, or have an affinity for, HSA (Gay et al., 2010).

The hydrolysis of diclofenac-AG during incubation with HSA follows similar kinetics to those described for several other AGs (Watt and Dickinson, 1990; Dickinson and King, 1991; Dubois et al., 1994). Following an initial rise in liberated aglycone, rate of hydrolysis slows and often eventually stops before the end of the incubation, resulting

in a plateau in the concentration of liberated aglycone. Careful investigation of the diclofenac-AG degradation data reveals that little further AG hydrolysis occurs following the three hour time point. At this time point, the concentration of the 1- β isomer in the incubation mixture is negligible. The 2-, 3- and 4- diclofenac-AG positional isomers remaining at relatively high exposure for the remaining 13 hours of the experiment with little further hydrolysis suggests that esterase-like action of HSA towards diclofenac-AG may be selective to the 1- β isomer. Selective hydrolysis of the 1- β isomer of AGs is well documented for the specialist ester hydrolase β -glucuronidase (Dickinson, 2011), and the incubation of isolated 1- and 2- isomers of naproxen-AG with HSA revealed a near doubling of the hydrolysis rate constant of the 1- isomer when compared to the 2- isomer (Iwaki et al., 1999). Other published studies showing a similar pattern of attenuated hydrolysis following loss of the 1- β isomer have not been found. It must not be ruled out that covalent modification of HSA may result in inhibition of the protein's esterase-like activity (Lockridge et al., 2008).

Further increased hydrolysis of diclofenac-AG was evident following incubation in pooled human plasma. This resulted in complete hydrolysis over the 16 hour time course at both 400 μ M and 2mM diclofenac-AG. Extensive hydrolysis of AG metabolites *ex vivo* by unidentified hydrolases/esterases has been reported for several conjugates (Ruelius et al., 1986; Volland et al., 1991; Williams et al., 1992; Akira et al., 2002; Shipkova et al., 2003; Karlsson et al., 2010), and has been hypothesised as a potential de-toxification pathway of AG metabolites (Mizuma et al., 1999; Zhang et al., 2011). The relative contributions of tissue and plasma hydrolases/esterases to the clearance of drug AG from the plasma and recycling of parent compound are still

largely unknown but the plasma concentration of valproic acid is determined substantially by the glucuronidase activity of a hepatic hydrolase (Nakajima et al., 2004; Suzuki et al., 2010). Human plasma contains three esterases in high enough concentrations to significantly contribute to ester hydrolysis: butyrylcholinesterase, paraoxonase and albumin (Li et al., 2005). However the relative contributions of these enzymes and of plasma β -glucuronidase (Soltaninejad et al., 2007) to the hydrolysis of drug AG are somewhat obscure. Additionally, there are substantial species differences between esterase expression and hydrolase activity in mammalian plasma (Bahar et al., 2012). This uncertainty over the influences of β -glucuronidase and esterases on the pharmacokinetics of AGs has led to the development of animal models where non-specific esterase inhibitors have been used to perturb the disposition of AGs (Smith et al., 1990b). Although treatment of guinea pigs with phenylmethylsulphonyl fluoride (PMSF) increased the plasma exposure of zomepirac-AG, this effect might have resulted principally from inhibition of tissue esterases and other intracellular hydrolases rather than the inhibition of plasma enzymes. These esterases and hydrolases, and not β -glucuronidase, are known to play a dominant role in the intracellular hydrolysis of certain drug AG in humans (Suzuki et al., 2010; Iwamura et al., 2012). The β -glucuronidase in a solid tissue can have an appreciable influence on aglycone exposure/elimination (Whiting et al., 1993; Tobin et al., 2006) but overall remarkably little is known about the enzymology, regulation and physiological effects of AG hydrolysis (Fukami and Yokoi, 2012).

It was observed that on reduction of the concentration of diclofenac-AG incubated with human plasma from 2mM to 400 μ M an increased rate of hydrolysis was again obtained. This may be through the same mechanism of enzyme saturation suggested

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for HSA incubations. The esterase activity of human plasma was clearly not specific to the 1- β isomer, as following loss of the 1- β isomer, hydrolysis of the positional isomers continued. An apparent parallel decline of the 2-, 3- and 4- isomer exposure emphasises this susceptibility, as no isomer appears to be resistant to hydrolysis.

Incubation of diclofenac-AG in human plasma also appeared to enhance the rate of acyl migration. This was revealed by maximal exposure of the 2-, 3- and 4- isomers remaining either similar to or above those measured in either phosphate buffer or HSA solution, whereas the time taken to reach those concentrations was reduced. This phenomenon has also been seen for incubation of oxaprozin-AG and fenpropfen-AG (Ruelius et al., 1986; Volland et al., 1991).

Following *in vitro* incubation of diclofenac-AG with HSA, an alkaline hydrolysis technique was used to assess covalent binding of the AG. This technique allows quantitative assessment of AG-mediated protein modification through measurement of liberated aglycone, and thereby assays acetylation and glycation adducts collectively (Smith et al., 1990a). It has been used in numerous *in vitro* and *in vivo* studies (Smith et al., 1986; Hyneck et al., 1988b; Munafo et al., 1990; Dubois et al., 1993b; Mayer et al., 1993; Castillo et al., 1995; McGurk et al., 1996; Presle et al., 1996; Sallustio et al., 1997) but invariably yields only approximate values because the fractional recovery of covalently bound carboxylic acid is unknown. Both the 150 and 750 μ M incubations followed a similar kinetic pattern, whereby a rise in covalent modification of HSA was observed over early time points of the incubation. However, between the 6 and 16 hour time points, it is clear that this rise in modification had ceased. It is, however, unclear whether following the six hour time point there is no change in modification, or if this is decreased. This trend follows the kinetics of several other *in vitro*

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incubations of differing AGs with HSA (Smith et al., 1986; Munafo et al., 1990; Watt and Dickinson, 1990; Mayer et al., 1993; Dubois et al., 1994; Castillo et al., 1995; McGurk et al., 1996; Sallustio et al., 1997). Despite these incubations being run for 24-70 hours, few of them appear to clearly show a definitive drop in the amount of covalent binding over the time course. Only the incubation with the AG of mefenamic acid showed evidence of definitive reduction in covalent binding over its 24 hour course (McGurk et al., 1996), with a suggestion of a reduction from the incubation with ketoprofen-AG (Presle et al., 1996). It is difficult to determine whether a decrease in binding is observed between the later time points because of the imprecision in the assay. Changing the concentration of AG in the incubation mixture did not appear to elicit a change in the profile of adduct formation, as was previously seen with ketoprofen-AG (Dubois et al., 1994; Presle et al., 1996).

It is clear, however, that the rate of covalent modification of HSA by diclofenac-AG does slow at later time points. Slowing of modification is at a lower level of modification for the 150 μ M than the 750 μ M incubation. This suggests a factor other than saturation of binding sites is responsible.

The maximum % of AG covalently bound following incubation with HSA under standard conditions has been suggested as a quantity for ranking the protein reactivity of AG metabolites *in vitro* (Castillo et al., 1995). The published values are 1.5% for ibuprofen (Castillo et al., 1995), 2.3% for zomepirac (Smith et al., 1990a), 3.2% for ketoprofen (Dubois et al., 1993b) and 3.3% for etodolac (Smith et al., 1992). The calculated values for diclofenac of 1.78 (\pm 0.28)% in the 150 μ M HSA incubation and 0.62 (\pm 0.10)% in the 750 μ M incubation (Table 2. 5) place diclofenac-AG as the least reactive AG on this list. Having a shorter half-life of degradation than ibuprofen-AG, it would be expected

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that diclofenac-AG would be more protein reactive (Benet et al., 1993; Ebner et al., 1999; Stachulski et al., 2006). With different % binding found following incubation with the two concentrations of diclofenac-AG, as has also been seen with studies with ketoprofen-AG (Dubois et al., 1993a), this is evidently not a sound method for assessing protein reactivity, as the result depends heavily on the concentration of AG metabolite rather than just the intrinsic reactivity of the metabolite. Confirmation of this method's severe limitations is provided by the *in vitro* screening model of (Bolze et al., 2002), which yielded an excellent correlation between the extent of AG covalent binding to HSA and the aglycone appearance constant weighted by the percentage of isomerisation: diclofenac-AG was found to be much more protein reactive than ketoprofen-AG and ibuprofen-AG.

Tandem mass-spectrometric analysis of the modification of purified serum albumin by AGs has only previously been reported for incubations of HSA with tolmetin-AG (Ding et al., 1993; Ding et al., 1995) and benoxaprofen-AG (Qiu et al., 1998). However, the modification of HSA *in vitro* has been characterised in comparable molecular detail using other compounds (Kristiansson et al., 2003; Olsen et al., 2003; Alvarez-Sanchez et al., 2004; Aldini et al., 2006; Aleksic et al., 2007; Lockridge et al., 2008; Grigoryan et al., 2009; Jenkins et al., 2009; Frolov and Hoffmann, 2010; Meng et al., 2011; Whitaker et al., 2011; Deng et al., 2012). By combining tryptic peptide HPLC, MRM survey scanning and product ion scanning, this technique enables identification of the site and structure of the modification and hence the chemical route of modification. Because of its great abundance and long residence time in human blood (Tornqvist et al., 2002), HSA is seen as having an important place in the assessment of exposure to exogenous and endogenous electrophiles through the methodology of blood adductomics: the

characterisation and measurement of adducts formed by reactions between circulating electrophiles – such as drug AG – and blood nucleophiles (Rappaport et al., 2012).

Diclofenac-AG was found to selectively modify lysine residues of HSA *in vitro* at pH 7.4: adducts were identified on only eight of the protein's 59 lysines even when the AG was in 50-fold molar excess. Whilst the selective modification of several HSA lysine residues by AG *in vitro* has been reported previously, modifications of small numbers of serine and arginine residues by AG have also been reported (Ding et al., 1995; Qiu et al., 1998). The highest proportion of lysine modifications on HSA was shown to occur on the central part of the molecule, on subdomains IIA and IIIA. These subdomains contain the hydrophobic pockets Sudlow sites I and II (located on IIA and IIIA, respectively). Modifications on K195, K199 (site IIA) and K432 and 436 (site IIIA) were detected in these regions. Additionally, two adducted lysines, namely K190 and K351, were located in a cleft between these two subdomains, and outside of the Sudlow sites (Sudlow et al., 1975). A third subdomain on the HSA molecule, IIIB, contained the modified amino acids K525 and K541 (Ghuman et al., 2005). The high proportion of adducts located in or around these pockets suggest that hydrophobic non-covalent interactions may be an important pre-requisite for covalent modification.

The first indications of the importance of hydrophobic binding pockets for covalent modification of HSA by AGs came from the use of compounds known for their high-affinity non-covalent binding at these sites. For example, the covalent binding of diflunisal AG to HSA was decreased in the presence of diazepam (a Site II ligand) and diflunisal but increased in the presence of warfarin (a Site I ligand) (Williams and Dickinson, 1994); which suggests non-covalent binding of the conjugate at Site I is relatively unproductive of covalent adducts. Other experiments with site-selective

ligands also appeared to differentiate between low and high productivity non-covalent binding of NSAID AGs. Thus warfarin reduced the non-covalent binding of (R)- and (S)-carprofen AGs to a greater extent than did diazepam (Iwakawa et al., 1990); whereas diazepam displaced the unconjugated enantiomers to a greater extent than did warfarin (Iwakawa et al., 1990). It seems reversible binding of an NSAID and covalent binding of its AG can occur selectively at Site II while reversible binding of the AG occurs selectively at Site I. However, pre-incubation of HSA with diazepam, warfarin or tolmetin which did not significantly alter covalent binding of tolmetin-AG (Munafo et al., 1990). Although diclofenac binds preferentially at Site II (Rahman et al., 1993) – in common with many carboxylate NSAIDs – its binding is displaced by ibuprofen, a Site II specific drug (Yamasaki et al., 2000).

With the highest normalised ion counts for both transacylation and glycation adducts, K190 appears to be the most favourable lysine residue for modification by diclofenac-AG (Figure 2. 12). The location of K190 is in a cleft just outside Sudlow site I. K190 was adducted detectably by benoxaprofen-AG (Qiu et al., 1998) but not by tolmetin-AG (Ding et al., 1993; Ding et al., 1995). It is also adducted by a number of non-AG acetylating agents (Kristiansson et al., 2003; Jenkins et al., 2009; Meng et al., 2011; Whitaker et al., 2011), but not by several glycating (Aldini et al., 2006; Frolov and Hoffmann, 2010; Deng et al., 2012) or acylating (Olsen et al., 2003) agents. Apparent preferential binding of benoxaprofen-AG to K159 (Qiu et al., 1998) and tolmetin-AG to K199 (Ding et al., 1995) suggests that the preferential modification of lysine residues may differ between AGs. Whilst the UV absorbance and fluorescence of modified peptides was used for characterising the adduction selectivity tolmetin-AG and benoxaprofen-AG, respectively, differences between the ionisation and transmission

efficiencies of peptides mean that a greater mass spectral signal may not accurately reflect the abundance of a modified peptide.

Normalised ion count data derived from time course incubation of 2mM diclofenac-AG with HSA *in vitro* suggested the transacylation adducts were formed preferentially during the first two hours, whereas after this time point little further increase in transacylation adducts was detected, but a sustained increase in the formation of glycation adducts was apparent (Figure 2. 11). Comparing these data to the degradation of 2mM diclofenac-AG in HSA solution suggested that the 1- β isomer of diclofenac-AG may be responsible for early formation of transacylation adducts, with slower acyl migration and aldehyde-amine condensation inevitably retarding the formation of glycation adducts. However, the contribution of the positional isomers of AGs to the formation of transacylation adducts on proteins remains unclear. Additionally, due to the quantitative limitations of normalised ion counts, it is by no means certain that glycation is the predominant mechanism of protein modification by the positional isomers.

In an attempt to clarify this ambiguity over the direct protein reactivity of 1- β diclofenac-AG, synthetic 1- β diclofenac-AG was degraded in phosphate buffer to <10% exposure, with consequent formation of the acyl migration isomers. This mixture was then incubated with HSA in increasing AG concentrations, and modification of the protein compared to a parallel incubation containing undegraded diclofenac-AG. Extensive removal of 1- β diclofenac-AG from the incubation mixture with HSA resulted in a reduction of transacylation adducts. The consistent reduction of individual transacylated tryptic peptides (Figure 2. 12) provides definitive evidence for the contributory, and apparently disproportionate, role of the 1- β isomer in forming these

adducts because a direct comparison is made with the same modified peptides, which removes uncertainties relating to possible differential electrospray ionisation of different peptides. This means that the experimental difference between ion counts of a transacylated peptide is equitable with molar quantities of the peptide. In contrast, as expected, similar ion counts were seen for glycation adducts in the two incubations, probably due to similar exposure to the AG positional isomers in the pre-degraded and in-situ degraded solutions. Although the qualitative features of protein adduction by AG are now well characterised, the quantitative aspects are still somewhat obscure. In particular, the relative contributions of (i) acylation versus glycation, (ii) acylation versus glycation by the positional isomers and (iii) glycation by the individual positional isomers are essentially unknown. (Dickinson and King, 1991) found that the rank order of covalent binding of diflunisal-AG and its isolated positional isomers to HSA is C-4>C-3>C-2>C-1. The highest reactivity of the C-4 isomer is intuitively but hypothetically attributable to lowest steric hindrance by the acyl group of aldehyde-amine condensation (Figure 2. 2). However, the high reactivity of the C-4 isomer will be counterbalanced by the isomer's low abundance in solution (Figure 2. 6). In contrast, (Iwaki et al., 1999) found that HSA adduct formation from the 2-isomer of (S)-naproxen-AG proceeded more slowly than that from the 1- β -conjugate, which suggests particular structural features of AGs can be more influential than generic effects.

Through these experiments, diclofenac-AG has been shown to be chemically unstable and protein reactive *in vitro*. Whilst the % covalently binding of diclofenac-AG is lower than that of other NSAID AGs, the rapid rate of degradation, in the context of the relationships between degradation and HSA covalent binding derived by (Benet et al.,

1993) and (Bolze et al., 2002), indicates that covalent binding is likely to be relatively extensive. Consequently, these *in vitro* data suggest diclofenac-AG is a suitable drug AG to investigate potential hapten formation in experimental animals and patients. Incubations with pooled human plasma revealed extensive AG hydrolysis. *In vivo*, this may represent a mechanism resulting in reduced exposure to AG metabolites (Mizuma et al., 1999; Zhang et al., 2011), consequently reducing the potential for protein adduct formation. Good evidence was provided suggesting the role of the 1- β isomer, i.e. the biosynthetic product, in formation of acylation adducts. Time course data provided clear evidence that the formation of covalent adducts of HSA by transacylation and glycation follows distinct time-lines *in vitro*. The protein adducts generated with another electrophilic compound suggest adduction of lysine residues by AGs is likely to be a general phenomenon (Grigoryan et al., 2009).

Finally, from early studies on the adduction of plasma proteins in humans administered carboxylic acid drugs (Smith et al., 1986; McKinnon and Dickinson, 1989; Volland et al., 1991; Benet et al., 1993; Mayer et al., 1993; Zia-Amirhosseini et al., 1994; Sallustio et al., 1997), it is predicted that proteins modified by diclofenac-AG and/or other reactive metabolites of diclofenac will be present in the plasma of patients. Recent advances in mass spectrometric technologies have brought within reach the structural characterisation of circulating protein haptens derived from drugs and their metabolites in patients on standard therapeutic doses (Jenkins et al., 2009; Meng et al., 2011).

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CHAPTER THREE

THE USE OF A RAT MODEL TO STUDY ACYL GLUCURONIDE REACTIVITY

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3.1 INTRODUCTION

In chapter 2, the protein reactivity and chemical instability of diclofenac-AG *in vitro* were described. Diclofenac-AG was found to have a short half-life of degradation in phosphate buffer, pH 7.4, and was found to covalently bind to HSA in these incubations. Consequently, with the reported 75% turnover of diclofenac into its AG metabolite in an incubation with human liver microsomes supplemented with UDPGA (Kumar et al., 2002), diclofenac would fit criteria outlined by the FDA in their MIST guidance (FDA, 2008) as necessitating ‘additional safety assessment’.

The chemical instability and protein reactivity of diclofenac-AG defined in chapter 2 could potentially result in cessation of development of the compound if it was a novel chemical entity, developed as a new NSAID for the market today, due to fears over the potential toxicity of its AG. Despite this consideration, diclofenac has remained on the market as the most widely prescribed NSAID, accounting for over 35% of NSAID prescriptions in the Netherlands as long ago as 1990 (Leufkens et al., 1990), and 31.8% of NSAID prescriptions in general practice in the UK (Seager et al., 2000), resulting in an estimated 11 million people being exposed to the drug in the UK in the last 20 years (Jick et al., 2007). Consequently, diclofenac clearly represents a pharmaceutical providing efficacious therapy with advantageous risk-benefit profile. So much so that diclofenac is now available as an over-the-counter therapeutic in the UK (Hasford et al., 2004). Despite its extensive therapeutic use, its chemically unstable and protein reactive AG still attracts frequent attention from toxicologists (Aithal et al., 2004; Sallustio et al., 2006; Lagas et al., 2010; Koga et al., 2011; Mueller et al., 2012; Pickup et al., 2012).

Toxicological fears over acyl glucuronides are largely centred on their potential to act as haptens, modifying proteins in the host organism, resulting in inappropriate immune stimulation (Spahn-Langguth and Benet, 1992; Shipkova et al., 2003). As described in chapter 2, protein reactivity and chemical instability of AG metabolites do appear to be ubiquitous characteristics of these phase II metabolites, and are very well characterised following *in vitro* incubation (Munafo et al., 1990; Volland et al., 1991; Spahn-Langguth and Benet, 1992; Ding et al., 1993; Ding et al., 1995; Qiu et al., 1998; Ebner et al., 1999; Iwaki et al., 1999; Mortensen et al., 2001; Walker et al., 2007). Evidence for the translation of this protein reactivity from *in vitro* incubation systems to *in vivo*, however, is much less abundant (Benet et al., 1993; Bailey and Dickinson, 1996), despite considerable interest in this research over the last 30 years (Faed, 1984; Spahn-Langguth and Benet, 1992).

Experimental investigations into AG reactivity *in vivo* have focussed on the administration of the parent carboxylate to test subjects, both animals and humans, and measuring chemically undefined covalent modifications of unfractionated plasma proteins or partly characterised hepatic proteins. Correlation of the covalent binding of carboxylate compounds with plasma exposure of AGs provides the most compelling evidence of AG's responsibility (Smith et al., 1986; Hynneck et al., 1988a; Castillo et al., 1995; Bailey and Dickinson, 1996). However, whilst plausible, this does not provide definitive evidence of AG's responsibility, as the analytical techniques are unable to differentiate protein adducts derived from distinct CRMs. Consequently, with most carboxylate compounds, including diclofenac, metabolised to other CRMs capable of forming covalent protein adducts, whether acyl glucuronidation is responsible for covalent binding of carboxylate drugs to proteins *in vivo* is not necessarily proven.

A challenging limitation to previous *in vivo* research into AGs was the inaccessibility of pure AG, in large quantities, with AGs for experimentation usually obtained through isolation from liver microsome incubations (Smith et al., 1990a; Dubois et al., 1993b), or isolated from rat (Dickinson et al., 1994) or human (Munafa et al., 1990; Watt et al., 1991; Castillo et al., 1995; Ding et al., 1995) urine samples or alternatively rat bile (Williams et al., 1992; Ebner et al., 1999). These sources do not yield large quantities of pure material, meaning biosynthesis of AGs for *in vivo* investigation using these techniques is highly time consuming and potentially expensive. Only recently has chemical synthesis of AGs resulted in the preparation of these metabolites for research at relatively high yields (Kenny et al., 2004; Stachulski et al., 2006). Consequently, access to chemically synthesised diclofenac-AG created amongst the first opportunity to properly characterise the AGs reactivity with protein in an *in vivo* model.

Only a few experiments have ever administered AGs to experimental animals, investigating the plasma clearance of AGs (Watt et al., 1991; Iwaki et al., 1995; Dong et al., 2005). All of these experiments found AG metabolites to be rapidly cleared from the plasma, with clearances exceeding those of their aglycone. All of these experiments also showed extensive hydrolysis of AGs. The extensive AG hydrolysis observed in bile duct-ligated animals implies that hydrolysis is a consequence of plasma and/or tissue hydrolysis, rather than enterohepatic recirculation (Watt et al., 1991; Iwaki et al., 1995; Dong et al., 2005). Consequently, this observation suggests that not only is plasma AG clearance rapid, as would be expected for most Phase II metabolites, but also that plasma hydrolysis causes AG clearance to exceed what would be expected of a stable Phase II metabolite. This suggestion implies that rapid AG clearance from plasma may preclude extensive protein conjugation *in situ*.

However, this idea remains speculative, as none of these experiments investigated the protein reactivity of AGs *in vivo*.

To address this issue, experiments were undertaken as described in this chapter to investigate the applicability of the rat to study diclofenac-AG reactivity with serum albumin proteins *in vivo*, firstly through the characterisation of the covalent modification of RSA *in vitro*. Following this study, chemically synthesised diclofenac-AG was administered intravenously to a rat to investigate whether covalent protein adducts could be detected in plasma. The final experiments in the rat investigated the plasma disposition of diclofenac-AG. All of these factors combined may allow prediction of the risk of protein adduct formation from a drug AG in the rat.

3.2 MATERIALS AND METHODS

3.2.1 Materials

Acetonitrile (LC-MS grade), hydrochloric acid (HCl), methanol (LC-MS grade) and trifluoroacetic acid (LC-MS grade) were purchased from Fisher Scientific, Leicestershire. Bio Rad Bradford reagent was purchased from Bio Rad, Hertfordshire, UK. Modified trypsin was purchased from Promega, Hampshire, UK. Acetic acid (LC-MS grade), ammonium hydrogencarbonate, diclofenac sodium salt, dimethyl sulfoxide (DMSO), dithiothreitol, formic acid (LC-MS grade), HSA (approx. 99% pure, essentially globin free and fatty acid free), iodoacetamide, RSA ($\geq 99\%$ pure, lyophilized powder, essentially fatty acid free, essentially globulin free), sodium chloride, tris(hydroxymethyl)aminomethane (TRIS), urethane and zomepirac sodium salt were purchased from Sigma-Aldrich, Dorset, UK.

0.1M phosphate buffer pH 7.4 was made using 0.3117% monosodium phosphate monohydrate, 2.0747% disodium phosphate, heptahydrate w/v in distilled water.

All other reagents were purchased from Sigma-Aldrich (Dorset, UK) unless otherwise stated.

3.2.2 Animals

Male rats of a Wistar substrain were purchased from Charles River UK Limited (Margate, Kent, UK) and were of body weight between 200-250 grams. Animals were housed in groups and acclimatised to their surroundings for a minimum of five days prior to experiments. All experiments were undertaken in accordance with criteria outlined in a license granted under the Animals (Scientific Procedures) act of 1986 and approved by the Animal Ethics Committee of the University of Liverpool.

3.2.3 Covalent modification of human and rat serum albumin by diclofenac acyl glucuronide during *in vitro* incubation

Concentration-dependent modification of HSA and RSA by diclofenac-AG was investigated by *in vitro* incubation. Increasing concentrations of 1- β diclofenac-AG (400nM, 4 μ M, 40 μ M, 400 μ M and 2mM) were incubated with 40 μ M HSA or RSA in 0.1M phosphate buffer, pH 7.4, at 37°C for 16 hours. The reaction was stopped and protein precipitated and separated through immediate vortex mixing with 900 μ l ice-cold methanol and subsequent centrifugation at 24,000g at 4°C for 15 minutes. The methanolic supernatant was removed, and the pellet washed three times with 60 μ l ice-cold methanol. The precipitated protein was dissolved in 50 μ l of 0.1M phosphate buffer, pH 7.4, reduced with dithiothreitol (10mM) for 15 minutes at room temperature and alkylated with iodoacetamide (55mM) for a further 15 minutes at room temperature. The protein mixture was then precipitated and washed with ice-cold methanol as before. The protein pellet was re-dissolved in ammonium hydrogencarbonate solution (50 μ M, 30 μ l), assayed for protein content using the Bradford assay (Bradford, 1976), and 30 μ l aliquots of 3.2mg/ml protein were digested with 5 μ g trypsin overnight. The digests were desalted using 0.6 μ l bed C18 Zip-Tip pipette tips (Millipore, Cork, Republic of Ireland) as per the manufacturer's instructions, eluted with 10 μ l 50% acetonitrile, 0.1% trifluoroacetic acid in deionised water and dried by centrifugation under vacuum (SpeedVac, Eppendorf UK Ltd, Cambridge, UK) and stored at 4°C for a maximum of 2 weeks prior to LC-MS/MS analysis. For LC-MS/MS analysis, aliquots of 3 μ L sample were delivered into a QSTAR Pulsar i hybrid mass spectrometer (AB Sciex) by automated in-line liquid chromatography (integrated LCPackings System, 5mm C18 nano-precolumn and

75µm x 15cm C18 PepMap column (Dionex, California, USA)) via a nano-electrospray source head and 10µm inner diameter PicoTip (New Objective, Massachusetts, USA). A gradient from 5% acetonitrile/0.05% TFA (v/v) to 48% acetonitrile/0.05% TFA (v/v) in 60mins was applied at a flow rate of 300nL/min, and MS and MS/MS spectra were acquired automatically in positive ion mode using information-dependent acquisition (IDA) (Analyst, AB Sciex). Database searching was performed using ProteinPilot version 3 (AB Sciex) against the latest version of the SwissProt database, with biological modifications allowed and with the confidence level set to 10%. DAG was included as a high probability user-defined modification of Lys and carboxamidomethyl as a high probability user-defined modification of Cys. The data were also assessed manually for the presence of a dominant fragment ion of 278amu, indicative of cleavage of the transacylated hapten, and fragment ions of 215 and 250amu, indicative of cleavage of the glycosylated hapten.

3.2.4 Investigations into the modification of serum albumin proteins of the rat following intravenous bolus administration

A 264 gram rat was anaesthetised through intraperitoneal administration of 14% (w/v) urethane in 0.9% (w/v) saline, 1ml/kg. Following induction of anaesthesia, the trachea was cannulated to maintain ease of respiration of the animal throughout the experiment. The femoral vein was cannulated with a cannula flushed and filled with heparinised saline (250 U/ml). A 60mg/kg (127 µmol/kg) dose of diclofenac-AG (made up as a 60mg/ml solution in 50% DMSO (v/v)) was administered as an intravenous bolus through the femoral cannula. Two hours following drug administration, at a time point when the AG was expected to have been eliminated from literature (Watt et al., 1991; Iwaki et al., 1995; Dong et al., 2005), the animal was exsanguinated through

cardiac puncture, and killed. Plasma was isolated from blood following centrifugation at 2000g for 10 minutes at 4°C, and stored at -80°C until analysis. RSA was isolated from 60 µl aliquots of plasma using affinity chromatography (Greenough et al., 2004; Jenkins et al., 2009b). RSA was captured on a POROS anti-HSA affinity cartridge installed on a PerSeptive BioSystems Vision Workstation (Applied Biosystems, Foster City, CA, USA). It was eluted with HCl (12 mM). Eluted protein fractions were immediately neutralised with 0.1M Tris buffer, pH7. Protein fractions were precipitated through vortex mixing with nine fold volumes of ice-cold methanol and separated by centrifugation at 14,000 rpm. The methanolic supernatant was removed, and the pellet washed three times with 60µl ice-cold methanol. The precipitated protein was dissolved in 50µl of 0.1M phosphate buffer, pH 7.4, reduced with dithiothreitol (10mM) for 15 minutes at room temperature and alkylated with iodoacetamide (55mM) for a further 15 minutes at room temperature. The protein mixture was then precipitated and washed with ice-cold methanol as before. The protein pellet was re-dissolved in ammonium hydrogencarbonate solution (50µM, 30µl), assayed for protein content using the Bradford assay (Bradford, 1976), and 50µl aliquots of 3.2mg/ml protein were digested with 5µg trypsin overnight. The digests were desalted using 0.6 µl bed C18 Zip-Tip pipette tips (Millipore, Cork, Republic of Ireland) as per the manufacturer's instructions, eluted with 10µl 50% acetonitrile, 0.1% trifluoroacetic acid in deionised water, and dried by centrifugation under vacuum (SpeedVac, Eppendorf UK Ltd, Cambridge) and stored at 4°C prior to LC-MS/MS analysis within 2 weeks of sample preparation. Mass spectrometric analysis was performed as outlined above for analysis of *in vitro* peptide samples.

3.2.5 Pharmacokinetic experiments

Rats were anaesthetised through intraperitoneal administration of 14% (w/v) urethane in 0.9% (w/v) saline, 1ml/kg. Following induction of anaesthesia, the trachea was cannulated to maintain ease of respiration of the animal throughout the experiment. The carotid artery and femoral vein were cannulated, with cannulae flushed and filled with heparinised saline (250 U/ml). At desired time points, blood samples (100µl) were taken through the carotid artery cannula. Diclofenac-AG (5mg/kg, 10.59µmol/kg) or diclofenac (3.36mg/kg, 11.35µmol/kg) dissolved in 0.1M phosphate buffer, pH 5 was administered as an intravenous bolus through the femoral vein cannula (each dose group contained three animals). The blood samples were taken 5 minutes before drug administration, and subsequently at 5, 10, 15, 20, 25, 30, 45, 60 and 120 minutes post-dose. Blood samples were immediately centrifuged at 2100g for 10 minutes at room temperature, with AG in the separated plasma stabilised through the addition of 2M acetic acid, 4% (v/v). Plasma samples were stored at -80°C until analysis (Sparidans et al., 2008).

3.2.6 Analysis of diclofenac and diclofenac-AG in plasma samples

Stored plasma samples were thawed at room temperature and processed immediately. Samples were diluted using acidified blank rat plasma, kindly provided by Julie Eakins, AstraZeneca, Alderley Park. To 50 µl aliquots of acidified plasma 10µl of 3µM zomepirac internal standard was added in acetonitrile-water (1:1, v/v) containing 0.1% formic acid. Protein was precipitated through addition of four times volume of ice-cold acetonitrile. Precipitated protein was pelleted by centrifugation at 14,000g for 5 minutes at 4°C. Supernatant was removed, and filtered through 0.45-µm low-binding hydrophilic PTFE filter plates (Multiscreen Solvinert filter plates, Millipore,

Cork, Republic of Ireland) according to the manufacturer's instructions to remove any remaining particulate material. The filtrate was evaporated to dryness at 37°C under a constant stream of nitrogen, and reconstituted in 60µl of acetonitrile-water (1:1, v/v) containing 0.1% formic acid. A 10 µl aliquot was injected onto the HPLC column. Analyte separation was performed at room temperature using a Zorbax Eclipse XDB-C18 column (150 x 2.1 mm, 5µm; Agilent Technologies, Santa Clara, CA, USA) connected to a Dionex Ultimate 3000 HPLC system (Dionex Ltd., Macclesfield, Cheshire, UK) and a 4000 QTRAP hybrid quadrupole mass spectrometer (AB Sciex, Foster City, CA, USA). Samples were maintained at 4°C in the autosampler. Analytes were eluted using a gradient of acetonitrile containing 0.1% formic acid against 0.1% formic acid in water: 50-95% over 10 minutes. The eluent flow rate was 210µl/minute. Under these conditions diclofenac 1-β AG and its isomers eluted as a single peak at 2.7 minutes; zomepirac eluted at 5.2 minutes and diclofenac at 7.0 minutes (Figure 3.1). MS operating parameters for the multiple reaction monitoring (MRM) analyses of diclofenac and diclofenac-AG are shown in Table 3. 1. No endogenous or artefactual materials interfering with analyte and internal standard signals in the selected MRM channels were detected in control plasma samples. The lower limit of detection of diclofenac and the 1-β AG spiked into pooled rat plasma, as defined by signal to noise ratio > 3 was below 10nmol for both compounds. The corresponding lower limits of quantification, as defined by accuracy of between 80-120%, and precision (coefficient of variation) < 20% was 50nM for diclofenac (accuracy 80.6%, precision 19.2%), and 30nM for diclofenac-AG (accuracy 99.0%, precision 19.8%). Three accuracy and precision replicates were run before sample analysis, and

one accuracy and precision run was also run for mass spectrometric assay for diclofenac and diclofenac-AG quantification.

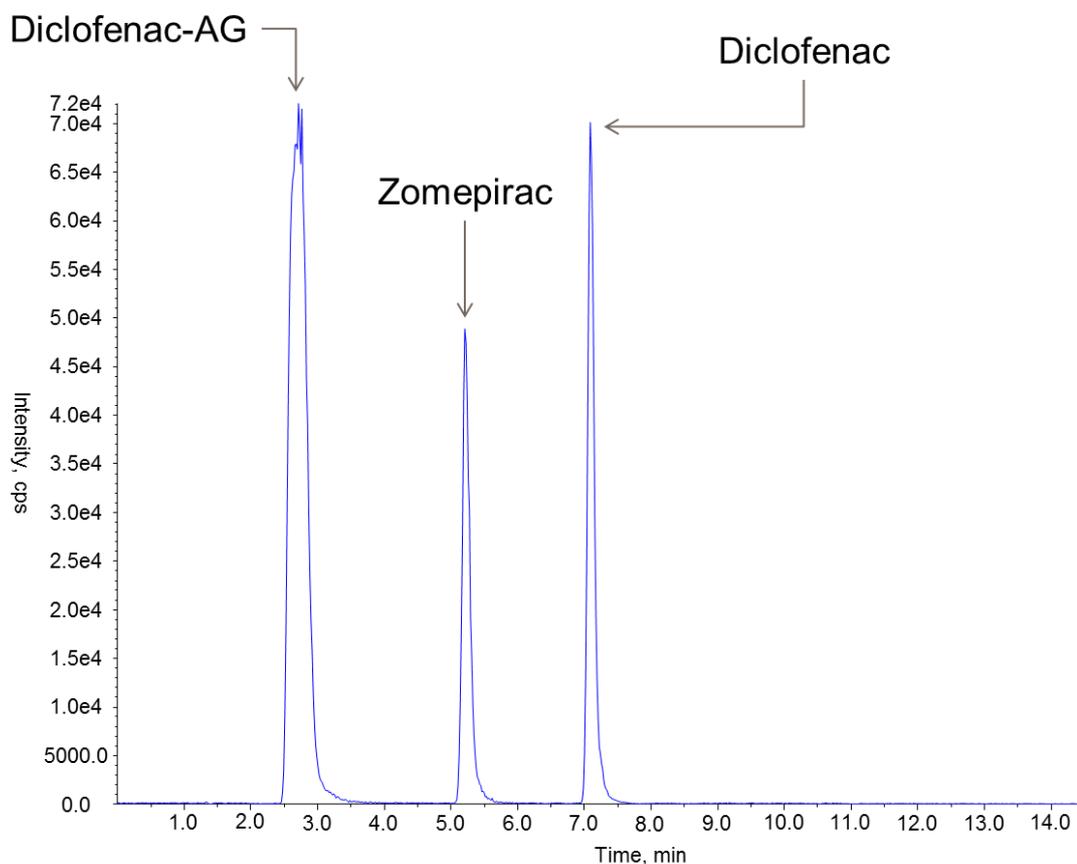


Figure 3. 1: Total ion current chromatogram representing separation of diclofenac-AG, zomepirac (internal standard) and diclofenac in rat plasma assays run on the API 4000 QTrap mass spectrometer.

Table 3. 1: API 4000 QTrap mass spectrometric operating parameters for MRM assays of diclofenac-AG and diclofenac. Parent ions are [M + H]⁺. ** Internal standard.

Parameters	Diclofenac-AG	Diclofenac	Zomepirac**
Fragmentation transition (m/z)*	472.0→296.1	296.1→215.1	292.0→139.0
Declustering potential (V)	37.00	31.00	40.00
Entrance potential (V)	10.00	10.00	10.00
Collision energy (eV)	14.00	41.00	27.00
Collision exit potential (V)	15.00	15.00	15.00
Dwell time (ms)	200	200	200

3.2.7 Pharmacokinetic analysis

Pharmacokinetic analysis of data was performed using Phoenix Winonlin (Version 5.2, Pharsight, St Louis, Missouri, USA). Non-compartmental analysis was used to allow the AUC to be calculated for diclofenac and diclofenac-AG using the trapezoidal rule. Plasma clearance (CL_p) was determined by the equation $CL_p = \text{Dose}/AUC_{0-\infty}$.

3.2.8 Statistical analysis

To test if statistical significance was achieved between pharmacokinetic parameters for the plasma clearance of diclofenac or diclofenac-AG following intravenous bolus drug administration, data were assessed for normality using a Shapiro-Wilk test. Data found to follow a normal distribution were assessed for significance using an unpaired t-test. Data found to not to follow a normal distribution was assessed for significance using a Mann-Whitney Rank Sum test. A statistically significant difference between groups was adjudged to be found if $p < 0.05$. Statistical analyses were performed using Sigmaplot for Windows Version 11.0.

3.3 RESULTS

3.3.1 Comparison of the sensitivity of the API5500 QTrap mass spectrometer and the QStar I Pulsar hybrid mass spectrometer

To allow assessment of the protein reactivity of diclofenac-AG towards RSA, *in vitro* incubations were undertaken. At the time of this experimentation, no access to an API 5500 QTrap (QTrap) mass spectrometer was available (chapter 2). Consequently, mass spectrometric analysis of the covalent modification of RSA was undertaken using an ABI QStar Pulsar i hybrid (QStar) tandem mass spectrometer (AB Sciex, Foster City, CA, USA). Diclofenac-AG was also incubated with HSA under the same conditions, with covalent modifications also analysed using the QStar mass spectrometer, to allow comparisons of modifications between RSA and HSA. The QStar has been used successfully to characterise the adduction of HSA by β -lactams *in vitro* (Jenkins et al., 2009a).

On comparison of the data for the covalent modification of HSA by diclofenac-AG acquired by QStar and QTrap mass spectrometric analysis, it was apparent that the QTrap had greater sensitivity. The lowest concentration of diclofenac-AG to produce a covalent adduct of HSA detectable by QStar analysis was 40 μ M, 100 fold higher than was achieved on the QTrap, which could detect a glycation adduct of K190 following incubation of 400nM diclofenac-AG with HSA. The identification of greater numbers of modified lysine residues of HSA following incubation with diclofenac-AG at 4 μ M, 40 μ M and 400 μ M by QTrap than QStar analysis (3, 5 and 4 more lysine residues respectively) provided further evidence for the greater sensitivity of the QTrap. Similar detection of HSA lysine modifications, however, was achieved following incubation with diclofenac-AG at the highest concentration of 2mM.

3.3.2 Investigation into the covalent modification of rat serum albumin following *in vitro* incubation

To assess the covalent modification of RSA (Table 3. 2), and its comparison to HSA (Table 3.2), diclofenac-AG was incubated for 16 hours at pH 7.4 and 37°C with 40µM RSA or HSA at increasing concentrations, from 4µM to 2mM. Due to the differences of amino acid sequence between RSA and HSA, and consequently the production of differing peptides by tryptic digestion, definitive assertions of the selectivity of modification cannot be made following mass-spectrometric analysis because of the differential ionisation of peptides as discussed in chapter 2. It is noticeable, however, that the numbers of lysine residues detected as modified by diclofenac-AG were far lower for RSA in comparison with HSA (4 in comparison to 10 respectively). It is also noticeable that the lowest concentration of diclofenac-AG producing detectable covalent adducts was higher in incubations with RSA than HSA (400µM compared to 40µM respectively). Consequently, this suggests that RSA may be less susceptible to modification by diclofenac-AG than HSA at physiological pH *in vitro*, and certainly identification of RSA modifications by tandem mass spectrometry was lower.

Table 3. 2: Covalent modifications of 40 μ M rat serum albumin (A) or 40 μ M human serum albumin (B) during incubation with synthetic diclofenac-AG at increasing concentrations. G represents a covalent adduct formed through the glycation pathway, T represents a covalent adduct formed through the transacylation pathway. The covalent modifications were identified by LC-MS/MS analysis of tryptic peptides. The adducted amino acid is represented by an asterisk. See figure 2.2 for comparison.

Lysine residue	Peptide sequence	Concentration of diclofenac-AG in the incubation mixture			
		4 μ M	40 μ M	400 μ M	2mM
K199	MK*CSSMQR			G	G
K212	AFK*AWAVAR				G
K317	EVCK*NYAEK				T
K525	K*QTALAELVK			T	T

Table 3. 2B

Lysine residue	Peptide sequence	Concentration of diclofenac-AG in the incubation mixture			
		4 μ M	40 μ M	400 μ M	2mM
K137	K*YLIEIAR				G
K162	YK*AAFTECCQAADK				G
K190	LDELRDEGK*ASSAK			G	GT
K195	ASSAK*QR				GT
K199	LK*CASLQK		G	G	GT
K351	LAK*TYETLEK			G	G
K432	NLGK*VGSK				G
K436	VGSK*CCK				GT
K525	K*QTALVELVK				GT
K541	ATK*EQLK				GT

3.3.3 Investigation of covalent binding of diclofenac acyl glucuronide to serum albumin in the rat

Following intravenous bolus administration of 60mg/kg diclofenac-AG to a single rat, albumin was isolated using affinity chromatography, and tryptic digests of the protein were analysed using a QStar mass spectrometer. Following exhaustive analysis of spectra, no diclofenac-related modifications could be detected.

3.3.4 Plasma clearance of diclofenac and diclofenac acyl glucuronide from the rat: non-compartmental analysis

The leading hypothesis for the mitigation of AG metabolites forming covalent adducts with proteins *in vivo* is their rapid elimination, and especially their rapid hydrolysis (Mizuma et al., 1999; Zhang et al., 2011). Potentially this provides an explanation for the absence of detectable covalent HSA adducts in the rat that received bolus intravenous administration of a large (60mg/kg) dose of diclofenac-AG. To test this hypothesis, the plasma clearance of diclofenac-AG administered as an intravenous bolus was investigated, and compared to that of diclofenac administered at a molar equivalent dose.

Following bolus administration of diclofenac (Figure 3. 2) and diclofenac-AG (Figure 3. 3) to the rat, both compounds showed an exponential fall in plasma concentration typical of those seen following bolus administration of drugs (Clarke and Smith, 2001). The mean plasma clearance of diclofenac-AG was found to be approximately 5.5 fold greater than that of diclofenac, resulting in plasma exposure of diclofenac as calculated by the mean AUC_{0-120} and $AUC_{0-\infty}$ to be 5.4 and 6.2 fold higher than that of diclofenac-AG (Table 3. 3).

Although diclofenac-AG was found in mouse plasma at low concentrations following administration of the aglycone (Sparidans et al., 2008), metabolism of diclofenac to its AG could not be quantified at any time point throughout the experiment. Dilutions of plasma samples, up to 1 in 40, were required for quantification of diclofenac. Due to the possibility of overloading the mass spectrometer, more concentrated plasma samples were not run in the attempt to assess metabolism of diclofenac to diclofenac-AG.

In vivo hydrolysis of diclofenac-AG to diclofenac, however, represented a considerable elimination pathway, with plasma concentrations of diclofenac surpassing those of diclofenac-AG by 15 minutes following AG administration. Plasma exposure of diclofenac following AG administration over the 120 minute experiment, as calculated by AUC_{0-120} , was two-fold greater than that of the AG.

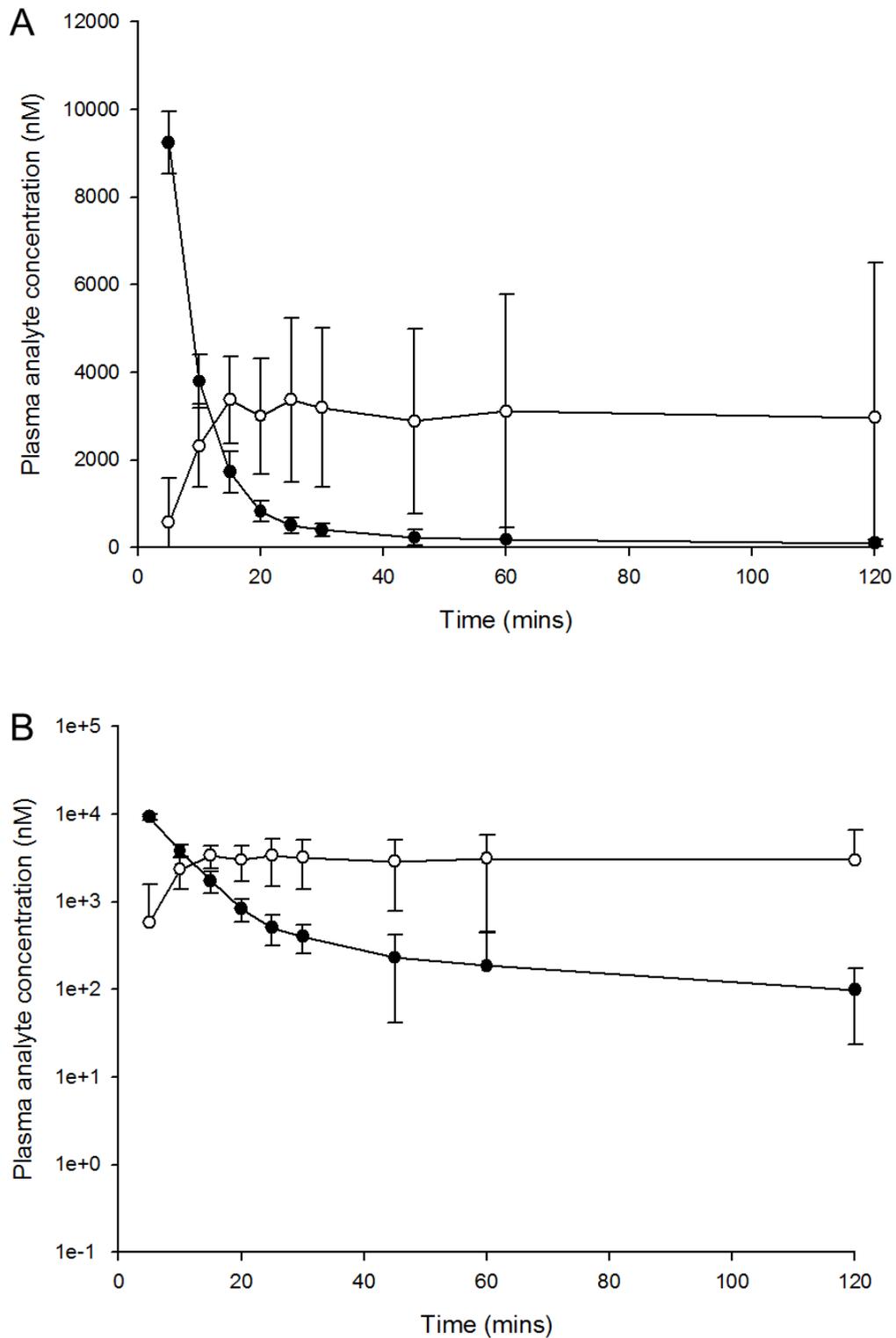


Figure 3. 2: (A) Plasma clearance of diclofenac-AG in the rat (filled circles) following iv administration of 5mg/kg and its hydrolysis to diclofenac (hollow circles). (B) These data shown on a log scale for comparison with published related studies of drug and drug-AG clearance in rats (Watt et al., 1991; Iwaki et al., 1995; Dong et al., 2005). Data presented as mean \pm standard deviation (n=3).

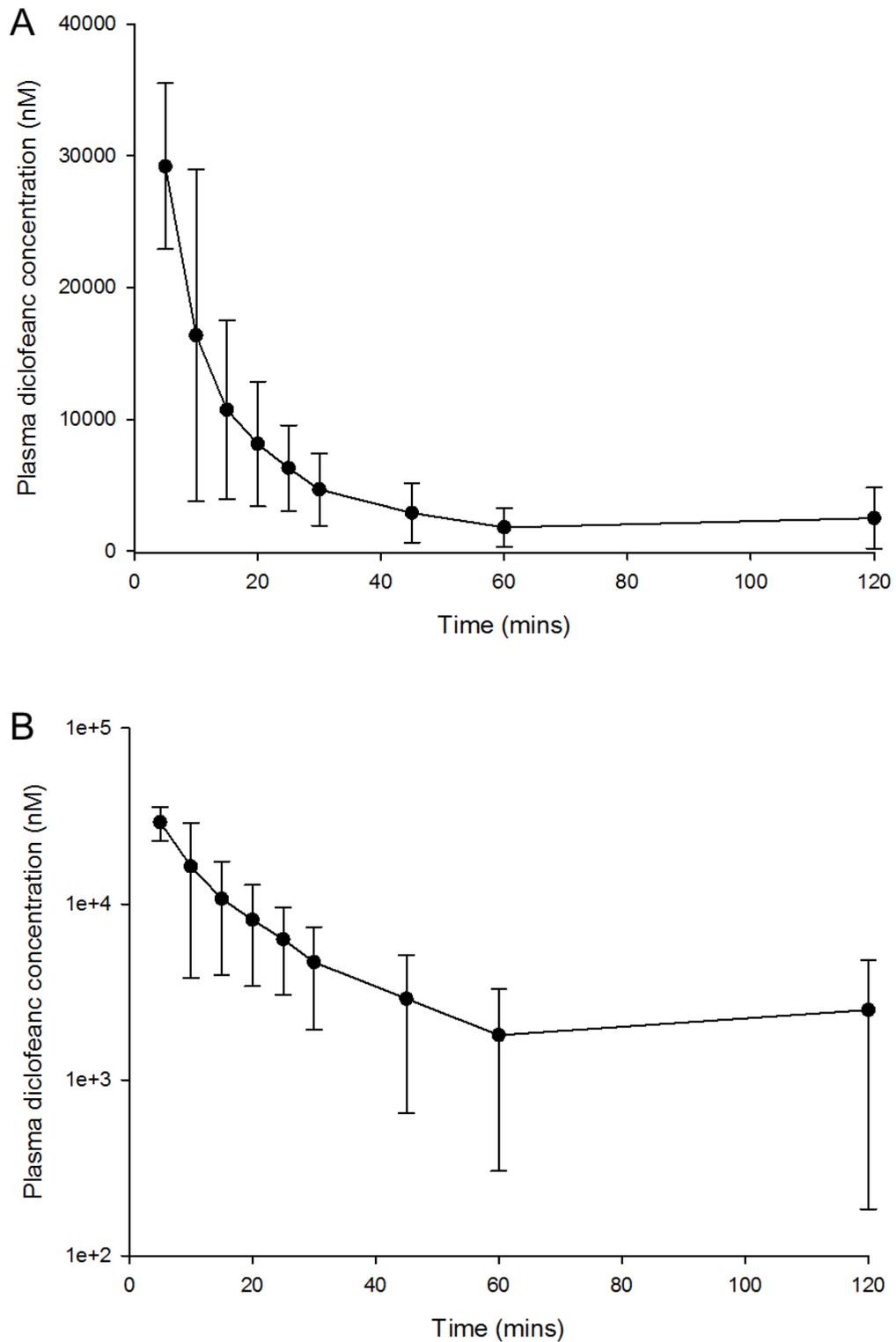


Figure 3.3: (A) Plasma clearance of diclofenac from the rat following iv bolus administration of 3.36 mg/kg. (B) These data shown on a log scale for comparison with published related studies of drug and drug-AG clearance in rats (Watt et al., 1991; Iwaki et al., 1995; Dong et al., 2005). Data presented as mean \pm standard deviation (n=3).

Table 3. 3: Pharmacokinetic parameters derived from non-compartmental modelling of data displayed in Figure 3. 2 and Figure 3. 3. Statistically significant differences between the pharmacokinetic parameters of diclofenac-AG and diclofenac following intravenous bolus administration of the compounds is analysed by either an unpaired t-test or a Mann-Whitney rank sum test (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$). n/a represents not applicable.

Drug administered	Analyte	$t_{1/2}$ (mins)	AUC_{0-120} (mmol min L ⁻¹)	$AUC_{0-\infty}$ (mMol min L ⁻¹)	Plasma Clearance (ml min ⁻¹ kg ⁻¹)
Diclofenac	Diclofenac	12.69 (± 4.51)	849.59 (± 134.4)	988.45 (± 261.9)	12.00 (± 2.98)
Diclofenac-AG	Diclofenac-AG	4.12 (± 0.58)*	154.6 (± 26.7)***	160.3 (± 33.1)**	67.81 (± 12.83)**
	Diclofeanc	n/a	344.07 (± 275.2)	n/a	n/a

3.4 DISCUSSION

The aims of this work were to investigate the adduction of RSA by diclofenac-AG, *in vitro*, ascertain whether these covalent adducts could be detected *in vivo* following bolus administration of diclofenac-AG to the rat, and finally determine the pharmacokinetic properties of diclofenac-AG following intravenous administration.

As with the investigations of HSA adduction by diclofenac-AG *in vitro* as described in chapter 2, diclofenac-AG was found to modify both RSA and HSA specifically at lysine residues. Despite exhaustive analysis of the mass spectra, no modifications (acylations) of serine or arginine residues were detected, as have been reported for reactions of benoxaprofen-AG and tolmetin-AG with HSA (Ding et al., 1993; Ding et al., 1995; Qiu et al., 1998). Reasons for these marked differences between adductions of HSA by AGs are not clear, but most likely they reflect in combination the electrophilic and steric properties of the conjugates (Berry et al., 2009; Potter et al., 2011). However, modifications of non-lysine residues were few in all of these experiments (Ding et al., 1995; Qiu et al., 1998); in every case, greater numbers of modifications of lysine residues were detected.

On comparison of the modification of HSA and RSA by diclofenac-AG, it is clear to see that greater numbers of lysine residues were detected as modified on HSA (ten lysine residues) in comparison to RSA (four lysine residues) following incubation with 2mM diclofenac-AG. Additionally, the lowest concentration of diclofenac-AG yielding detectable covalent modification of albumin was 10-fold lower for HSA (a glycation adduct detected at K199 following incubation with 40 μ M AG) than for RSA (a glycation adduct at K199 and a transacylation adduct at K525 following incubation with 400 μ M AG) (Table 3. 2, Table 3.3). These findings may suggest that covalent modification of

HSA is preferential to modification of RSA, although definitive conclusions as to the selectivity of the modifications cannot be made due to the potential for differential ionisation of tryptic peptides as discussed in section 2.28.

Few investigations have attempted to determine the relative reactivity of AG metabolites with HSA and RSA, however, Watt and Dickinson (1990) found a 74.7% greater modification of HSA compared to RSA following incubation with diflunisal-AG at a molar ratio of 0.16:1 (AG:HSA) compared with 0.3:1 (AG:RSA) (Watt and Dickinson, 1990), suggesting the lesser modification of RSA is a real result. Whilst little difference was observed following incubation at lower AG concentrations, this may have been due to the low concentrations used.

HSA and RSA, and other mammalian serum albumins (Ahmad et al., 2011) are comprised of generally well-conserved amino acid sequences; HSA and RSA sharing 80% sequence homology (Carter et al., 1989; Kosa et al., 1997). However, variations in their non-covalent binding sites have been reported. Whilst non-covalent interactions of the Sudlow Site I ligands warfarin and phenylbutazone were similar between RSA and HSA, interactions were greatly reduced for the Sudlow site II ligands ibuprofen and diazepam (Kosa et al., 1997). Species differences in the non-covalent interactions of an unconjugated carboxylic acid with RSA and HSA have been reported for the leukotriene D4-antagonist MK-571, whose S- isomer preferentially formed non-covalent interactions with HSA, whereas the R- isomer preferentially interacted with RSA (Lin et al., 1990). With hydrophobic sites shown to be important in the covalent modification of HSA by AGs, as described in chapter 2 and by others (Munafo et al., 1990; Williams and Dickinson, 1994; Ding et al., 1995; Qiu et al., 1998), species differences resulting in lower affinity for AGs at these sites are likely to have an effect on covalent

interactions. With bovine serum albumin *in vitro*, the covalent modification of tolmetin-AG was much less than binding to HSA, although the rate of adduct formation was the same as with HSA (Munafo et al., 1990). BSA lacks inter alia K195 and K199 (Ahmad et al., 2011), both of which in HSA are adducted by tolmetin AG *in vitro* (Ding et al., 1995).

However, the absence of 6 of the 10 lysine residues of HSA modified by diclofenac-AG from the primary sequence of RSA is the most likely explanation for the reduced identification of modified lysine residues in RSA (sequences from (Sargent et al., 1981; Minghetti et al., 1986)). The absence of these six lysine residues (K137, K162, K190, K195, K432 and K541) from RSA is likely to have a drastic effect on the extent of covalent modification. Consequently, the appropriateness of RSA as an adduction target in any attempt to predict the covalent binding of drugs and metabolites to plasma proteins in humans may be questioned.

Despite this observation, qualitative assessments of modifications of RSA by carboxylate drugs and their AGs is still useful. An identification of AG-derived glycation adducts on RSA *in vivo* may at least suggest corresponding adductions of HSA *in vivo*, *pro rata* with metabolic formation of the AG, will be more abundant and/or numerous, due to the generically slower rate of plasma clearance of drugs in humans than rats (Chiou et al., 1998) and the more numerous sites for covalent modification of HSA than RSA (Table 3. 2, Table 3.3). Consequently, to investigate if diclofenac-AG can modify RSA *in vivo*, a single intravenous bolus dose of diclofenac-AG was administered to an anaesthetised rat, and isolated serum albumin was analysed using peptide mass spectrometry to detect any covalently modified residues. With two covalent adducts detected on lysine residues K199 and K525 following *in vitro* incubation of 400µM

diclofenac-AG with 40 μ M RSA, which represented a relative molar exposure of 10:1 (AG:RSA), it was decided to administer diclofenac-AG to the rat at a dose, which, ideally, would produce a similar exposure of the conjugate to RSA.

Taking the estimated total plasma volume of the 264 g rat used here to be 10.22 ml (Bijsterbosch et al., 1981), a 60mg/kg bolus intravenous dose would theoretically result in a maximum plasma diclofenac-AG concentration of approximately 3.3mM. With the plasma concentration of RSA estimated at 22.3mg/ml (337.8 μ M) (Papet et al., 2003), the molar ratio of diclofenac-AG to RSA immediately following bolus administration is estimated at 9.76:1 (AG:RSA). This is approximately the relative exposure of AG that produced detectable covalent adducts of RSA during a 16 hour *in vitro* incubation (Table 3. 2).

Despite exhaustive analysis of the tryptic digests of RSA isolated from plasma samples taken from the rat 2 hours following AG administration, no covalent adducts could be detected using QStar mass spectrometry. This finding would appear to conflict with non-mass spectrometry studies suggesting AGs are able to form covalent adducts with plasma proteins in the rat following administration of the parent carboxylic acid (Sallustio and Foster, 1995; Liu et al., 1996). It must be emphasised, however, that because these covalent binding assays rely on essentially indiscriminate alkaline hydrolysis of adducts, they do not permit attribution of the drug metabolites responsible for covalent modification of plasma proteins *in vivo*.

Glucuronidation of xenobiotics is traditionally considered a process acting to nullify the adverse chemical and physiological activities of unusable compounds and hasten their elimination from the biological system (Caldwell, 1982; Miners and Mackenzie, 1991; Spahn-Langguth and Benet, 1992). With their generic potential for covalent adduct

formation *in vivo*, AGs clearly do not represent model products of glucuronidation. Therefore the exceptionally rapid elimination of AG metabolites can be regarded as an essential counterbalance to the metabolite's chemical reactivity, which mitigates their potential to exert toxicological effect.

Despite assumptions of AG's rapid elimination, measurements of AG clearance *in vivo* have not been undertaken extensively. Most investigations into the rates of AG clearance *in vivo* employed measurement of the conjugate's renal clearance in humans, following administration of the parent drug. In all of these cases, renal clearance of the AG metabolite was found to be greater than for the parent drug (Smith et al., 1985; Castillo et al., 1995; Vree et al., 1995). Experiments in the rat, where the AGs of benoxaprofen, flunoxaprofen, diflunisal and the R- and S- isomers of naproxen were directly administered as an intravenous bolus also reported rapid AG elimination in comparison with their parent carboxylates (Watt et al., 1991; Iwaki et al., 1995; Dong et al., 2005). Consequently, it was decided to investigate whether plasma clearance of diclofenac-AG in the rat is also rapid, potentially providing an explanation for not detecting covalent adducts *in vivo*.

As expected, the plasma clearance of diclofenac-AG was found to be significantly greater than that of diclofenac (by approximately six-fold), resulting in a shorter plasma half-life of diclofenac-AG of 4.12mins in comparison to 12.69mins for diclofenac (Table 3. 3). The greater clearance of diclofenac-AG may be a result of its greater affinity for export transporters. The major route of elimination of diclofenac in the rat has been shown to be biliary elimination (Peris-Ribera et al., 1991), with TR^{-/-} (MRP2 deficient) rats revealing the importance of the export transporter MRP2 for the elimination of diclofenac acyl glucuronide from the rat (Seitz et al., 1998). In knockout

mice not expressing MRP2, no statistically significant reduction of diclofenac biliary elimination could be detected. However, elimination of the AG was vastly increased (Lagas et al., 2010); suggesting that elimination of the AG is dependent on export hepatic export transporters, whereas that of diclofenac is not. Further evidence for this difference was derived from *in vitro* studies using transfected MRP-2 that suggested that diclofenac is not transported by this protein (Lagas et al., 2009).

As a consequence of the greater rate of AG clearance in comparison to diclofenac, plasma exposure of the AG was significantly lower by 5.5-fold, supporting the hypothesis that rapid clearance of AGs, resulting in reduced AG exposure may be a mitigating factor in the covalent modification of endogenous macromolecules.

The plasma clearance of diclofenac-AG ($67.81 \pm 12.83 \text{ ml min}^{-1} \text{ kg}^{-1}$, Table 3.3) being approximately double the $35.5 \pm 5.2 \text{ ml min}^{-1} \text{ kg}^{-1}$ hepatic blood flow of urethane-anaesthetised rats (Hiley et al., 1978) indicates that mechanisms other than hepatic metabolism and elimination contribute to the conjugate's clearance from the circulation. The major mechanisms which would be expected to account for extra-hepatic AG eliminations would generally be considered to be renal elimination, or extra-hepatic AG hydrolysis.

Whilst investigations into the renal and biliary elimination of AGs was not undertaken in these studies, extra-hepatic hydrolysis of diclofenac-AG to its parent carboxylate appeared to represent an important route of clearance in the rat. This has also been shown for the AGs of benoxaprofen, flunoxaprofen, diflunisal and naproxen (Watt et al., 1991; Iwaki et al., 1995; Dong et al., 2005). High variability in plasma or tissue hydrolysis of diclofenac-AG was detected in this experiment, most likely due to inter-animal variation. This explanation is represented by one animal having consistently

lower values for plasma hydrolysis than the other two in the experiment. Whilst correlations between hepatic blood-flow and AG clearance, as described above, suggest that the detection of plasma diclofenac is largely as a consequence of extra-hepatic hydrolysis, the contribution of enterohepatic circulation was not defined in this experiment. The relative contributions of plasma and tissue hydrolases/esterases to the clearance of drug AG from plasma are still largely unknown (Fukami and Yokoi, 2012). Human plasma contains three esterases in high enough concentrations to contribute significantly to ester hydrolysis: butyrylcholinesterase, paraoxonase and albumin (Li et al., 2005). However, there are substantial species differences between esterase expression and hydrolase activity in mammalian plasma (Bahar et al., 2012), and carboxylesterase is absent from human plasma (Li et al., 2005). Extensive enterohepatic recirculation of several NSAIDs including diclofenac has been found in the rat (Stierlin and Faigle, 1979; Lin et al., 1985; Dietzel et al., 1990). However, the extensive hydrolysis of the AGs of flunoxaprofen and benoxaprofen in rats with cannulated bile ducts (Dong et al., 2005), which consequently were unable to undergo enterohepatic recirculation, combined with the extensive hydrolysis of AGs incubated in isolated plasma as shown in chapter 2 and by others (Ruelius et al., 1986; Volland et al., 1991; Williams et al., 1992; Akira et al., 2002; Shipkova et al., 2003; Karlsson et al., 2010), suggest hydrolysis is primarily located in the tissues and/or plasma. If this is the case, plasma hydrolysis of AGs does represent an additional elimination pathway, complimenting rapid direct elimination, reducing the risk of AG-mediated adduction of macromolecules *in vivo*.

From this work, it is clear that species differences, expressed as a 20% variance in the primary structure between RSA and HSA (Sargent et al., 1981; Minghetti et al., 1986;

Carter et al., 1989; Kosa et al., 1997), result in a marked disparity of covalent adducts detected by QStar mass-spectrometry. This finding is likely to be principally a consequence of the absence from RSA of 60% of the lysine residues of HSA known to be modified by diclofenac-AG *in vitro*. Despite the identification of two covalent adducts following incubation of 400 μ M diclofenac-AG with 40 μ M RSA, no AG derived adducts were identified on RSA isolated from a rat administered diclofenac-AG at a dose estimated to produce the same exposure. Rapid elimination of diclofenac-AG from plasma was hypothesised to be a mechanism mitigating covalent adduct formation by the AG metabolite in the rat. To investigate this proposition, the plasma clearance of an intravenous bolus dose of diclofenac-AG was determined. Plasma clearance of the AG was rapid, resulting in a measured half-life of clearance of only 4 minutes, which was three fold faster when compared to diclofenac. Hydrolysis of the administered AG represented a considerable elimination pathway, resulting in three-fold higher exposure of diclofenac than the AG over the 120 minute experiment. Whilst it cannot be definitively identified in this experiment whether enterohepatic recirculation or plasma/tissue hydrolysis was primarily responsible for the extent of diclofenac-AG hydrolysis detected in rats, the incubations of AGs with isolated human plasma described in chapter 2 do suggest that plasma hydrolysis of diclofenac-AG would be extensive. This would constitute a significant elimination pathway, greatly reducing the adduction of proteins by diclofenac-AG *in vivo*, and consequently, may also be considered a detoxification pathway.

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CHAPTER FOUR

PHARMACOKINETICS AND TOXICITY ASSESSMENT OF DICLOFENAC FOLLOWING CONTINUOUS INTRAVENOUS INFUSION TO THE RAT

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4.1 INTRODUCTION

The most convenient and widely used route for the administration of pharmaceuticals is via oral ingestion of either liquid or capsule formulations. However a variety of other routes of drug administration are available and may be used when episodic oral administration is considered inappropriate or undesirable. Continuous infusion allows continuous administration of drugs, usually into the circulatory system. Clinically, various routes of continuous infusion are used, including skin patches, where infusion is maintained by an osmotic gradient as is used in rotigotine administration in the treatment of Parkinson's disease (Sanford and Scott, 2011). Alternatively, in a hospital setting continuous intravenous drug infusion may be used, allowing continuous and consistent drug plasma concentrations. This is used for compounds including antibacterial agents, flucloxacillin (Leder et al., 1999; Howden and Richards, 2001), and chemotherapeutics such as 5-fluorouracil (Howell et al., 1997; Recchia et al., 2001). These routes of infusion allow consistent and careful control of drug plasma concentrations. This is particularly important for compounds where consistent plasma concentrations are required for efficacy.

Plasma kinetic profiles of drugs following continuous infusion usually follow 1st order kinetic principles, where drug clearance is proportional to drug plasma concentration. Following commencement of drug infusion drug plasma concentrations are low. Consequently clearance is low, and below that of the rate of infusion, resulting in a rise in drug plasma concentration. As drug plasma concentration increases, so does the rate of drug clearance. This continues until the rate of drug clearance matches that of drug infusion. At this point plasma drug concentrations remain constant, and are consequently maintained (steady-state pharmacokinetics are said to have been

reached) until cessation of drug administration (Clarke and Smith, 2001; Hill, 2004).

Whilst continuous intravenous infusion is a heavily used technique in the delivery of drugs in a clinical setting, and consequently the pre-clinical model is well-established in development of these drugs, application of the continuous intravenous infusion model to *in vivo* toxicology studies is not.

Commonly, pre-clinical investigations of drug pharmacokinetics and drug safety are undertaken in rat models. The quicker rate of drug elimination in rats vs man is a major limitation. With 52 of 54 drugs tested showed a faster rate of plasma clearance in the rat than man, ranging from 1.3 times faster for ketoprofen to 123 times faster for diazepam. Only cyclosporine and nifedepine are cleared faster in man than the rat (Chiou et al., 1998). Consequently, even repeat drug administrations in the rat are unlikely to suitably model the clinical situation, as increased drug clearance may result in periods of 'drug holiday' of minimal drug exposure which is not observed in man. Consequently, it is hypothesised that continuous drug infusion, reaching steady state drug exposure may better model the clinical situation.

Alternatively, continuous intravenous infusion may also be considered a useful tool in toxicological investigations of compounds. Whilst allowing better correlation with clinical exposure, continuous infusion may also be used as a technique allowing 'forced' increased drug (and potentially) metabolite exposure. This increased drug and/or metabolite exposure may unmask mechanisms of toxicity not detectable following bolus drug administration techniques which are currently frequently used in current drug toxicity studies. Acute dosing of compounds also has the potential to produce toxicities in secondary organs to the primary ones being studied. Diclofenac represents a good example of this, where the dose-limiting toxicity is damage to the

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gastrointestinal tract (Menasse et al., 1978; Seitz and Boelsterli, 1998), which limits doses which may be used for investigation of hepatotoxicity. Continuous infusion may present a model by which these secondary toxicities may be avoided or managed to allow further investigation of toxicities of intended interest.

To test the applicability of continuous infusion in a toxicological setting, diclofenac was chosen. For some disorders including rheumatoid and osteo-arthritis diclofenac may be administered daily over many years, with dosing only ceased on suspicion of a serious ADR. Due to the necessity of continuous drug efficacy, diclofenac is often administered either twice or three times a day, at daily doses reaching 150mg, sometimes in slow release formulations. Further to this, the rat exhibits 3.7 times faster plasma clearance of diclofenac than man (15.7ml/min/kg against 4.2 ml/min/kg respectively) (Chiou et al., 1998). Consequently, as described above, single bolus drug administrations or repeat bolus drug administration studies in the rat are unlikely to represent the clinical situation.

Diclofenac is associated with the generation of idiosyncratic hepatotoxicity, with approximately 6.3 patients out of 100,000 requiring hospital treatment (de Abajo et al., 2004). Further to this, transient ALT rises are observed in patients receiving drug treatment in approximately 15% of patients, with approximately 3-5% of patients experiencing ALT rises above 3x the upper limit of normal (Banks et al., 1995; Laine et al., 2009). To date, no *in vivo* models have allowed prediction or mechanistic understanding of either of these two types of drug toxicities.

Incubations with human liver microsomes supplemented with UDPGA has suggested that approximately 75% of a dose of diclofenac is directly glucuronidated, forming its acyl glucuronide metabolite, with the remaining dose oxidised by CYP2C9 into its 4-OH

metabolite (Leemann et al., 1993; Tang et al., 1999c; Kumar et al., 2002). The acyl glucuronide metabolite can be further oxidised into 4-OH diclofenac catalysed by CYP2C8 (Kumar et al., 2002). Alternatively diclofenac may be oxidised by CYP3A4 into its 5-OH metabolite (Shen et al., 1999). Both the 4-OH and 5-OH diclofenac metabolites have been reported to be further oxidised into quinone-imine metabolites (1', 4'-quinoneimine and 2', 5'-quinoneimine metabolites respectively) (Poon et al., 2001). CYP2B, 2C and 3A isoforms have been implicated with oxidative metabolism in the rat (Tang et al., 1999a). Bioactivation of diclofenac into its reactive metabolites has been postulated to play a key role in the eliciting of idiosyncratic ADRs associated with the parent drug (Boelsterli, 2003; Aithal, 2004).

Presentation of hepatotoxic ADRs associated with diclofenac is usually delayed, with 76% of cases presenting after 1 month following introduction to the compound (Banks et al., 1995). This combined with case reports of liver failure following diclofenac re-administration to patients already suffered a delayed hepatotoxic reaction to the drug (Helfgott et al., 1990; Greaves et al., 2001) suggest an immunological pathogenesis. Whilst production of drug-protein adducts is hypothesised to be a critical process in the pathogenesis of immune-mediated ADRs, the provision of 'danger signals' from stressed, necrotic or apoptotic cells is also required for immune stimulation (Matzinger, 1994; Curtsinger et al., 1999). Mechanisms for generation of danger signals in drug, and diclofenac-induced liver injury are largely undefined. Several mechanisms have been hypothesised for the production of danger signals, including translocation of bacteria or endotoxins from the GI tract to the liver (Deng et al., 2006; Deng et al., 2008) and cytokine release as a result of cell-death elicited as a consequence of underlying disease, including osteoarthritis (Banks et al., 1995).

Alternatively, with transient plasma ALT rises detected in 3-5% of patients receiving diclofenac therapy (Banks et al., 1995; Laine et al., 2009), it can be hypothesised that danger signal release may be a direct response to drug exposure. We hypothesise that continuous infusion of diclofenac to the rat would unmask these mechanisms of hepatotoxicity, as a result of forced-increased drug-exposure.

Individual variations in phenotype, resulting in increased drug exposure have also been associated with increased susceptibility to off-target hepatotoxicity associated with diclofenac. Genotyping studies have revealed an increased abundance of polymorphisms in the ABCC2 gene in patients who have retrospectively experienced an off-target ADR to diclofenac treatment, with an odds ratio of 5.0 ($p=0.05$). This gene encodes the hepatobiliary export pump MRP2. Whilst the functional outcome of the 24C>T ABCC2 polymorphism is not fully defined (Daly et al., 2007), it has been associated with identified associated with reduced mRNA expression of the gene (Haenisch et al., 2007), suggesting that increased drug or metabolite exposure may play a role in the clinical onset of diclofenac induced liver injury.

Further associations with polymorphisms in the genes encoding CYP2C8 and UGT2B7 were also found to produce increased risk of developing idiosyncratic hepatotoxicity following treatment with diclofenac (Daly et al., 2007), further suggesting accumulation of drug metabolites may represent a mechanism for the pathogenesis of idiosyncratic hepatotoxicity towards diclofenac. Continuous infusion of diclofenac provides a tool by which this may be more closely modelled.

Through this chapter we investigate the use of the continuous intravenous infusion system for its applicability for toxicological assessment of diclofenac hepatotoxicity in the rat. This chapter aims to investigate whether diclofenac reaches steady state

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following continuous intravenous infusion, and consequently if this results in initiation of hepatotoxicity in the rat, and possibly identification of mechanisms responsible.

4.2 MATERIALS AND METHODS

4.2.1 Materials

Medfusion 3500 syringe pump (Smiths Medical, Ashford, Kent, UK), PhysioCath small animal vascular catheter (Data Sciences International, Minnesota, USA), Covance infusion harness (Instech Laboratories, Pennsylvania, USA), Vet-bond adhesive (3M, Minnesota), Water for injections (Hameln Pharmaceutical, Gloucestershire, UK and Fresenius Kabi, Cheshire), Sterile 0.9% (w/v) saline and Sterile 5% (w/v) Mannitol (Fresenius Kabi, Runcorn, UK), Vet-bond adhesive (3M Animal Care, Minnesota, USA), Diclofenac sodium salt (Sigma-Aldrich, Dorset, UK), Roche P modular analyser and standard Roche reagents (Roche Diagnostics, West Sussex, UK), Bio Rad Bradford reagent (Bio Rad, Hertfordshire, UK), MRX microplate reader with Max Revelations 3.04 software (Dynotech Laboratories, West Sussex, UK). 3,3'-diaminobenzidine, Background Blocker with Casein and X-cell plus polymer HRP (A.Menarini diagnostics, Berkshire, UK), Carazzi's Hematoxylin, polyclonal rabbit anti-catalase (2363-1, Epitomics, California, USA), Rabbit anti-Superoxide Dismutase-2 (ab:13534, Abcam, Cambridgeshire, UK), polyclonal rabbit anti-CYP2C19 (HPA015066, SigmaAldrich, Dorset, UK), goat anti rat CYP1A1 (423635, Daiichi Pure Chemicals, Japan), rabbit anti-BSEP (PC-064, Kamiya, Washington, USA), mouse anti-MRP2 (ab3373, Abcam, Cambridgeshire, UK), biotinylated rabbit anti mouse secondary (E0464, Dako, California, USA). All other reagents were purchased from Sigma-Aldrich (Dorset, UK) unless otherwise stated.

4.2.2 Animals

Male rats of Wistar Hannover substrain RccHan:WIST of 10 weeks of age were provided from Harlan Laboratories, UK. Animals were housed in groups and acclimatised to

their surroundings for a minimum of 5 days prior to surgery for cannulation. Following surgery, animals were housed singly. Food and water was provided ad libitum to all animals throughout the study. Experiments undertaken were in accordance with the criteria outlined in a license granted under the Animals (Scientific Procedures) Act of 1986. All cannulation surgery and sampling was performed by trained animal surgeons and technicians at AstraZeneca, Alderley Park, UK. All analysis and sample work-up was performed in Liverpool.

4.2.3 Cannulation procedure

Initial induction of anaesthesia of rats was achieved by placing animals in an anaesthesia chamber containing 3-4% isoflurane/100% medical oxygen. Following anaesthesia induction, the dorsal scapula and left ventral groin regions of the animals were shaved. A small incision was made at the scapula region to facilitate cannula exteriorisation. Proprietary skin cleansers were used on the shaved regions and the animals were transferred to the surgical table. Each animal was placed on a homeothermic operating table over a sterile cloth and placed under 2-3% isoflurane inhalation. A sterile drape was placed over the animal and a small incision (approximately 1cm) is made in the inguinal region adjacent to the peritoneal cavity. Following blunt dissection the femoral vein was exposed through the incision ligatures used to control blood flow. Following this, an incision in the femoral vein was made, through which a PhysioCath catheter was inserted to a distance where the cannula tip sits within the vena cava. The catheter was tied in place using silk ligatures and flushing of the catheter was carried out to ensure blood flow. The free end of the catheter was led subcutaneously from the groin to the incision at the scapula region. The groin and neck incisions were closed using surgical staples and vet-bond

adhesive. The free end of the catheter was led through a steel spring protector attached to an animal infusion harness. The animal was then placed in the harness and the catheter was attached to the syringe pump via an infusion tether and swivel joint. Following surgery, animals were maintained on continuous infusion of sterile saline at a flow of 1ml/hour/animal for approximately 24 hours. This was then reduced to 0.5ml/hour/animal until 24 hours before drug dosing. 24 hours before drug dosing, animals were administered 5% mannitol vehicle at a flow rate of 2ml/kg/hour, prior to administration of the test compound.

4.2.4 Dose escalation arm

To allow selection of the most appropriate dose for the study, a dose raising investigation was undertaken to investigate the maximum tolerated dose. Using 2 animals per group, diclofenac infusion of 12mg/kg/day using a vehicle of 5% mannitol (w/v) was well tolerated in animals. A subsequent increase in dose to 24mg/kg/day was poorly tolerated. Consequently, a 12mg/kg/day infusion of diclofenac was used for the main study.

4.2.5 Continuous intravenous infusion of diclofenac at a dose of 12mg/kg/day

A 0.25mg/ml solution of diclofenac was made up in 5% (w/v) sterile mannitol solution, and was administered to rats at a rate of 2ml/kg/hour. This equates to a dose of 12mg/kg/day. A control group was administered 5% (w/v) sterile mannitol solution at the same rate of infusion. Drug infusion was maintained for 48hours. Following the 48 hour time point animals were disconnected from infusion equipment and cannulae were tied off. Animals were subsequently maintained for 72 hours before termination of the study. At desired time points, blood samples were obtained from animals

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through tail vein knicks. A composite sampling approach was chosen to ensure blood sampling is maintained below the 20% blood volume throughout the in-life phase of the study in accordance with the criteria in the Home Office Project License. Group sizes were selected at n=8 for diclofenac infusion and n=6 for vehicle infusion. Large group sizes were selected to allow for non-drug related losses from the study which are known to occasionally occur in continuous infusion experiments, including escape of animals from infusion equipment and development of blood clots in cannulae, preventing infusion. The vehicle control group contained two less animals due to technical difficulties in the surgery procedure. The diclofenac group contained one less animal than intended as one received its 24 hour dose in 24 minutes, and hence data achieved from this animal was discounted from analysis. At the end of the in-life phase of the study, animals were killed by administration of halothane. At the end of the experiment, a 3-5mm transverse section of the left lateral lobe of the liver was removed and stored in 10% buffered formalin and prepared for light microscopy assessment. A further 3-5mm section from the left lateral lobe was isolated and immediately snap-frozen in liquid nitrogen and stored at -80°C for assessment of glutathione content.

Table 4. 1: Blood sampling time points and volume (ml) for animals in diclofenac continuous infusion study.

Dose group	Number of animals in group	Predose	1hr	2hr	6hr	24hr	48hr	72hr	96hr	Terminal sample (120hr)
Vehicle infusion	3	0.2		0.2		1.2		1.8		3
	3	0.2	0.2		0.2		1.4		1.8	3
Diclofenac 12mg/kg/day	4	0.2		0.2		1.2		1.8		3
	3	0.2	0.2		0.2		1.4		1.8	3

4.2.6 Isolation of plasma from blood samples

Blood samples were collected from animals using tail vein knicks into heparinised tubes (Table 4. 1). Plasma was isolated from these blood samples through centrifugation at 2100g for 10 minutes at 4°C.

4.2.7 Analysis of diclofenac and diclofenac acyl glucuronide in plasma samples

48µl of plasma were collected at 1hr, 2hr, 6hr, 24hr, 48hr, 72hr and 96hr following initiation of infusion, as well as immediately before sampling, using a composite sampling approach as outlined in Table 4. 1. Plasma was immediately acidified through the addition of 2µl of 2M acetic acid in dH₂O (4% v/v) to stabilise any acyl glucuronide metabolite. Samples were subsequently immediately stored at -80°C until analysis (Sparidans et al., 2008).

Plasma concentrations of diclofenac and diclofenac-AG were quantified as described in chapter 3.

4.2.8 Pharmacokinetic analysis

Non-compartmental analysis of diclofenac pharmacokinetics was assessed using Phoenix Winnonlin (Version 5.2, Pharsight, Missouri). This allowed the area under the curve to be calculated.

4.2.9 Clinical chemistry assessment

Clinical chemistry assessment was undertaken at AstraZeneca, AlderleyPark, UK. In brief, 600µl heparinised blood samples were isolated from animals at 72hr, 96hr and terminal (120hr) time points, and analysed for plasma ALT, glutamate dehydrogenase (GLDH), bile acid, total bilirubin, conjugated bilirubin and unconjugated bilirubin using

a Roche P modular analyser and standard Roche reagents, as defined by manufacturer's instructions.

4.2.10 Histopathological assessment of hepatotoxicity

Immunohistochemical preparation of slides was undertaken at AstraZeneca, Alderley Park UK. All immunohistochemistry was performed on formalin-fixed paraffin-embedded sections. From these paraffin-embedded section 4µm sections were cut, mounted on glass slides, dewaxed, and rehydrated. All antibody washes and dilutions were undertaken utilising Tris Buffered Saline (TBS) containing 0.1% Tween (v/v). To allow visualisation of antibodies, sections were incubated with 3,3'-diaminobenzidine and counterstained with Carazzi's Hematoxylin. Sections were subsequently dehydrated, cleared and mounted. Appropriate positive and negative controls were utilized for analysis.

For catalase and superoxide dismutase immunohistochemistry, heat-induced epitope retrieval was performed using 0.01M citrate buffer pH 6.0. Endogenous peroxidase was blocked using 3% H₂O₂ (v/v) for 10 minutes. Non-specific immunoglobulin (Ig)-binding sites were blocked using Background Blocker with Casein for 20 minutes at room temperature (RT). Sections were incubated in either polyclonal rabbit anti-catalase (1:100 dilution) or Rabbit anti-SuperOxide Dismutase-2 (1:2000 dilution) for 60 minutes at room temperature. Following primary antibody incubation, sections were washed and incubated with a ready-to-use peroxidase-labelled secondary reagent, X-cell plus polymer HRP for 15 minutes at RT and subsequently washed again. For CYP2C19 immunohistochemistry, heat-induced epitope retrieval was achieved following incubation with proteinase K for 2 minutes. Endogenous peroxidase was blocked using 3% H₂O₂ (v/v) for 10 minutes. Non-specific Ig-binding sites were blocked

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using Background Blocker with Casein for 20 minutes at RT. Sections were incubated in either polyclonal rabbit anti-CYP2C19 (1:500 dilution) for 60 minutes at room temperature. Following primary antibody incubation, sections were washed and incubated with a ready-to-use peroxidase-labelled secondary reagent, X-cell plus polymer HRP for 15 minutes at RT and subsequently washed again.

For CYP1A1 staining, sections were immersed in 0.01M citrate pH 6.0 buffer and heated to 100°C in RHS-2 processor for 5 minutes. Endogenous peroxidase was blocked with 3% H₂O₂ for 10 minutes, and non-specific Ig-binding sites were blocked using Background blocker with Casein for 20 minutes at RT. Sections were incubated in goat anti rat CYP1A1 (1:3000) for 60 minutes at RT. Following this, a goat probe and goat HRP-polymer (Biocare Medical GHP516H) were applied for 15 minutes each, with sections then washed.

For BSEP, heat-induced epitope retrieval was achieved following incubation of sections with 10mM EDTA, pH 8.0. Endogenous peroxidase was blocked with 3% H₂O₂ for 10 minutes, and non-specific Ig-binding sites were blocked using Background Blocker with Casein for 20 minutes at RT. Sections were incubated in rabbit anti-BSEP (1:30 dilution) for 60 minutes at RT. Following primary antibody incubation, sections were washed and incubated with a ready-to-use peroxidase-labelled secondary reagent, X-cell plus polymer HRP for 15 minutes at RT. Thereafter, sections were washed again.

For MRP2, heat-induced epitope retrieval was again achieved following incubation of sections with 10Mm EDTA, pH 8.0. Endogenous peroxidase was blocked with 3% H₂O₂ for 10 minutes, and non-specific Ig-binding sites were blocked using Background Blocker with Casein for 20 minutes at RT. Sections were incubated with mouse anti-MRP2 (1:300 dilution, Abcam: ab3373) for 60 minutes at RT. A biotinylated rabbit anti

mouse secondary (1:400 dilution) was then applied for 30mins. Following secondary antibody incubation, sections were washed and incubated with X-cell plus polymer HRP for 15 minutes at RT. Thereafter, sections were washed again in buffer.

Sections were kindly read by John Foster, AstraZeneca, and scored on a scale from Grade 1 to Grade 4, as defined by Table 4. 2.

Table 4. 2: Scoring method used for histopathological analysis of sections

Grading	Severity
Grade 1	Minimal / Very few/ Very small
Grade 2	Slight / Few / Small
Grade 3	Moderate / Moderate number / Moderate size
Grade 4	Marked / Many / Large

4.2.11 Hepatic glutathione concentration

Hepatic total glutathione levels (reduced glutathione (GSH) + oxidised glutathione (GSSG)) were determined using a microtitre plate assay. In brief, approximately 50mg of hepatic tissue isolated from the medial lobe were isolated, and homogenised in 200µl of 6.5% 5-sulphosalicylic acid (w/v) and 800µl glutathione stock buffer (143 mM NaH₂PO₄, 6.3mM EDTA, pH7.4 using 5M NaOH). Homogenised tissue was incubated on ice for 10 minutes before centrifugation at 14,000 rpm for 5 minutes. The supernatant was removed and used to determine total glutathione content spectrophotometrically at 412nm using 5,5'-dithio-bis(2-nitrobenzoic acid), NADPH and GSH reductase, as described by (Vandeputte et al., 1994). The results were compared to GSH standards (0-40 nMol/ml), and were normalised to protein content in the protein pellet.

4.2.12 Hepatic protein concentration for glutathione normalisation

Protein concentration in pellets achieved from centrifugation in the glutathione assay was achieved using BioRad protein assay reagent as described by (Bradford, 1976). Protein standard curves were prepared using bovine serum albumin fraction V (0.5mg/ml).

4.2.13 Statistical analysis

To test if a significant difference could be observed in plasma diclofenac concentration between the 24hour and 48 hour time points (Figure 4. 1) the data were first assessed for normality using a Shapiro-Wilk test. As this found that the data could be described using a normal distribution, an unpaired t-test was used to investigate statistical significance.

To test if a significant difference between vehicle control and diclofenac infused animals for clinical chemistry assays at each time point (Figure 4. 2), data was tested for normality using a Shapiro-Wilk test. If data was found to follow a normal distribution an unpaired t-test was used to investigate significance, and a Mann-Whitney Rank Sum test was used to test significance for non-normal data.

For both tests a statistical significance was adjudged to have been found if $p < 0.05$.

Statistical analyses were assessed in SigmaPlot for Windows Version 11.0.

4.3 RESULTS

4.3.1 Investigation into the maximum tolerated dose to be used for continuous intravenous infusion of diclofenac to the rat using a dose raising study

To allow the best opportunity for hepatotoxicity and detection of covalent adducts to plasma proteins, an initial dose-raising study was run to ascertain the maximum tolerated dose of diclofenac infusion.

An estimated maximum tolerated continuous intravenous dose of diclofenac was derived from the literature. It is widely reported that the dose limiting toxicity of diclofenac is due to gastro-intestinal toxicity. The LD₅₀ following an oral dose of diclofenac to the rat was reported at 250mg/kg (Menasse et al., 1978). With the bioavailability of diclofenac in the rat following an oral dose being reported at 79% following an oral dose of 1.25mg (Peris-Ribera et al., 1991), it was estimated that the LD₅₀ of an intravenous dose of diclofenac would be 197.5 mg/kg. Therefore, for an initial dose in a dose finding continuous intravenous infusion study it was decided to use a dose at least 10 times less than this. A dose of 12mg/kg/day was used for the first investigation.

This dose of 12mg/kg/day was well tolerated, with both animals in the test group surviving to the end of the in-life phase. No clinical signs of adverse effects due to drug treatment were observed during the in-life phase of the study. Upon necropsy, dark brown/black discolouration of areas of the cecum was observed, however, the dose was generally considered to be well tolerated. Therefore, a higher dose of 24mg/kg/day was investigated.

This higher dose of 24mg/kg/day was not tolerated in one of the two animals investigated. This animal was prematurely removed from the study approximately 28 hours into the 48 hour infusion. This is due to exhibition of piloerection, reduced motor activity and reduced respiration rate combined with increased respiration depth. On necropsy, dark red discolouration of the jejunum and black colouration of the cecum were observed. The second rat in this group completed the infusion protocol, but exhibited piloerection, weight loss and low food consumption. The 24mg/kg/day dose was not considered to be tolerated, and a 12mg/kg/day infusion dose was selected for the main study.

4.3.2 Investigation into plasma pharmacokinetics of diclofenac and diclofenac-AG following continuous intravenous infusion of 12mg/kg diclofenac/day.

Plasma concentrations of both diclofenac and its AG metabolite were monitored throughout the study, using the same analytical assay described in Chapter 3, meaning that the lower limit of quantification was maintained at 50nM for diclofenac and 30nM for diclofenac-AG. Lowest limits of detection were below 10nM for both diclofenac and diclofenac-AG.

Plasma concentrations of diclofenac rose rapidly following initiation of infusion (Figure 4. 1), reaching a concentration of $1.19 \pm 0.37\mu\text{M}$ over the first 6 hours of the experiment. Plasma diclofenac concentrations subsequently appeared to stabilise between the 6 and 24 hour time points (24 hour concentration was $1.22 \pm 0.20\mu\text{M}$), with no statistical difference detected between the plasma concentration between these timepoints. A statistically significant decrease in plasma diclofenac concentration was observed between the 24 and 48 hour time points, with plasma

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concentration decreasing to approximately 423 ± 101 nM. This reduction in plasma concentration whilst infusion was maintained suggests an adaptive change in animals resulting in enhanced drug clearance. This is supported by plasma clearance of diclofenac increasing by a multiple of 1.76 (from $13.37 \text{ ml kg}^{-1} \text{ min}^{-1}$ for 0-24hr time points to $23.48 \text{ ml kg}^{-1} \text{ min}^{-1}$ for the 0-48hr time points, Table 4. 3). Following cessation of dosing, diclofenac concentrations remained above the lower limit of quantification at the 72 hour and 96 hour time points ($119.83 \pm 9.64 \text{ nM}$ and $76.08 \pm 23.14 \text{ nM}$ (mean \pm stdev) respectively).

Throughout the experiment, diclofenac-AG could not be detected in plasma from the dose rats. Due to the dilutions required for quantification of plasma diclofenac concentrations, it was not possible to use less diluted samples to investigate whether very low levels of diclofenac-AG might have been present, due to the need to avoid possible overloading of the HPLC-MS/MS with diclofenac.

No diclofenac or diclofenac-AG could be detected in any plasma samples isolated from vehicle control animals.

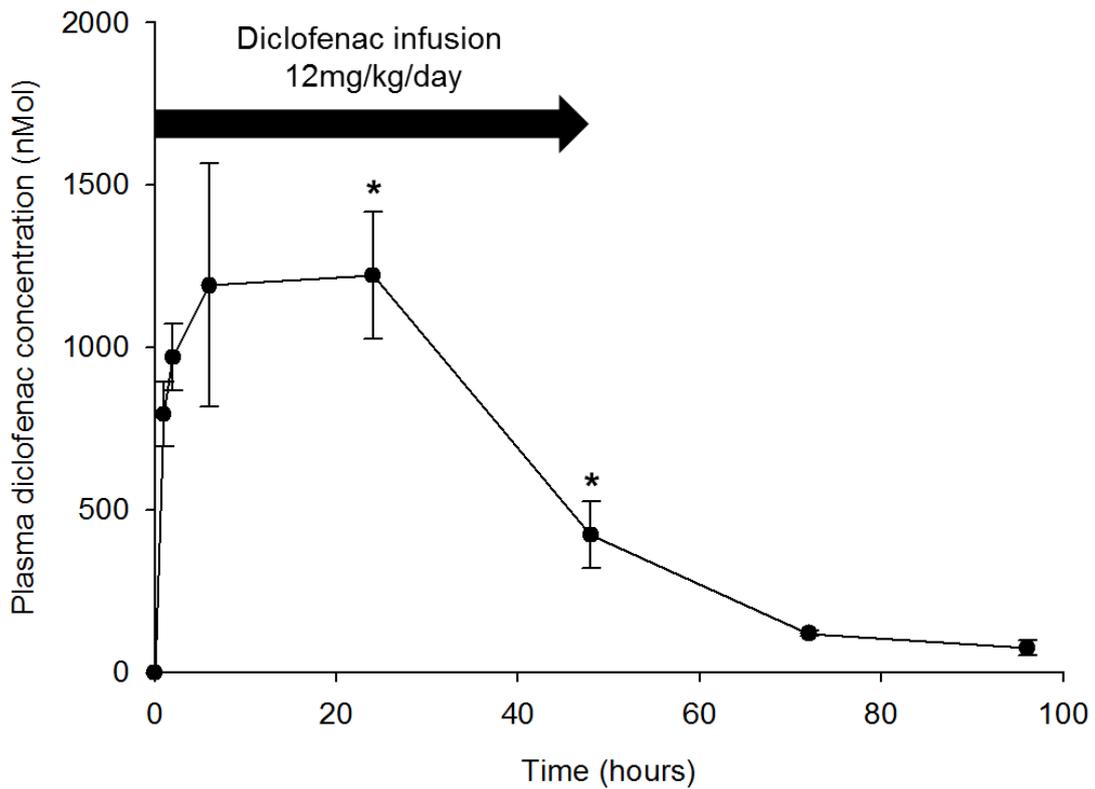


Figure 4. 1: Pharmacokinetic profile of diclofenac following continuous intravenous infusion to the rat for 48 hours (mean \pm stdev). * represents significant difference between the two points ($p \leq 0.001$) assessed using an unpaired t test.

Table 4. 3: Pharmacokinetic parameters of diclofenac following continuous intravenous infusion to the rat. Due to composite sampling used in the study, data was analysed from mean parameters

Infusion timepoint	Dose (nmol/kg)	AUC (nmol hr L ⁻¹)	Plasma clearance (ml kg ⁻¹ min ⁻¹)
Diclofenac ₀₋₂₄	37724	47030.4	0.802
Diclofenac ₀₋₄₈	75448	53544.7	1.409
Diclofenac ₀₋₉₆	75448	55893.5	1.350
Diclofenac _{0-∞}	75448	58022.0	1.300

4.3.3 Assessment of hepatotoxicity induced by continuous intravenous infusion of diclofenac

A combination of histopathology, immunohistochemistry and plasma clinical chemistry assays were used to assess whether continuous infusion of diclofenac resulted in hepatotoxicity.

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Plasma samples taken at 72, 96 and 120 hour time points (all after cessation of dosing) were analysed for alanine transaminase, lactate dehydrogenase, total bile acid concentration, concentrations of both free and conjugated bilirubin, and glutamate dehydrogenase. No statistically significant differences between diclofenac and vehicle treated animals could be detected for any of these clinical chemistry assays at any time point assayed throughout the investigations (Figure 4. 2). These studies therefore did not provide evidence that administration of diclofenac had elicited hepatotoxicity. Following the end of the in-life phase of the study, livers were isolated from animals and assessed for hepatic glutathione content. No difference between diclofenac and vehicle treated animals was detected (Figure 4. 2).

Further liver sections were isolated at the end of the study, and prepared for histopathological and immunohistochemical analysis by a trained veterinary pathologist. Haematoxylin and eosin (H & E) staining was used for assessment of alterations between diclofenac and vehicle treated animals. Intrahepatocyte depletion of glycogen stores was observed in 4 out of 7 diclofenac treated animals in the study, as observed by the loss of intrahepatocyte vacuoles in H&E sections. Glycogen depletion was graded as grade 2 severity in 3 animals, and grade 1 severity in 1. Glycogen depletion was not observed in any of the animals receiving vehicle infusion.

On necropsy a growth was observed on the liver of one diclofenac treated animal, which (approximately 10mm width x10mm length x10mm height). Upon histopathological staining, this was identified as an adhesion encapsulated haematoma. In addition, the right medial lobe of the same animal appeared large upon necropsy. Following H&E assessment, the lobe was identified as being hypertrophied. Furthermore, this animal along with one other diclofenac treated

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animal also showed an area of inflammatory cell infiltration and one further distinct diclofenac treated animal showed an area of necrosis with inflammatory cell infiltration (Table 4. 4).

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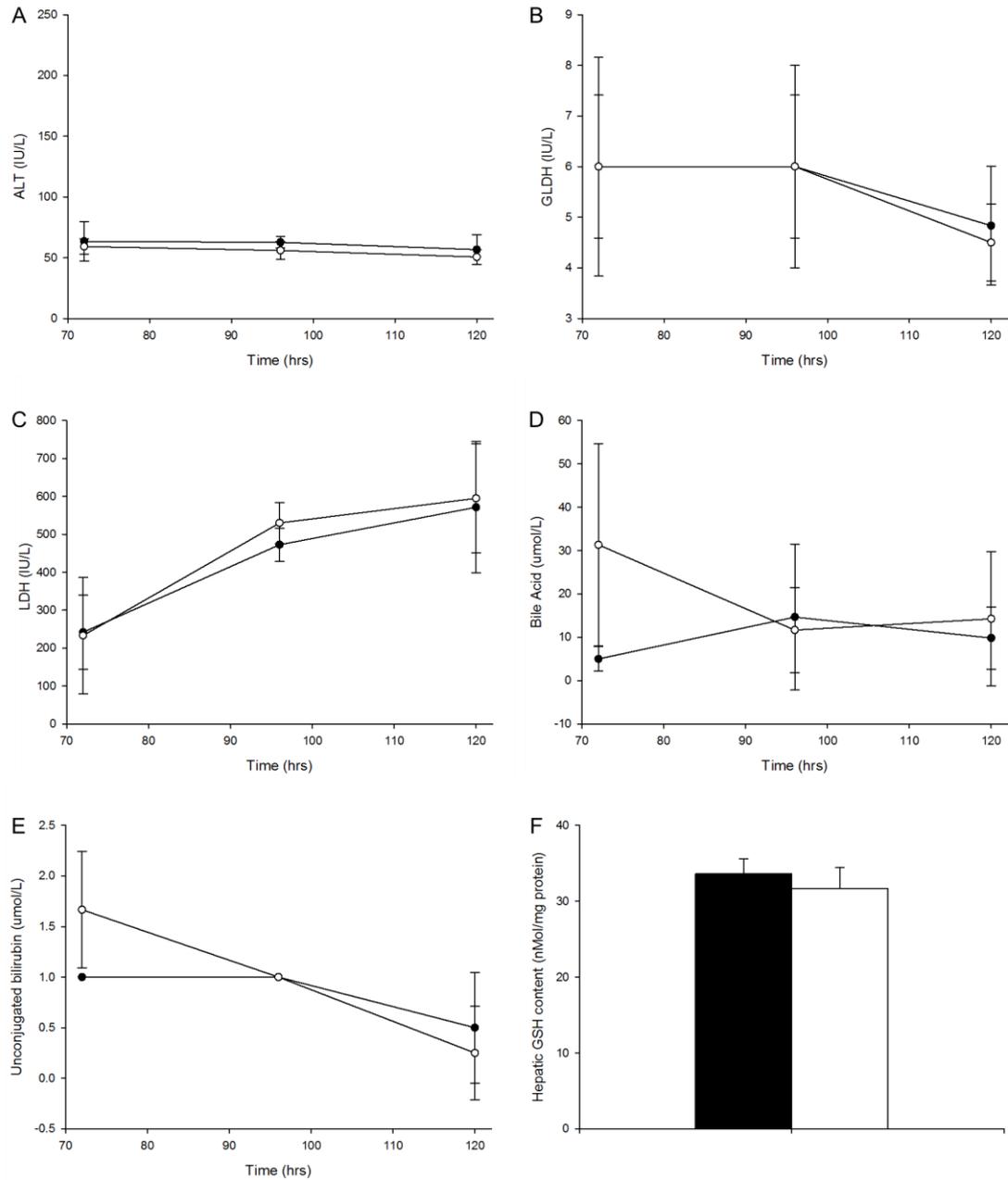


Figure 4. 2: Plasma biomarker assessment of hepatotoxicity and hepatic glutathione content. Black filled in figures represent vehicle control, hollow white figures represent diclofenac infusion (mean \pm stdev). (A) Plasma ALT activity (B) Plasma GLDH activity (C) plasma LDH activity (D) plasma bile acid concentration (E) total plasma bilirubin concentration (F) hepatic glutathione content from livers isolated from animals at the end of the study (120 hrs following commencement of diclofenac infusion). No statistical significance was observed between diclofenac or vehicle control treated animals.

Table 4. 4: Histopathological assessment of livers sections, allowing comparison between vehicle control and diclofenac infused animals.

Histopathological finding	Vehicle (5% mannitol) infusion	Diclofenac continuous infusion (12 mg/kg/day)
Glycogen depletion	Not detected in any animals	Grade 1 : 1 animal Grade 2 : 3 animals
Necrotic foci with inflammatory cells	Not detected in any animals	Grade 2 : 1 animal
Mixed inflammatory cell infiltration	Grade 1 : 2 animals	Grade 1 : 2 animals
Hypertrophied right median lobe	Not detected in any animals	1 animal
Adhesion encapsulated haematoma	Not detected in any animals	1 animal

4.3.4 Immunohistochemical assessment of liver sections

It was hypothesised that either induction of cytochrome P450 enzymes or hepatic export transporters could have contributed to the reduced plasma concentrations that were evident at time intervals beyond 24 h of continuous infusion. Consequently, hepatic sections were immunohistochemically stained to assess expression of the cytochrome P450 isozymes CYP1A1 and 2C19 as well as the hepatic export transporters MRP2 and BSEP.

Following analysis of sections, no consistent induction of the CYPs 1A1 or 2C19 could be found. In addition, no consistent induction of the export transporters MRP2 and BSEP could be ascertained as reported in Table 4. 5.

Table 4. 5: Scored results of sections stained for immunohistochemistry. 6 animals were in the vehicle control group, and 7 animals were in diclofenac treated animals.

Hepatic staining	Vehicle (5% mannitol) infusion	Diclofenac continuous infusion (12 mg/kg/day)
CYP1A1 IHC	Grade 1 : 3 animals Grade 2 : 3 animals	Grade 1 : 4 animals Grade 2 : 3 animals
CYP2C19 IHC	Grade 1 : 1 animal Grade 2 : 2 animals Grade 3 : 3 animals	Grade 1 : 1 animal Grade 2 : 5 animals Grade 3 : 1 animal
BSEP IHC	Grade 1 : 2 animals Grade 2 : 4 animals	Grade 1 : 5 animals Grade 2 : 2 animals
MRP2 IHC	Grade 1 : 0 animals Grade 2 : 6 animals	Grade 1 : 5 animals Grade 2 : 2 animals
Catalase IHC	Grade 1: 5 animals Grade 2 : 1 animal	Grade 1 : 1 animals Grade 2 : 6 animals
Superoxide dismutase-2 IHC	Grade 1 : 5 animals Grade 2 : 1 animal Grade 3 : 0 animals	Grade 1 : 1 animal Grade 2 : 1 animal Grade 3 : 5 animals

Diclofenac and its oxidative metabolites have been shown to elicit mitochondrial stress following incubation with isolated mitochondria and hepatocytes (Bort et al., 1999; Gomez-Lechon et al., 2003a; Gomez-Lechon et al., 2003b; Lim et al., 2006). To test whether induction of oxidative stress had occurred in livers from rats dosed with diclofenac by continuous infusion, hepatic sections were stained for the expression of catalase and superoxide dismutase. Continuous infusion of diclofenac resulted in expression of elevated levels of both of these enzymes, when compared to vehicle control animals (Figure 4. 3 and Figure 4. 4).

Hepatic sections isolated from animals which had been infused with the dose vehicle infusion exhibited greater expression of SOD-2 in hepatocytes distributed around the central vein, although protein expression was also evident in in portal hepatocytes. Continuous infusion of diclofenac resulted in increased intensity of SOD-2

immunostaining around the vasculature. This may be an indicator of oxidative stress. Conversely, midzonal hepatocytes appeared to have reduced SOD2 expression following diclofenac infusion, when compared to centrilobular or periportal hepatocytes.

Catalase expression in rats receiving vehicle was evident primarily in portal areas, with reduced intensity of staining in centrilobular regions. Intracellular catalase intensity was highest around peroxisomes (Figure 4. 3C). Catalase staining in animals receiving diclofenac infusion was not apparent in portal areas, but was induced in centrilobular hepatocytes. It was also noted that following diclofenac treatment, peroxisomes appeared enhanced in size (Figure 4. 3D).

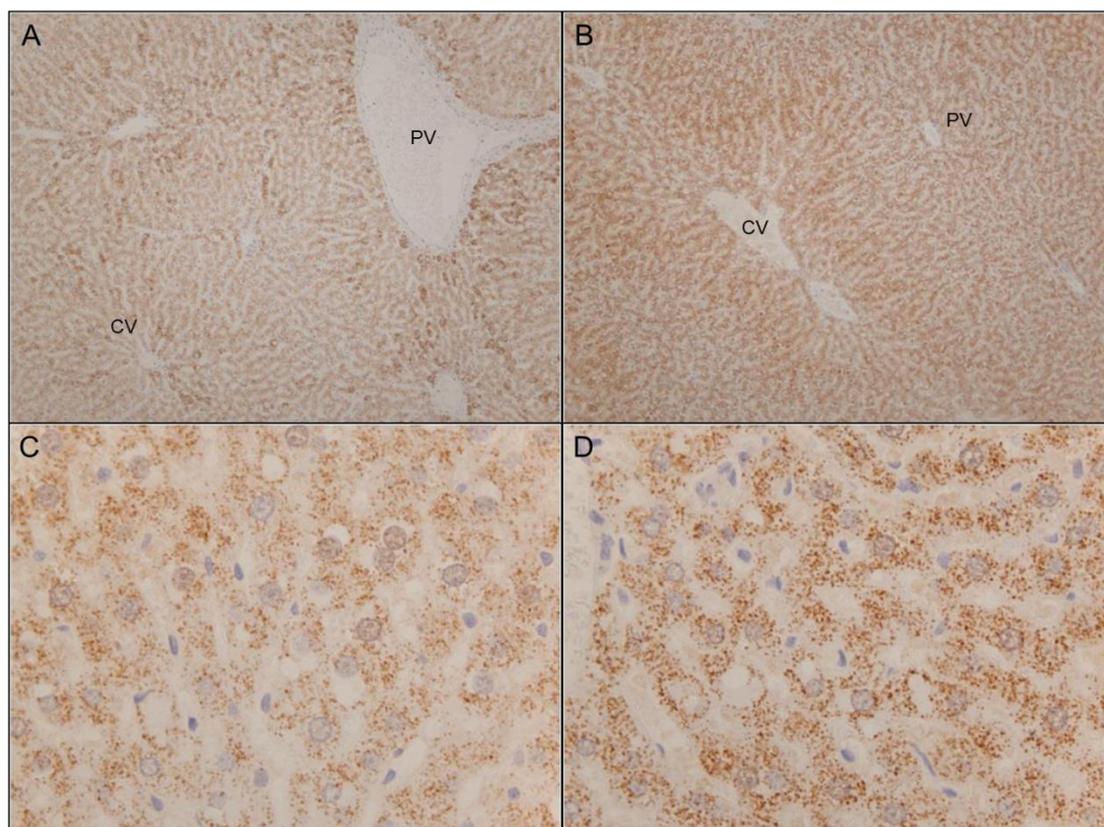


Figure 4. 3: Continuous infusion of diclofenac induces expression of catalase in the liver. (A+C) vehicle control animal (B+D) diclofenac infusion 12mg/kg/day for 48 hours. CV represents central vein, PV represents portal vein. (A+B) at 10x magnification (C+D) at 40x magnification.

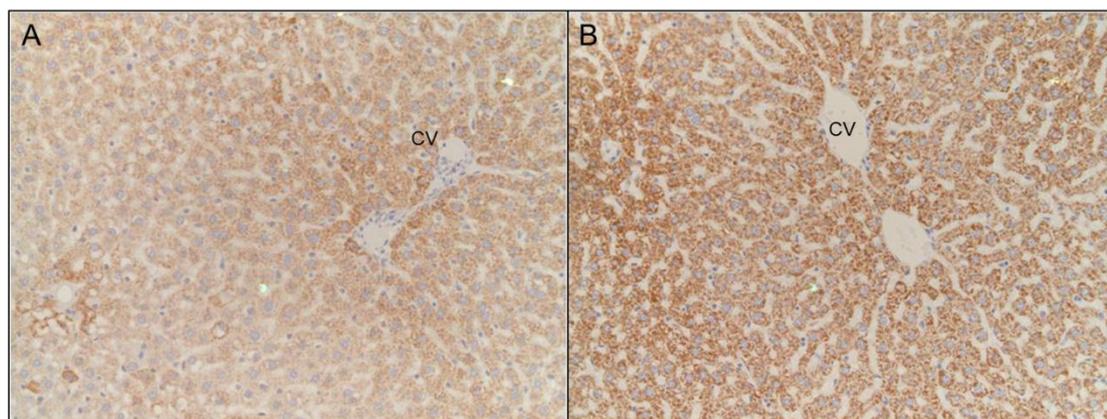


Figure 4. 4: Continuous infusion of diclofenac induces hepatic superoxide dismutase immunostaining in cetntral vein (CV) areas. (A) vehicle control (B) diclofenac infusion. Both figures at 10x magnification.

**4.3.5 Identification of protein binding following continuous intravenous
infusion of diclofenac to the rat.**

No evidence of covalently modified rat serum albumin adducts could be detected in plasma from diclofenac treated rats, using LC-MS technology.

4.4 DISCUSSION

Currently, no investigations of the continuous infusion of diclofenac to the rat have been reported in the literature. However, continuous intravenous infusion of diclofenac to humans, at a dose of 0.96mg/kg/day, has been reported to control fever in patients with acute cerebral damage and in neurosurgical critical care (Cormio et al., 2000; Cormio and Citerio, 2007).

Continuous intravenous infusion of diclofenac to the rat followed first order kinetics over the first 24 hours of infusion. A rapid increase in diclofenac plasma concentration was observed initially, which subsequently slowed, culminating in steady-state diclofenac exposure between the 6 and 24 hour time points following commencement of infusion (Figure 4. 1). Similar pharmacokinetic profiles have been observed for numerous other compounds following continuous intravenous infusion to either the rat or man, where the infusion has been maintained for sufficient time for plasma concentration to induce a rate of clearance matching that of continuous infusion (Bowersox et al., 1997; Arens and Pollack, 2001; Boselli et al., 2003)

Subsequently, between the 24 and 48 hour time points a statistically significant decrease in plasma diclofenac concentrations was found. Plasma diclofenac concentrations declined by 65% (from $1.220 \pm 0.195\mu\text{M}$ to $0.423 \pm 0.102\mu\text{M}$ respectively). This decline occurred whilst continuous intravenous infusion of diclofenac was maintained. The pharmacokinetic analysis indicated that this decrease in plasma diclofenac concentration was indicative of a 76% increase in diclofenac plasma clearance, from the 0-24hr clearance of 13.37 to $23.48 \text{ ml kg}^{-1} \text{ min}^{-1}$ to 0-48hours. Little evidence of equivalent pharmacokinetic changes following continuous infusion of other compounds could be identified in the literature, with

most drugs being found to maintain steady-state plasma concentrations until infusion was ceased. This suggests that this increased clearance is not a result of the infusion technique, but rather is likely to be due to an adaptive change resulting from continuous diclofenac exposure.

The primary mechanisms for drug plasma clearance are drug metabolism and drug export via active transport. Consequently, it was hypothesised that continuous infusion of diclofenac induced either drug metabolism or drug clearance, resulting in its enhanced plasma clearance between the 24 and 48hour timepoints.

Diclofenac is metabolised into 3 primary metabolites in most species, including the rat and man, namely CYP mediated oxidation into the 4-OH and 5-OH metabolites and diclofenac-AG. In the rat, the CYP enzymes 2B, 2C and 3A isoforms have been associated with diclofenac oxidation (Tang et al., 1999b), with glucuronidation catalysed by UGT2B1 (King et al., 2001). Diclofenac-AG is transported into bile via the active biliary efflux transporter Multi-Drug Resistance Protein Type 2 (Mrp2), following which it is eliminated via bile in faeces, is hydrolysed to liberate parent diclofenac which undergoes enterohepatic cycling and /or is metabolized by CYPs to hydroxy-AG metabolites (Seitz et al., 1998). On analysis of hepatic sections immunohistochemically stained using an MRP2 antibody, no induction could be identified on comparison between diclofenac and vehicle infused animals. In the literature, whilst MRP2 has been shown to be inducible (Courtois et al., 1999; Payen et al., 2001; Choi et al., 2007), its induction by diclofenac has not been reported. Genetic associations with mutations in the ABCC2 gene encoding MRP2 have suggested that reduced function of MRP2 is a risk factor in developing diclofenac hepatotoxicity (Daly et al., 2007), suggesting accumulation of diclofenac or its metabolites may be involved in the pathogenesis of

hepatotoxicity, and consequently, induction of this enzyme on exposure to diclofenac may be desirable cellular defence pathway. However no identification of its induction could be detected in this study.

Staining was also added to investigate whether diclofenac infusion resulted in hepatic induction of the bile salt export pump (BSEP). Several case-reports of off-target hepatotoxicity elicited by diclofenac have been associated with generation of cholestasis in patients (Dunk et al., 1982; Banks et al., 1995; Hackstein et al., 1998), however it is not clear whether diclofenac induced cholestasis is a causative factor in diclofenac hepatotoxicity, or is a consequence of other hepatotoxic mechanisms of the drug. To investigate the potential for diclofenac to elicit obstructive cholestasis plasma bile salt and bilirubin concentrations were assessed combined with investigation of induction of BSEP transcription. Through this experiment, no evidence of BSEP induction or induction of cholestasis could be found.

Induction of oxidative metabolism was also hypothesised to be a mechanism responsible observed increased plasma clearance of diclofenac. Many CYP enzymes have been found to be inducible, usually following a drugs activation of gene pathways resulting in the stimulation of RNA synthesis (Guengerich, 2001).

Oxidative metabolism of diclofenac is catalysed by CYP2B, 2C and 3A families, with CYP2C11 and CYP2C7 identified as subtypes involved in diclofenac metabolism (Tang et al., 1999a). As no synthetic standards of 4-hydroxy and 5-hydroxy diclofenac were available to allow accurate method development and mass-spectrometric quantification, immunohistochemical staining of liver sections for the CYP isoforms 1A1 or 2C19 were used to investigate induction of these enzymes as a possible mechanism for enhanced plasma diclofenac clearance following its continuous

intravenous infusion. No CYP3 family immunohistochemical stains were available for the investigation of CYP induction.

No consistent induction of CYP enzymes could be identified in hepatic sections isolated from diclofenac infused animals when compared with vehicle controls (Table 4. 5). This is consistent with published literature where no induction of either CYP enzymes or UGTs by diclofenac has been reported using either *in vitro* or *in vivo* assays. Whether this reflects an absence of investigation into diclofenac's ability to induce CYP enzymes remains unclear.

For these immunohistochemical studies, however, it should also be noted that the timepoint for isolating the liver for stabilisation and immunohistochemical stabilisation was 72 hours following cessation of diclofenac delivery. During this 3 day period it is possible that hepatocytes may have recovered, subsequently degrading induced CYP protein, with the cell returning to a normal phenotype. It should also be noted that the absence of CYP2B and 3A immunohistochemistry, that induction of these enzymes may also have been responsible for increased diclofenac clearance following infusion. However, no evidence for their induction by diclofenac either has been reported in the literature. Consequently no evidence was attained identifying induction of metabolism as a mechanism for increased diclofenac plasma clearance.

Plasma concentrations of diclofenac acyl glucuronide were monitored throughout the experiment; however this metabolite could not be detected in plasma at any time point of sampling. It should be noted that samples were diluted either 1 in 2, or 1 in 4, prior to analysis. Consequently the lower limit of AG detection at these dilutions would be 20nM or 40nM. Undiluted samples could not be assayed due to the sensitivity of the mass-spectrometer. Due to the known concentration of diclofenac in

these samples, there was a high chance of overloading the system if undiluted samples had been analysed.

In vitro investigations using liver microsomes have predicted that acyl glucuronidation accounts for approximately 70% of the clearance of diclofenac in the rat (Kumar et al., 2002), however the rate of acyl glucuronidation by rat UGT2B1 is 3 time slower than that of human UGT2B7 (King et al., 2001).

AG metabolites have been detected in plasma following single bolus administrations of the aglycone to the rat for several compounds including diflunisal and suprofen (Dickinson et al., 1989; Smith and Liu, 1995), potentially continuous intravenous infusion of diclofenac at a rate of 0.5mg/hour may be insufficient to allow turnover by UGTs to form the for AG metabolism in the rat to levels detectable in the plasma. AG metabolites are largely biliary excreted in the rat (Peris-Ribera et al., 1991), with TR^{-/-} rats deficient in MRP2 showing the importance of this transporter in the efflux of diclofenac-AG across the cannalicular membrane (Seitz et al., 1998). This export pump driven efflux of AG metabolites has been associated with eliciting high up-concentrations of AG metabolites along the cannalicular membrane and away from the basolateral membrane (Sallustio et al., 2000), resulting in reports of biliary concentrations of gemfibrozil-AG 50 to 5000 times higher than the circulatory perfusate (Sabordo et al., 1999; Sabordo et al., 2000). Active up-concentrations of AG metabolites are also thought to be maintained by active transport across the basolateral membrane back from the blood into the hepatocyte (Sallustio et al., 2000). Consequently for AGs to reach the plasma, it is likely that they would need to be formed in concentrations sufficient to overwhelm this hepatic concentration-driven efflux.

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It was further hypothesised that continuous intravenous infusion of diclofenac, resulting in continuous exposure of the drug and/or its metabolites might elicit hepatotoxicity in the rat. However, throughout the study, no identification of diclofenac hepatotoxicity could be identified using either clinical chemistry assessment (Figure 4. 2) although possible diclofenac induced alterations in liver histopathology were observed in some diclofenac treated animals following assessment of H&E stained liver sections (Table 4. 4). Restriction of blood sampling volumes, as dictated by the project license and the desire for close monitoring of plasma diclofenac concentrations, meant that time points for clinical chemistry assessment of hepatotoxicity were not ideal. Since the half-life of alanine transaminases has been estimated to be 8 hours in the rat (Ennulat et al., 2010), it cannot be ruled out that raised ALTs may have occurred in diclofenac treated animals at time intervals been cleared before clinical chemistry sampling at the 72 hour time point. No evidence of cell death was evident on examination of H&E stained liver sections. This observation is in line with the findings of Sallustio, who found no induction of hepatotoxicity following daily oral administration of diclofenac at a dose of 15mg/kg (Sallustio and Holbrook, 2001). With the reported bioavailability of diclofenac at 79% (Peris-Ribera et al., 1991), Sallustio's bioavailable dose is calculated at 11.85mg/kg, close to our 12mg/kg/day dose. Intraperitoneal administration of diclofenac at bolous doses of 100mg/kg to the rat and 80mg/kg to the mouse has been shown to elicit hepatotoxicity (Gomez-Lechon et al., 2003b; Deng et al., 2008). These doses far exceed the 24mg/kg dose of diclofenac received by rats over the 48 hour period of this infusion study.

Translocation of bacteria and endotoxins from the GI tract has been suggested to play a key role in heightening immune responses and exacerbating toxicity. Evidence of this for diclofenac has been shown from the attenuation in plasma ALTs by pharmacological sterilisation of a rat's GI tract prior to diclofenac administration (Deng et al., 2006). The dose selected for this infusion was designed to elicit minimal toxicological stress on the gastro-intestinal tract of rats, translocation of gastro-intestinal bacteria and endotoxins to the liver may have been abated, and may provide an explanation by which hepatotoxicity observed in the single large doses reported in the literature was abated. However, clinical evidence supporting this 'germ theory' is lacking, as histopathological examination of liver biopsies from patients undergoing an idiosyncratic adverse drug reaction to diclofenac as yet has not revealed bacterial hepatic translocation.

Despite this, the immune system does appear to have a critical role in the eliciting of diclofenac induced hepatotoxicity. Pharmacogenetic studies have revealed a 5-fold increased risk in development of diclofenac induced hepatotoxicity in patients expressing polymorphisms in genes encoding the interleukins IL-4 and IL-10 (Aithal et al., 2004), and associations with HLA11 (Berson et al., 1994). Detection of circulating antibodies recognising diclofenac-modified rat hepatic proteins in 100% of patients experiencing diclofenac induced hepatotoxicity, and 60% of patients receiving diclofenac therapy without hepatotoxicity, combined with identification of diclofenac-modified hepatic proteins in a liver isolated from a patient experiencing diclofenac induced hepatotoxicity provide good evidence for the immune system and the role of drug-protein adducts. Further evidence has been achieved from mouse models, where lymph nodes isolated from diclofenac sensitised mice were stimulated on *ex vivo*

exposure to the hydroxyl metabolites of diclofenac (Naisbitt et al., 2007), and selective killing of diclofenac-exposed hepatocytes by splenocytes isolated from mice sensitised to diclofenac (Kretz-Rommel and Boelsterli, 1995). The source of danger signals, which are hypothesised to be necessary for propagation of delayed ADRs to diclofenac, however remain unclear.

In vitro investigations have associated diclofenac with eliciting cell death through perturbation of mitochondrial function. Two separate mechanisms have primarily been hypothesised in eliciting diclofenac mitochondrial disruption. Due to its weakly acidic properties, combined with its lipophilic nature, diclofenac has been hypothesised to readily cross the outer membrane of mitochondria, acting to uncouple mitochondrial oxidative phosphorylation (OXPHOS) (Whitehouse, 1967; Boelsterli and Lim, 2007). Further to this, the diphenylamine structure of NSAIDs including diclofenac has been suggested to be important in this function (Masubuchi et al., 1999; Masubuchi et al., 2000). Oxidative metabolites of diclofenac have also been suggested to act synergistically to mitochondrial toxicity elicited through the parent compound through inducing increased cellular calcium concentrations, subsequently inducing mitochondrial permeability transition (mPT), and eliciting cell death (Lim et al., 2006). Experimental evidence for diclofenac eliciting mitochondrial stress to hepatocytes has only ever been shown following *in vitro* incubation, with no direct *in vivo* evidence. Further to this, diclofenac has only been identified as cytotoxic to hepatocytes following incubation at super-physiological concentrations, with the exception of one recent study where repeat administration of diclofenac to primary human hepatocytes to elicit hepatotoxicity at 6.5µM concentration (Mueller et al., 2012). With 99.5% of a

dose of diclofenac plasma bound (Chamouard et al., 1985), the potential of diclofenac to elicit mitochondrial stress *in vivo* remains controversial (Boelsterli and Lim, 2007).

Histopathological assessment of H&E stained liver sections however, did not reveal any mitochondrial swelling, indicative of mitochondrial stress as a consequence of opening of the mPT pore. H&E staining however did reveal glycogen depletion from hepatocytes in 4 animals of the 7 receiving diclofenac infusion. Glycogen depletion in three of these animals was classified as grade 2. Metabolism of glycogen in stores may represent a mechanism for replenishing energy production lost as a consequence of diminished ATP provision due to mitochondrial stress. To further test for mitochondrial stress, hepatic sections were immunohistochemically stained for catalase and superoxide dismutase as markers of reactive oxygen species released as a consequence of mitochondrial dysfunction.

Immunohistochemical assessment of liver sections revealed an induction in both catalase and superoxide dismutase staining following continuous infusion of diclofenac (Figure 4. 3, Figure 4. 4 and Table 4. 5). This suggests that diclofenac treatment may increase hepatic exposure to reactive oxygen species. Both catalase and superoxide dismutase staining was found most intense in the areas surrounding the central vein. This is where cytochrome P450 enzymes are most abundantly expressed (Lindros, 1997), potentially providing further evidence of the importance of oxidative metabolism in eliciting mitochondrial stress.

In summary, following continuous intravenous infusion of 12mg/kg/day for 48 hours, no hepatotoxicity could be detected using clinical chemistry assays and histopathological assessment of hepatic sections. Induction of mitochondrial stress was suggested by depletion of glycogen stores and induction of the ROS scavenging

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enzymes superoxide dismutase and catalase. These adaptive mechanisms appear to be protective mechanisms, abating cell death. Further to this, protection against hepatotoxicity also appears to have been elicited through induction of unidentified mechanisms 24hours following commencement of diclofenac infusion, resulting in enhanced plasma clearance of the parent drug.

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CHAPTER FIVE

FORMATION OF COVALENT PROTEIN ADDUCTS BY DICLOFENAC ACYL GLUCURONIDE METABOLITES IN PATIENTS

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5.1 INTRODUCTION

The biotransformation of drugs to reactive intermediates, able to covalently modify endogenous macromolecules, is hypothesised to play a key role in the pathogenesis of delayed hypersensitivity reactions and other off-target ADRs (Kalgutkar and Soglia, 2005; Park et al., 2005; Park et al., 2011). Consequently, pharmaceutical companies will usually preferentially avoid the incorporation of chemical structures capable of being metabolically bioactivated into CRMs in drug design, and routinely incorporate screens into pre-clinical drug development and candidate selection to assess a molecule's covalent binding towards macromolecules (Evans et al., 2004; Kalgutkar and Soglia, 2005; Nakayama et al., 2009; Thompson et al., 2012). However, covalent binding assays using radiolabelled compounds almost invariably have a major mechanistic limitation: if they employ simple hepatic microsomal incubations to generate reactive metabolites, they are usually restricted to assessing oxidative bioactivations (Evans et al., 2004); if they employ complex preparations, such as isolated hepatocytes (Thompson et al., 2012), which should express all of the various bioactivation pathways, they invariably create a substantial requirement for additional studies if the metabolic basis of a positive finding is to be identified.

Metabolic activation of carboxylate compounds to intermediates able to covalently modify proteins is also considered a critical stage in the pathogenesis of off-target ADRs associated specifically with this class of compounds (Boelsterli, 2002; Skonberg et al., 2008; Aithal, 2011). The covalent binding of carboxylate compounds *in vivo* is well established, as shown by their modification of plasma proteins in volunteers and patients (Smith et al., 1986; Hyneck et al., 1988; Volland et al., 1991; Munafo et al., 1993; Dickinson et al., 1994; Castillo et al., 1995) and in experimental animals (Smith et

al., 1990b; Sallustio and Foster, 1995; Liu et al., 1996; Dong et al., 2005). Modification of hepatic proteins has also been revealed in both humans (Aithal et al., 2004) and animals (Pumford et al., 1993; Hargus et al., 1994; Wade et al., 1997; Wang and Dickinson, 1998; Wang and Dickinson, 2000; Wang et al., 2002; Aithal et al., 2004). However, none of these investigations determined either the precise structural type or the location of the covalent modification. Proof of the adduction of plasma proteins rested solely on liberation of the parent drug from the unfractionated protein samples by non-specific alkaline hydrolysis. This method is assumed to release the carboxylic acid and any oxygenated metabolites (Hermening et al., 2000) selectively from (i) amine and ester (acylation/transacylation) adducts and (ii) ester linkages within glycation adducts derived exclusively from AG metabolites (Figure 2.2), and thereby assays acylation and glycation adducts collectively and indiscriminately (Smith et al., 1990a). While these assumptions are reasonable in respect of the parent carboxylate, oxygenated metabolites released by alkaline hydrolysis might have been adducted via alkylation reactions to cysteine residues through thioether bonds (Deng et al., 2011). Limited identification and quantification of modified hepatic proteins was achieved using Western blotting with anti-drug antibodies (Wade et al., 1997; Wang et al., 2001; Aithal et al., 2004). Radiotracer experiments in rats confirmed the covalent binding of carboxylate drugs – including diclofenac – to plasma and hepatic proteins (Masubuchi et al., 2007; Takakusa et al., 2008) but did not provide any additional insights into either the mechanisms or sites of bioactivation or the sites of protein adduction. Nevertheless, it should be noted that several drugs not known to form AGs also adducted plasma proteins.

The importance of haptentation in eliciting immune responses has been shown specifically for carboxylate compounds. Circulating antibodies to diclofenac metabolite-modified liver protein adducts were found in all patients diagnosed with diclofenac-induced liver injury, 60% of patients receiving diclofenac therapeutics, but in none of the healthy controls (Aithal et al., 2004). The specific killing of hepatocytes exposed to diclofenac (and showing protein modifications) by lymphocytes derived from spleens isolated from diclofenac-immunised mice provides further evidence for the importance of covalent modification in immune-mediated off-target ADRs associated with carboxylate compounds (Kretz-Rommel and Boelsterli, 1995).

Direct glucuronidation of carboxylate compounds to AGs has received the most attention as the bioactivation pathway potentially responsible for generation of these off-target ADRs. Many AGs have been shown to be chemically unstable (Table 2.1) and protein reactive (Munafo et al., 1990; Volland et al., 1991; Spahn-Langguth and Benet, 1992; Ding et al., 1993; Ding et al., 1995; Qiu et al., 1998; Ebner et al., 1999; Iwaki et al., 1999; Mortensen et al., 2001; Walker et al., 2007) during *in vitro* incubation. With evidence of the covalent binding of carboxylate compounds to plasma proteins in volunteers and patients correlating to AG plasma exposure (Smith et al., 1986; Hyneck et al., 1988; Volland et al., 1991; Benet et al., 1993; Munafo et al., 1993; Dickinson et al., 1994; Castillo et al., 1995), acyl glucuronidation has been designated a metabolic pathway of toxicological concern, producing uncertainty in the pharmaceutical industry as to how to progress AG-forming compounds safely (Faed, 1984; Spahn-Langguth and Benet, 1992; Boelsterli, 2002; Shipkova et al., 2003; FDA, 2008).

As a result of this anxiety, pharmaceutical companies often feel the need to provide evidence mitigating the potential risk of drugs found to form AGs in humans, often

deriving reassurance from the identification of AGs that are relatively stable *in vitro* and thereby possess low protein reactivity (Benet et al., 1993). For example, following the identification of citalopram's metabolism to a carboxylic acid which undergoes acyl glucuronidation, the authors felt the need to emphasise that the drug was administered at low doses (<40mg daily) and the AG was not a major metabolic product (Dalgaard and Larsen, 1999). Following identification of the extensive metabolism of the ester pro-drug BIBF 1120 to an AG, reassurance was derived from the conjugate's long degradation half-life at pH 7.4 (10.5 hours), and consequently it was claimed that the AG 'has a low tendency to form covalent adducts to protein due to its higher stability [*in vitro*] and thus should not be the cause of safety concerns', despite the identification of its acyl migration isomers circulating in plasma (Stopfer et al., 2011).

Further to these experiences, efforts have been made to investigate the applicability of pre-clinical tests to predicting the clinical risks of carboxylic acid drugs and their AG, with correlations between drug risk and AG instability *in vitro* (Sawamura et al., 2010), predictions of AG instability and protein adduction (Bolze et al., 2002; Wang et al., 2004; Karlsson et al., 2010) and extensive investigations into structure-activity relationships of AG (Vanderhoeven et al., 2004a; Vanderhoeven et al., 2004b; Baba and Yoshioka, 2009a; Baba and Yoshioka, 2009b; Yoshioka and Baba, 2009) being undertaken. Medicinal chemists are also investigating mechanisms to remove carboxylic acid structures from the pharmacophore (Crosignani et al., 2011a), or incorporating α -carbon moieties to sterically hinder acyl glucuronidation or provide increased stability to the resulting AG (Crosignani et al., 2011b; Gallant et al., 2011).

Anxiety over drug acyl glucuronidation, however, may be unjustified. To date, no evidence has been reported showing covalent modification of protein *in vivo* is definitely attributable to AGs, either in animal models, or in man. Moreover, rates of renal and biliary clearance of AGs in man are high (Smith et al., 1985; Verbeeck et al., 1988; Castillo et al., 1995; Balani et al., 1997; Hofmann et al., 2005; Wang et al., 2006), with the conjugate's clearance from biological systems further increased by their spontaneous and enzymatic hydrolysis in both plasma (Ruelius et al., 1986; Volland et al., 1991; Williams et al., 1992; Akira et al., 2002; Karlsson et al., 2010) and tissues, including the liver (Brunelle and Verbeeck, 1996; Iwamura et al., 2012). Consequently *in vivo* blood exposure of AGs may actually be relatively low in many cases, and certainly considerably reduced in comparison with exposures predicted by liver microsome incubations (Table 2.1). These factors have led to debate as to whether AG metabolites actually do form covalent protein adducts *in vivo*, and consequently whether they actually are of toxicological concern (Bailey and Dickinson, 2003; Shipkova et al., 2003; Regan et al., 2010). The most compelling evidence of protein modification by a xenobiotic AG in a complex biological system is the observation that covalent binding of diclofenac to proteins in isolated rat hepatocytes was greatly reduced by inhibitors of glucuronidation but was not affected by inhibitors of P450 (Kretz-Rommel and Boelsterli, 1993). Nevertheless, metabolic AG formation does not translate inevitably into measurable covalent protein binding in either isolated hepatocytes or *in vivo* (Levesque et al., 2007). If AG metabolites do not produce protein adducts *in vivo*, it would have to be assumed that the adduction of plasma and hepatic proteins by carboxylic acid drugs was due entirely to reactions with the diverse electrophilic products of oxidative bioactivation (Chen et al., 2006; Masubuchi et al.,

2007; Takakusa et al., 2008) and thioester conjugation (Grillo and Benet, 2002; Grillo, 2011) that many such drugs, including diclofenac (Shen et al., 1999; Poon et al., 2001; Grillo et al., 2003; Masubuchi et al., 2007; Grillo et al., 2008a; Grillo et al., 2008b; Waldon et al., 2010), are known to form.

To address this issue HSA, isolated from patients receiving conventional therapeutic doses of diclofenac, was analysed in the form of tryptic peptides, using tandem mass spectrometry. Survey scanning was used to search specifically for acylation adducts and adducts derived exclusively from AG metabolites; namely the simple, predictable, glycation adducts of lysine residues that were found following incubation of the protein with synthetic 1- β diclofenac-AG (chapter 2). In addition to glyating HSA in simple buffered incubations, diclofenac-AG has also been shown to glycate proteins in the much more complex environment of rat liver microsomes (Kretz-Rommel and Boelsterli, 1994). However, HSA was pragmatically selected for investigation of AG binding in these studies as it has a well characterised structure (Ghuman et al., 2005), is accessible and abundant, and has a long residence time in the circulation (Nicholson et al., 2000; Tornqvist et al., 2002), which favours accumulation of adducted protein (Zia-Amirhosseini et al., 1994), but it is also a physiologically relevant target for covalent modification by drug AG because these metabolites frequently circulate in plasma (Volland et al., 1990; Benet et al., 1993; Zia-Amirhosseini et al., 1994; Hermening et al., 2000; Zhang et al., 2011; Klepacki et al., 2012). HSA has a significant experimental advantage in that it can be readily extracted from plasma and greatly enriched by affinity chromatography (Greenough et al., 2004; Jenkins et al., 2009b). Additionally, even relatively unsophisticated analyses of circulating HSA for adducts of drug and non-drug xenobiotics and their metabolites have identified HSA as a common

and tractable target of diverse acylating and alkylating species (Noort et al., 1999; Johannesson et al., 2001; Johannesson et al., 2004; Damsten et al., 2007; McCoy et al., 2008). HSA has an established place in protein adductomics: the assessment of exposure to exogenous and endogenous electrophiles through characterisation and measurement of adducts (Rappaport et al., 2012). In certain cases (Johannesson et al., 2001; Johannesson et al., 2004), there is evidence that HSA is adducted selectively. Several undefined plasma proteins were modified by unidentified reactive metabolites of zomepirac and diflunisal in rats but the major modified protein was serum albumin (Bailey and Dickinson, 1996). While none of the studies that found carboxylic acid drugs bound covalently to circulating human plasma proteins identified the modified proteins, it is known that ketoprofen-AG, which circulates at relatively high concentrations versus the parent compound (Grubb et al., 1999), binds selectively to HSA in vitro (Dubois et al., 1993); there being no detectable covalent binding to fibrinogen and gamma globulins, and only low-level binding to alpha and beta globulins. These instances of selective adduction might be attributable to HSA's abundance of nucleophilic side chains (Kristiansson et al., 2003).

Due to tandem mass spectrometry of modified peptides enabling the sequence location and incremental mass of the modification to be identified simultaneously, even when only small samples of human protein are available (Jenkins et al., 2009b; Meng et al., 2011; Whitaker et al., 2011), it was anticipated this technique may allow the glycation pathway of adduct formation from diclofenac to be revealed in patients. Identification of HSA adducts formed through the glycation pathway would provide unequivocal proof that an AG metabolite is responsible for at least a fraction of any

adductions of the protein that occur *in vivo*, due to retention of a glucuronic acid residue in the adducts (Figure 2.2).

5.2 MATERIALS AND METHODS

5.2.1 Materials

Acetonitrile (LC-MS grade), hydrochloric acid (HCl), methanol (LC-MS grade) and trifluoroacetic acid (LC-MS grade) were purchased from Fisher Scientific, Leicestershire. Bio Rad Bradford reagent was purchased from Bio Rad, Hertfordshire, UK. Modified trypsin was purchased from Promega, Hampshire, UK. Acetic acid (LC-MS grade), ammonium hydrogencarbonate, diclofenac sodium salt, dithiothreitol, formic acid (LC-MS grade), iodoacetamide, potassium phosphate (KH₂PO₄), potassium chloride (KCl), tris(hydroxymethyl)aminomethane (TRIS) and zomepirac sodium salt were purchased from Sigma-Aldrich, Dorset, UK.

0.1M phosphate buffer, pH 7.4 was made using 0.3117% monosodium phosphate monohydrate, 2.0747% disodium phosphate, heptahydrate w/v in distilled water.

All other reagents were purchased from Sigma-Aldrich, Dorset, UK, unless otherwise stated.

5.2.2 Recruitment of diclofenac patients, blood sampling and plasma stabilisation

Six patients were recruited to the study by Guru Aithal and Ira Pande at the Nottingham Digestive Diseases Centre, NIHR Nottingham Digestive Diseases Biomedical Research Unit, University of Nottingham and the Department of Rheumatology respectively, both at Nottingham University Hospitals NHS trust. The patients had taken combined 100-150mg doses of diclofenac per day for a minimum of one year (Supplementary tables 6 and 7). Diclofenac was taken variously as twice- or thrice-daily at 50mg or 75mg doses. Patients N01-N03 took their final dose of diclofenac in the clinic, and an 18ml blood sample was removed 1 hour later. Patients

N08-N10 took their final dose of diclofenac at home, and an 18ml blood sample was removed at the hospital 2.5 to 3 hours later. Blood samples were collected by Marie-Josèphe Pradere, at the Departmental of Rheumatology, Nottingham University Hospitals NHS Trust, into heparinised tubes on melting ice to minimise AG degradation and therefore protein adduction *ex vivo*, and centrifuged at 2000g for 10 minutes at 4°C. It is known that AG can be stabilised effectively at physiological pH through cooling alone (Xue et al., 2006; Matthews and Woolf, 2008; Klepacki et al., 2012). Plasma aliquots (60µl) for mass spectrometric analysis of covalent modifications of HSA by diclofenac were immediately frozen at -80°C in cryovial tubes. They were not acidified because mildly acidic conditions cause a selective loss of AG glycation adducts from serum albumin (Smith et al., 1990a). 100 µl plasma aliquots for analysis of diclofenac and diclofenac-AG were taken, and the AG was immediately stabilised by acidification (Kenny et al., 2004) through addition of 2M acetic acid, 4% (v/v) final concentration (Sparidans et al., 2008). Based upon the behaviour of other NSAIDs and their AGs (Ojingwa et al., 1994), reversible protein binding of diclofenac and diclofenac-AGs in the plasma sample will have been decreased significantly by acidification. Acidified plasma samples were immediately frozen and stored at -80°C. All samples were frozen at -80°C within 1 hour of blood collection. Sample stabilisation of samples N08, N09 and N10 was performed by Melanie Lingaya and Rawinder Bainwait, Nottingham Digestive Diseases Centre, NIHR Nottingham Biomedical Research Unit, Nottingham University Hospitals NHS Trust and University of Nottingham. Plasma samples were transported to the University of Liverpool, buried in dry ice, and were immediately stored at -80°C until they were analysed. The covalent binding samples were analysed between 3 and 39 weeks after blood

sampling, and the plasma samples for diclofenac and diclofenac-AG metabolite analysis were run between 12 and 13 weeks of blood sampling. Control plasma was obtained from 3 healthy volunteers who had never taken diclofenac.

5.2.3 Analysis of diclofenac and diclofenac-AG concentrations in plasma samples

Stored plasma samples were thawed at room temperature and processed immediately. To 50 μl aliquots of the acidified plasma 10 μl of 3 μM zomepirac internal standard was added in acetonitrile-water (1:1, v/v) containing 0.1% formic acid. Protein was precipitated through addition of four times volume of ice-cold acetonitrile. Precipitated protein was pelleted by centrifugation at 14,000g for 5 minutes at 4°C. Supernatant was removed, and filtered through 0.45- μm low-binding hydrophilic PTFE filter plates (Millipore, Cork, Republic of Ireland) according to the manufacturer's instructions to remove any remaining particulate material. The filtrate was evaporated to dryness at 37°C under a constant stream of nitrogen, and reconstituted in 60 μl of acetonitrile-water (1:1, v/v) containing 0.1% formic acid. A 10 μl aliquot was injected onto the HPLC column. Analyte separation was performed at room temperature using a Zorbax Eclipse XDB-C18 column (150 x 2.1 mm, 5 μm ; Agilent Technologies, Santa Clara, CA, USA) connected to a Dionex Ultimate 3000 Ultimate 3000 HPLC system (Dionex Ltd., Macclesfield, Cheshire, UK) and a 4000 QTRAP hybrid quadrupole mass spectrometer (AB Sciex, Foster City, CA, USA). Samples were maintained at 4°C in the autosampler. Analytes were eluted using a gradient of acetonitrile containing 0.1% formic acid against 0.1% formic acid in water: 50-95% over 10 minutes. The eluent flow rate was 210 $\mu\text{l}/\text{minute}$. Under these conditions diclofenac 1- β AG and its isomers eluted as a single peak at 2.7 minutes; zomepirac eluted at 5.2 minutes and diclofenac

at 7.0 minutes. MS operating parameters for the multiple reaction monitoring (MRM) analyses of diclofenac and diclofenac-AG are shown in Table 5. 1. No endogenous or artefactual materials interfering with analyte and internal standard signals in the selected MRM channels were detected in control plasma samples. The lower limit of detection of diclofenac and the 1- β AG spiked into pooled human plasma obtained from healthy volunteers, as defined by a signal to noise ratio > 3, was below 10nM for both compounds. Detection of analytes in the clinical samples was confirmed using enhanced product ion (EPI) scans (Hopfgartner et al., 2004); the MS/MS spectra obtained matched the spectra of the authentic compounds. The corresponding lower limits of quantification, as defined by accuracy of between 80-120%, and precision (coefficient of variation) < 20% was 50nM for diclofenac (accuracy 93.2%, precision 15.9%), and 30nM for diclofenac-AG (accuracy 108.7%, precision 3.9.). Three validation assays were run prior to analyte analysis, with further validation assays run each time sample analysis was performed.

Table 5. 1: MRM parameters for analysis of diclofenac and diclofenac-AG in plasma samples. *Parent ions are [M + H]⁺. ** Internal standard

Parameters	Diclofenac-AG	Diclofenac	Zomepirac**
Fragmentation transition (m/z)*	472.0→296.1	296.1→215.1	292.0→139.0
Declustering potential (V)	37.00	31.00	40.00
Entrance potential (V)	10.00	10.00	10.00
Collision energy (eV)	14.00	41.00	27.00
Collision exit potential (V)	15.00	15.00	15.00
Dwell time (ms)	200	200	200

5.2.4 Isolation of HSA from plasma obtained from diclofenac patients

Stored plasma samples were thawed at room temperature. HSA was immediately isolated from three 60µl aliquots of unacidified plasma samples from each of the six diclofenac patients using affinity chromatography at room temperature (Greenough et al., 2004; Jenkins et al., 2009a). HSA from patients N01, N02, N03 and N08 and also HSA from the pooled control plasma was captured on a POROS anti-HSA affinity cartridge (Applied Biosystems, Foster City, CA, USA). This cartridge expired before any more plasma samples could be processed, and could not be replaced because the manufacturer had discontinued production. Therefore HSA from patients N09 and N10 was captured using an Affinity Removal System column (HSA only; 4.6 x 50mm; Agilent Technologies, Santa Clara, CA, USA). Both columns were installed on a PerSeptive BioSystems Vision Workstation (Applied Biosystems). HSA was eluted with HCl (12 mM) for patients N01, N02, N03 and N08 (Greenough et al., 2004), and with a proprietary acidic elution buffer (Agilent Technologies) for the N09 and N10 samples. Eluted protein fractions were immediately neutralised through addition of 0.1M Tris buffer, pH 7. Protein fractions were precipitated immediately through vortex mixing with nine volumes of ice-cold methanol, and the precipitate was pelleted by centrifugation at 14,000 rpm. The supernatant was removed, and the pellet washed three times with 60µl ice-cold methanol. The precipitated protein was dissolved immediately in 50µl of 0.1M phosphate buffer, pH 7.4, reduced with dithiothreitol (10mM) for 15 minutes at room temperature and alkylated with iodoacetamide (55mM) for a further 15 minutes at room temperature. The protein was then precipitated and washed with ice-cold methanol as before. The protein pellet was re-dissolved in ammonium hydrogencarbonate solution (50µM, 50µl), assayed for protein

content using the Bradford assay (Bradford, 1976), and 50 μ l aliquots of 3.2mg/ml protein were digested with 5 μ g trypsin overnight. On the following day, the tryptic peptides were subjected to ion exchange chromatography on a PolySULFOETHYL A strong cation-exchange column (200 \times 4.6 mm, 5 μ m, 300 \AA ; PolyLC Inc, Columbia, MD, USA); a procedure that enhances substantially the sensitivity of the peptide analyses by LC-MS/MS (Jenkins et al., 2009b). Peptides were eluted with a linear gradient (0-50% over 75 min) of 10 mM KH_2PO_4 containing 1 M KCl and acetonitrile (3:1,v/v), pH <3, against 10 mM KH_2PO_4 and acetonitrile (3:1,v/v), pH <3, at a flow rate of 1 ml/min. The eluate was monitored at 214 nm. Approximately 15 peptide-containing fractions (2 ml) were collected per elution. They were dried by centrifugation under vacuum before being desalted using a Macroporous Reversed-Phase C_{18} High-Recovery column (4.6 \times 50 mm, Agilent Technologies) installed on a Vision Workstation and finally dried under vacuum for LC-MS/MS analysis. These analyses were carried out within 1 week.

5.2.5 Mass spectrometric characterisation of modified HSA isolated from patients receiving therapeutic doses of diclofenac

Modified tryptic peptides were detected on a 5500 QTRAP hybrid triple-quadrupole/linear ion trap instrument fitted with a Nanospray II source (AB Sciex). MRM transitions for peptides modified by diclofenac-AG by the transacylation or glycation pathways were selected as follows: the m/z values for all singly charged peptides with a missed trypsin cleavage at the modified lysine residue and mass additions of either 277 amu (for the transacylated peptide) or 453 amu (for the glycated peptide) were calculated. These values were paired with the m/z values of the dominant fragment ions of diclofenac, namely m/z 215 and m/z 250 (Figure 2.6) to complete the MRM transitions. Transitions for which a modified peptide was detected

in any of the experiments described here are listed in Supplementary tables 1 and 2, with cation exchange fractions shown in Supplementary table 3. Other transitions representing peptides modified by a range of drugs in our laboratory but not observed here are listed in Supplementary tables 4 and 5. Sample aliquots (2.4-5.0 pmol) were delivered into the mass spectrometer by an Ultimate 3000 HPLC System through a 5-mm C18 nano-precolumn, a C18 PepMap column (75 μm \times 15 cm; Dionex) and a 10- μm i.d. PicoTip ionspray emitter (New Objective, Woburn, MA, USA). The ionspray potential was set to 2200-3500 V, the nebuliser gas to setting 19 and the interface heater to 150°C. A gradient from 2% acetonitrile/0.1% formic acid (v/v) to 50% acetonitrile/0.1% formic acid (v/v) over 60 min was applied at a flow rate of 300 nL/min. MRM transitions were acquired at unit resolution in both Q1 and Q3 to maximize specificity. Collision energies were optimised for each MRM transition and dwell times were 20 ms. MRM survey scans were used to trigger up to three enhanced product-ion scans of modified peptides according to the MIDAS technique (Unwin et al., 2005; Unwin et al., 2009), with Q1 set to unit resolution and with dynamic fill of the trap.

Modified tryptic peptides were also analysed on an AB Sciex Triple TOF 5600 for confirmation. The higher resolution and broader mass range of this instrument allowed for more confident assignments of some peptide sequences and hapten structures. Peptide aliquots (2.4-5.0 pmol) were delivered into the mass spectrometer via a 10- μm i.d. PicoTip (New Objective) by a direct-flow nano-LC system (Eksigent, Dublin, CA, USA): a NanoLC-Ultra chromatograph linked to a cHiPLC-Nanoflex docking station, ChromXP C₁₈ trap column (200 μm \times 0.5 mm) and ChromXP C₁₈ column (75 μm \times 15 cm). The ionspray potential was set to 2200-3500 V, the nebuliser gas to

setting 5 and the interface heater to 150°C. A gradient from 2% acetonitrile/0.1% formic acid (v/v) to 50% acetonitrile/0.1% formic acid (v/v) in 90 min was applied at a flow rate of 300 nL/min. The instrument acquired data at 25 MS/MS spectra per cycle with an accumulation time of 100 ms each. The data were sorted in PeakView (AB Sciex) to highlight spectra with fragment ions of m/z 215 and m/z 250 and were then interpreted manually.

5.3 RESULTS

5.3.1 Covalent binding of diclofenac to human serum albumin isolated from patients receiving therapeutic doses of the drug

As found previously with *in vitro* incubations of benoxaprofen-AG and tolmetin-AG (Ding et al., 1993; Ding et al., 1995; Qiu et al., 1998), diclofenac was found to modify HSA in patients by both transacylation and glycation pathways, with representative mass spectra shown in Figure 5. 1. Whilst the MRM transitions utilised in MIDAS analysis of the modifications were designed specifically for adducted HSA lysine residues, no modifications of other amino acid residues, such as the arginines and serines adducted by benoxaprofen-AG and tolmetin-AG (Ding et al., 1995; Qiu et al., 1998), were identified by exhaustive analyses of selected cation exchange fractions on a 5500 QTRAP hybrid triple-quadrupole/linear ion trap mass spectrometer.

Reactive metabolites of diclofenac were found to have modified the HSA isolated from all six patients analysed (Table 5. 2), with transacylation adducts detected in all samples. Glycation adducts were detected in three of the six patient samples analysed (N01, N08 and N09). The finding of these glycation adducts revealed definitively for the first time that AG metabolites can modify proteins *in vivo*. None of these modifications were detected on tryptic peptides of the HSA isolated from pooled control plasma.

Whilst diclofenac was found to have modified at least one amino acid residue of HSA isolated from each patient analysed, the number of lysine residues identified as modified and chemical pathways of modification varied greatly between patients. The numbers of lysine residues modified through the transacylation pathway varied from one residue (K195) for patients N09 and N10, to five residues for patients N01, N02

and N08. Transacylation adducts to residue K195 were identified in all 6 patient samples analysed. K199 and K541 were the next most commonly modified lysine residues identified by the transacylation pathway in patients, N01, N02, N03 and N08.

Glycation adducts, however, were only detected in three out of the six patient samples isolated. Whilst four distinct lysine residues were identified as modified in HSA isolated from patient N08 (K195, K199, K432 and K525), only one modification was detected on albumin samples isolated from patients N01 (K199) or N09 (K195).

From these data it is apparent that greater numbers of modifications by the transacylation pathway were detected compared to glycation, suggesting this may be the more favourable chemical pathway of adduct formation *in vivo*. However, it cannot be discounted that differential ionisation of peptides, producing different lower limits of identification, may not allow accurate comparison.

Almost all the modified lysine residues of HSA detected in the patient albumin samples were also detected following *in vitro* incubation of diclofenac-AG with HSA (Chapter 2).

The only exception to this finding was the detected acylation of K436 (peptide ⁴³³VGSKCCK⁴³⁹). However, two of the eight lysine residues modified *in vitro* were not modified detectably *in vivo*, namely K137 and K351. Only three of the eight lysines glycosylated by diclofenac-AG *in vitro* were adducted with the complete AG structure (Figure 2.2) *in vivo*: K195, K199 and K432. All of these residues were transacylated both *in vivo* and *in vitro*. Two of the seven lysines transacylated and glycosylated *in vitro* were only transacylated *in vivo*: K190 and K541. Uniquely in peptide ⁵²⁵KQTALVELVK⁵³⁴ from the HSA of patient N08, K525, which was transacylated *in vitro* and *in vivo* and adducted with the complete AG structure *in vitro*, was adducted with glucuronic acid alone.

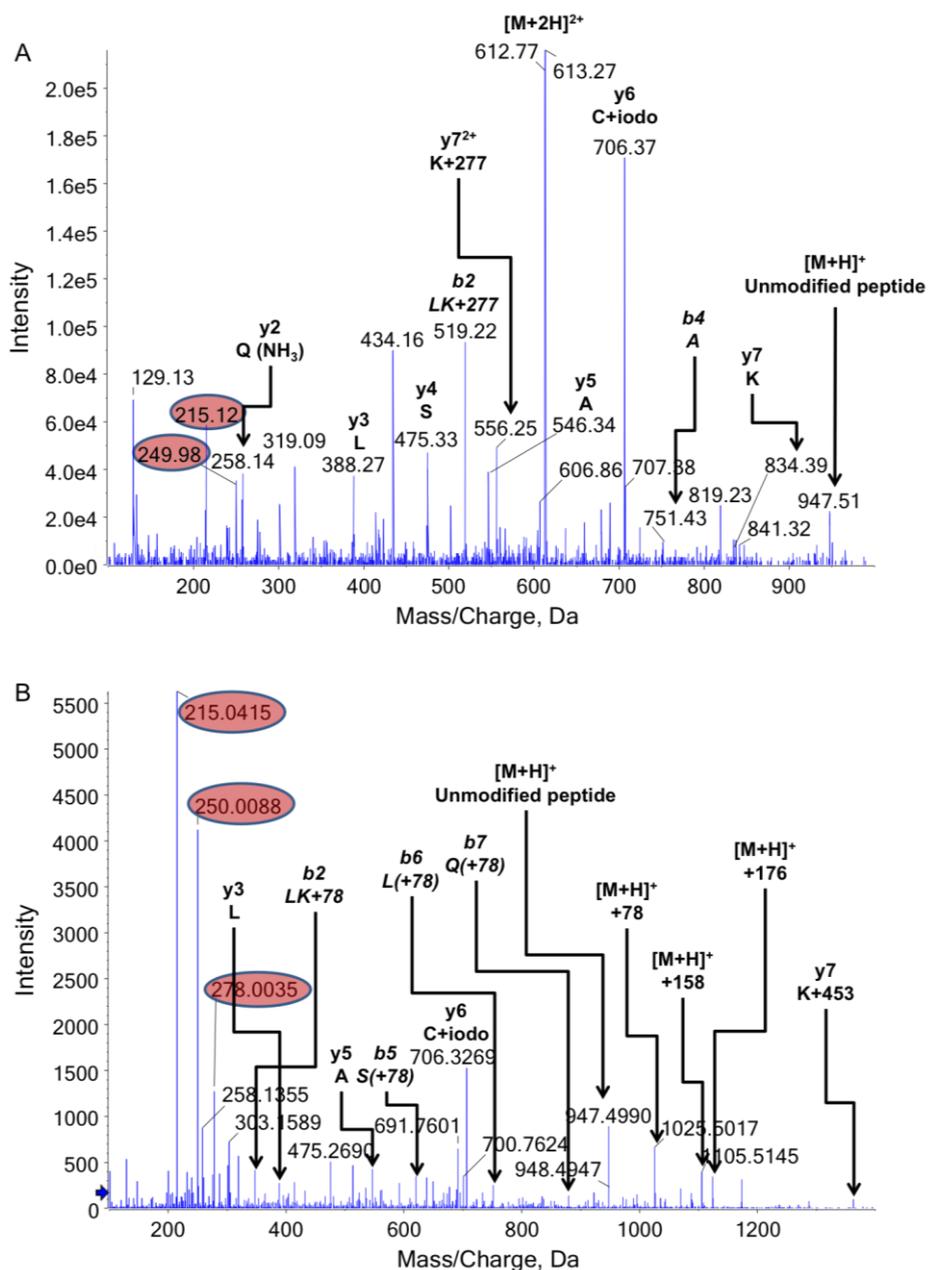


Figure 5. 1: Examples of product-ion spectra demonstrating covalent modification of K199 in the tryptic peptide ¹⁹⁸LKASLQK²⁰⁵ of HSA isolated from a patient (N08) receiving diclofenac: modification by the transacylation (A) or glycation (B) pathways. The modified peptides were analysed on a 5500 QTRAP instrument. Ions produced by fragmentation of the diclofenac and diclofenac-AG residues are enclosed by red ellipses (see Figure 2.6). The m/z values of the modified peptides and fragments correspond to the 35Cl₂ isobars. C+iodo=cysteine residue alkylated with iodoacetamide. Note, the modified and unmodified peptides were not resolved completely by LC. The assignments of the '+453,' '+277,' '+176,' and '+158' ions are given in Figure 2.6. The '+78' ion was commonly seen on analysis of spectra glycated by the AG, but the fragment could not be definitively identified. The MRM transitions for these peptides and the other adducted peptides are given in Supplementary tables 1 and 2.

Table 5. 2: Identification of modified lysine residues of HSA isolated from patients receiving therapeutic doses of diclofenac and their chemical routes of modification.

Patient identification number	Time of blood sampling following last dose of diclofenac	Chemical pathway of modification*	Modified lysine residues**
N01	1hr	Transacylation	K190, K195, K199, K432, K541
		Glycation	K199
N02	1hr	Transacylation	K190, K195, K199, K432, K541
N03	1hr	Transacylation	K195, K199, K541
N08	2 hr 52 mins	Transacylation	K195, K199, K436, K525, K541
		Glycation	K195, K199, K432, K525
N09	2hr 34 mins	Transacylation	K195
		Glycation	K195
N10	2hr 40mins	Transacylation	K195

5.3.2 Detection and quantification of diclofenac-AG in plasma samples

Plasma samples were also isolated and stabilised for qualitative analysis of diclofenac and diclofenac-AG and for quantitative analysis where possible. LLOQ of the analyses were defined, as described in the methods section, at 50nM for diclofenac and 30nM for diclofenac-AG. Sparidans *et al.* (2008) obtained corresponding figures of 68nM and 42nM respectively (Sparidans *et al.*, 2008). Analytes were determined as detected but not quantified when their concentration was below the LLOQ provided the MRM peak eluting at the correct retention time had a signal-noise ratio of >3. Further confirmation of analyte detection was provided through the use of multiple reaction monitoring-triggered EPI scans (Hopfgartner *et al.*, 2004). The survey scans triggered

MS/MS data capture automatically on detection of a signal for diclofenac (m/z 296.1→215.1) or diclofenac-AG (m/z 472.0→296.1). Analyte detection was confirmed by EPI scans if drug fragments were found in spectra captured at the known elution time of the analyte. The known mass spectrometric fragments of diclofenac (MW 295 for the $^{35}\text{Cl}_2$ isobar) are m/z 215, 250 and 278. The presence of diclofenac-AG was shown by identification of the known diclofenac fragments (the conjugate underwent conventional facile loss of the glucuronic acid residue), and occasionally by additional identification of the loss of water from diclofenac-AG giving an ion at m/z 454 (as shown by Figure 5. 2). The lower limit of detection of diclofenac and diclofenac-AG was identified at <10nM for both analytes.

Blood was taken from patients N01, N02 and N03 1 hour following the last administration of diclofenac. Diclofenac-AG was not detected in plasma from N01 and N02 (Table 5. 3). The concentrations of diclofenac from N01 and N02 were below the LLOQ. Diclofenac-AG was detected in plasma from patient N03, and confirmed using EPI scans, although it remained below the LLOQ. The plasma diclofenac concentration in this patient was 166.7nM.

The time point of blood sampling from patients N08, N09 and N10 was extended to about three hours following the last dose of diclofenac. Plasma concentration of diclofenac could be quantified in patients N08 and N10, at 423nM and 63.9nM, respectively, and the drug could be detected in patient N09. The only patient in whom diclofenac-AG could be quantified in was patient N08, at 90.78nM, although it could be detected in the N09 and N10 plasma samples.

Table 5. 3: Plasma diclofenac and diclofenac-AG concentration. ND=not detected (MRM signal-to-noise ratio <3 at confirmed R_t of the analyte). NQ=not quantified (analyte concentration >LLOD, <LLOQ).

Patient ID	Time of blood sampling	Diclofenac AG (nM)	Diclofenac (nM)
N01	1 h	ND	NQ
N02	1 h	ND	NQ
N03	1 h	NQ	166.7
N08	2 hr 52 mins	90.78	423.3
N09	2hr 34 mins	NQ	NQ
N10	2hr 40 mins	NQ	63.91

Chapter 5 – Formation of Covalent Protein Adducts by Diclofenac Acyl Glucuronide Metabolites in Patients

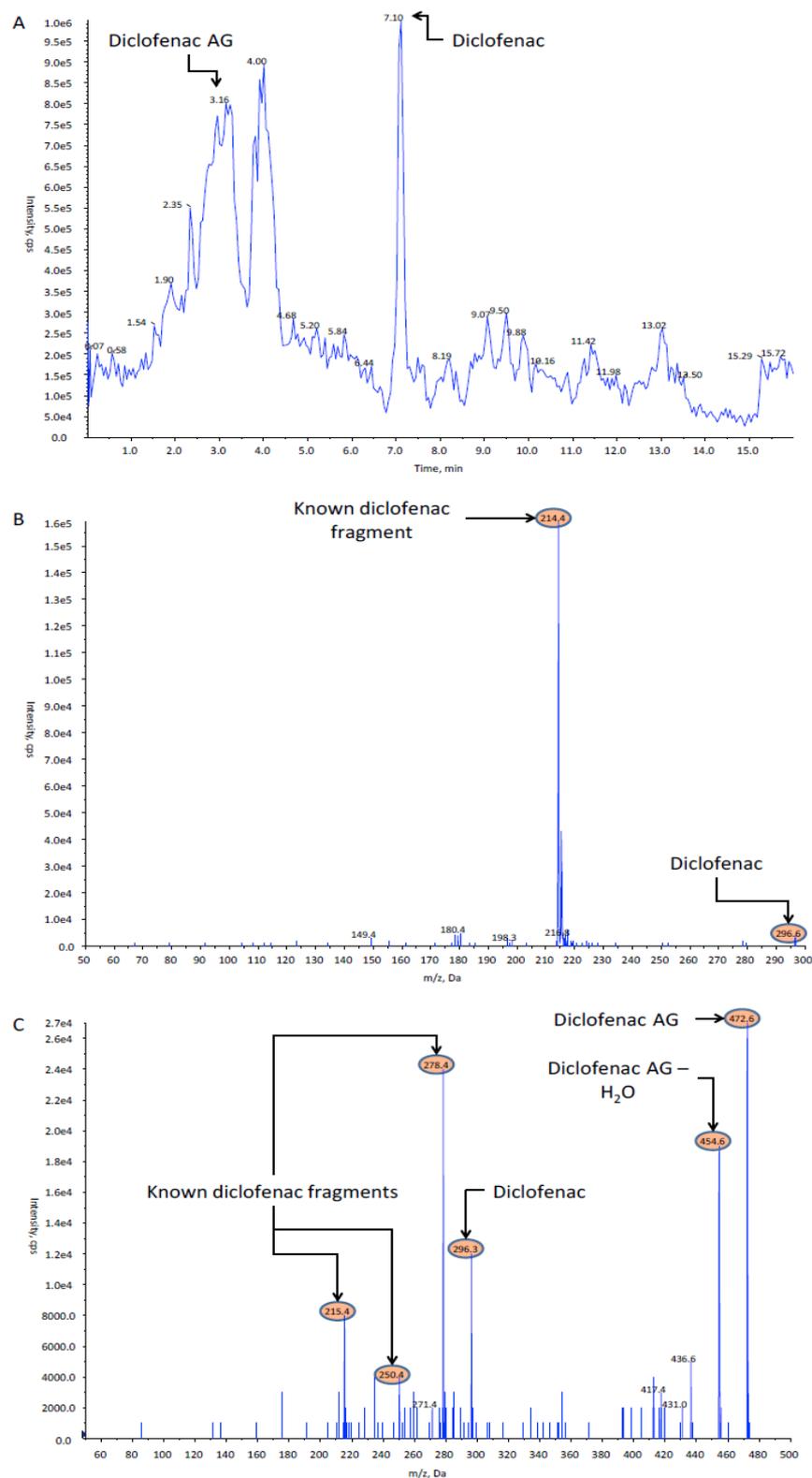


Figure 5. 2: Representative total ion current scan (A), and EPI scans showing identification of diclofenac (B) and diclofenac-AG (C) in plasma isolated from patient N03. The protonated molecules (m/z 296 and m/z 472, respectively) and the fragment ions are enclosed by red ellipses (see Figure 2.6). The fragment ion of the AG at m/z 454 is $[M+H-H_2O]^+$.

5.4 DISCUSSION

The main aim of this study was to investigate whether AG metabolites can form covalent adducts with a protein in patients receiving therapeutic doses of the drug, using mass spectrometric techniques proven *in vitro* for identification of the adducts. Identification of glycation adducts of HSA in plasma isolated from three of the six patients analysed (N01, N08 and N09) provides the first definitive evidence that AGs can form covalent adducts in humans. This finding reinforces the toxicological concerns held over AG metabolites over the last 30 years (Faed, 1984; Benet et al., 1993; Shipkova et al., 2003; Skonberg et al., 2008).

Transacylation adducts were formed in all of the six patients tested. Whilst AGs are known to covalently modify HSA through the transacylation pathway *in vitro* (Ding et al., 1995; Qiu et al., 1998), reactive ester metabolites other than AGs may also be responsible *in vivo*. Greater numbers of modifications in the clinical samples had occurred by the transacylation pathway than glycation. This is unlike the 16 hour incubations of diclofenac-AG with HSA, where modifications by glycation were detected at lower concentrations than transacylation (Table 2.7). Nevertheless, because of the high plasma concentration of HSA (Anderson and Anderson, 2002), the low molar ratio of AG to protein in these incubations bears some resemblance to the situation *in vivo*. It is hypothesised, because 1- β AGs form transacylation adducts exclusively and sequential acyl migration and aldehyde-amine condensation are required for formation of glycation adducts (Chapter 2) and (Smith et al., 1990a), that *in vivo* AGs may be cleared before extensive acyl migration and/or glycation occurs, resulting in increased transacylation adduct formation in comparison to glycation.

Clearance of AGs in humans has been shown to be rapid, with renal clearances of zomepirac-AG of 406 ± 110 ml/min (11.3 fold faster than its aglycone) in healthy volunteers, and ibuprofen-AG of 2.46 ± 0.82 ml/min/kg (307.5 fold faster than its aglycone) in elderly patients (Smith et al., 1985; Castillo et al., 1995). Biliary clearance of AG metabolites (Verbeeck et al., 1988; Balani et al., 1997; Hofmann et al., 2005; Wang et al., 2006) and their extensive spontaneous and enzymatic hydrolysis as shown in chapter 2 and the literature (Ruelius et al., 1986; Volland et al., 1991; Williams et al., 1992; Akira et al., 2002; Karlsson et al., 2010) might also contribute to rapid and extensive plasma clearances of AGs. This suggests that 1- β AGs may consequently be cleared from the plasma before extensive rearrangement into their positional isomers occurs, resulting in greater plasma exposure of the 1- β AG isomer than the positional isomers individually or collectively, and potentially greater formation of transacylation adducts than glycation.

Nevertheless, there are reports of AG positional isomers in human plasma (Hyneck et al., 1988; Stopfer et al., 2011). The extent of AG isomerisation in a patient's plasma *in vivo* may be assessed through the use of longer LC-gradient conditions, allowing complete separation of positional isomers, as was described in chapter 2. In the present study, all AG positional isomers were co-eluted in metabolite analyses to increase assay sensitivity. From these analyses, only plasma of one patient (N08) contained a sufficiently high AG concentration for a separation gradient to be run. Unfortunately time was not available to perform this assay.

However, it cannot be discounted that acyl-coenzyme A thioester (acyl-CoA thioester) conjugates of carboxylate compounds, which can also form covalent adducts through the transacylation pathway, and transacylate glutathione (Grillo and Benet, 2002;

Sidenius et al., 2004) and protein (Olsen et al., 2003) *in vitro*, may also be responsible for transacylation adducts detected in patient samples. Studies using isolated rat hepatocytes and selective conjugation inhibitors indicated adduction of intracellular proteins by xenobiotic carboxylic acids can be mediated by their acyl-CoA thioesters (Li et al., 2002; Li et al., 2003; Grillo and Lohr, 2009). Indeed the modification of proteins in rat hepatocytes by phenylacetic acid occurred in the absence of detectable AG formation (Grillo and Lohr, 2009). Unlike AG metabolites, which are considered to be relatively low reactivity CRMs but with high abundance, allowing them to leave hepatocytes, reaching the general circulation and extra-hepatic tissues, acyl-CoA thioester metabolites are generally considered to be high reactivity but low abundance CRMs (Boelsterli, 2002). To date no evidence has shown acyl-CoA thioesters escaping the cell into plasma. Consequently, if the diclofenac-CoA thioester is partly or entirely responsible for the transacylation adducts detected on albumin in this experiment, it would suggest protein modification is at least partly located in the liver.

This hypothetical mechanism of HSA adduction *in vivo* would appear to conflict with the findings of early studies of plasma protein modification by carboxylate drugs, where modification was found to correlate with plasma AG exposure (Smith et al., 1986; Hynneck et al., 1988; Benet et al., 1993). Due to their ability to reach the blood stream in appreciable concentrations (Grubb et al., 1999; Dockens et al., 2000; Hermening et al., 2000; Stangier et al., 2007; Stopfer et al., 2011; Zhang et al., 2011), if protein modification does occur in the plasma, it would suggest that AG metabolites are more likely to be responsible for transacylation adducts detected in patients. With extensive hepatic generation of albumin, at approximately 12g/day (Anderson and Anderson, 2002), its intrahepatic modification by transacylation or glycation before

secretion into the plasma cannot be discounted. Although serum albumin is synthesized largely in the liver, albumin gene expression has been detected in several other tissues (Shamay et al., 2005). In principle, any tissue that synthesizes the protein and glucuronidates diclofenac could produce modified albumin. Finally, the relative contributions of AGs and acyl-CoA thioesters to protein adduction *in vivo* might differ between carboxylic acids; as seems to be the case in isolated hepatocytes (Li et al., 2002; Li et al., 2003; Grillo and Lohr, 2009).

Any attempt at analysing protein adduction by AGs has to be qualified by an appreciation that even the use of advanced LC-MS/MS techniques has potentially significant limitations. In particular, the glycation reactions of sugar species are very complex and probably incompletely understood (Ahmed and Thornalley, 2002; Wa et al., 2007), and certainly not limited to the familiar lysine condensation reactions and Amadori rearrangements (Frolov and Hoffmann, 2010). No practical analysis of protein modification by a drug AG can allow for all of the potential glycation products; for example, cross-linking adducts derived from Amadori (2-ketoamine) structures (Acharya et al., 1988). Consequently, in comparison with the intrinsically simple acylation adducts, the number of glycation adducts formed *in vitro* and *in vivo* may have been underestimated.

Whilst no experiments have ever investigated plasma AG exposure following diclofenac administration to humans, the reported C_{\max} of diclofenac is approximately 1.93 µg/ml (6.5 µM) in volunteers, or 0.99 µg/ml (3.3 µM) in patients with rheumatoid arthritis (Crook et al., 1982), with the normal population range for the concentration of HSA in plasma between 35-50 mg/ml (530-758 µM) (Anderson and Anderson, 2002), although concentrations have been shown to be lower in patients with rheumatoid

arthritis (Ballantyne et al., 1971; Crook et al., 1982). Consequently, as plasma concentrations of diclofenac-AG are unlikely to exceed those of the aglycone C_{max} , the maximum expected molar ratio of AG : HSA in patient's plasma will be between 0.012:1 and 0.004:1. By analogy with the glycation of plasma proteins by endogenous species (Bhonsle et al., 2012), low albumin levels in patients might be associated with increased AG-mediated glycation because albumin competitively inhibits the natural glycation of less abundant proteins.

In vitro incubation of 400nM diclofenac-AG with 40 μ M HSA (molar ratio of 0.01 : 1) revealed only a single modification of HSA, through the glycation pathway, at K190. In contrast to this, several lysine modifications were identified on HSA isolated from patient's plasma samples. Cation-exchange enrichment of tryptic peptides during sample preparation may have increased the sensitivity of detection, revealing modifications undetectable in its absence, despite *in vitro* incubation for 16 hours. Alternatively, the long turnover time of HSA of approximately 21 days (Anderson and Anderson, 2002) or 14.6 days in rheumatoid arthritis patients (Ballantyne et al., 1971), may allow accumulation of covalent modifications. Reduction of plasma HSA concentrations by 23% (Ballantyne et al., 1971) or 16.6% (Crook et al., 1982) in patients with rheumatoid arthritis, resulting in an increased ratio of AG:HSA may also result in increased albumin modification.

Attempts were made to quantify plasma diclofenac and diclofenac-AG concentrations in patient's plasma samples. Restrictions in place for ethical approval of the study meant only one time point could be selected for blood sampling, meaning in-depth pharmacokinetic analyses could not be undertaken. Due to the long turnover time of serum albumin in human plasma (Anderson and Anderson, 2002), the time point of

sampling is not likely to greatly influence the detection of covalent modifications of albumin in patients who have taken diclofenac for many months. Consequently, time points were selected with the aim of allowing quantification of diclofenac and diclofenac-AG.

Previous studies of diclofenac pharmacokinetics in humans used the enteric coated formulation of the drug, with t_{\max} shown to be highly variable in these studies, varying from 1 hour to 5 hours in different individuals, but with a mean of between 2.0 and 2.5 hours (Willis and Kendall, 1978; Willis et al., 1979; Willis et al., 1980).

Whilst no restrictions were placed on the formulation of diclofenac for patients recruited to the study, it was known that most of the patients would be receiving enteric coated diclofenac-sodium formulations. The enteric coating is a pH-sensitive polymer, which remains intact in the acidic environment of the stomach, protecting the contents of the tablet. After passing through the stomach, the coating disintegrates in the alkaline environment of the small intestine. With absorption of enteric coated formulations delayed until later in the gastro-intestinal tract, it was assumed that t_{\max} of enteric coated formulations would be later than those of standard diclofenac sodium formulations. Blood samples were collected from the first few patients (N01-N03) 1 hour following DCF administration. This was selected to ensure patient compliance. However, with AG only detected in one sample and only minimal DCF detected in all three, it was decided to extend this time-point to three hours, and allow patients to take their final dose of DCF out of the clinic.

Diclofenac-AG could not be detected in plasma samples isolated from patients N01 and N02, who received enteric-coated diclofenac sodium. Plasma diclofenac was below the LLOQ, but could be detected. Patient N03, however, received a modified (slow)

release form of the enteric coated formulation of diclofenac. At this 1 hour time point, diclofenac could be quantified at 166.7nM, and although the AG remained below the LLOQ, its identification was confirmed by an EPI scan.

The time point of blood sampling was extended to 2.5-3 hours after the last administration of diclofenac for the second group of patients (N08-N10). This time point was selected to be closer to the reported plasma t_{max} of enteric coated formulations, in an attempt to facilitate identification and quantification of diclofenac and its AG metabolite. Diclofenac could be quantified in the plasma of two patients, namely N08 (at 423.3nM, following administration of enteric coated formulation) and N10 (at 63.39nM receiving diclofenac sodium). Diclofenac-AG could also be quantified in patient N08 (90.8nM), and whilst it could not be quantified, the AG was identified in plasma from patients N09 and N10 samples (Table 5. 3).

In each plasma sample assayed through this study, the diclofenac concentration was considerably lower than the C_{max} reported in the literature. Whilst the C_{max} of diclofenac has been reported to be reduced in rheumatoid arthritis and elderly patients compared to healthy volunteers (Willis and Kendall, 1978; Crook et al., 1982), values measured in this study remain lower than expected. The most likely reason for this disparity is the time point of blood sampling. The extensive variation of t_{max} values in patients receiving the same enteric coated formulation (Willis et al., 1980) means that consistent quantification with only one time point of sampling is difficult. Rapid absorption of diclofenac following an initial lag-time (Willis et al., 1979; Willis et al., 1980) suggests that the window of sampling within which diclofenac is detectable before C_{max} is reached may be small, although it remains unclear whether this phenomenon is specific to enteric coated formulations of diclofenac, or is a property of

diclofenac itself. This study, however, does provide confirmation that diclofenac is metabolised to its AG metabolite in patients, and the AG metabolite is present in plasma.

Through the detection of drug-derived glycation adducts of HSA isolated from patients receiving diclofenac therapy, these experiments have provided the first definitive confirmation that AG metabolites can form covalent protein adducts *in vivo*. This reaffirms the potential of these metabolites to act as haptens, potentially having a role in the initiation of delayed hypersensitivity ADRs associated with their parent carboxylate compounds.

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CHAPTER SIX

FINAL DISCUSSION

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6.1 INTRODUCTION

ADRs, accounting for 6.5% of hospital admissions in the UK (Pirmohamed et al., 2004), represent a major challenge for clinicians and healthcare systems alike. Representing the 2nd most common cause of attrition of NCEs before clinical trials (Kola and Landis, 2004), ADRs also play a large role in the reduced productivity and consequential profitability of the pharmaceutical industry. With an estimated cost for the development of an NCE into a marketed drug of \$802 million (DiMasi et al., 2003), late-stage drug withdrawal as a consequence of ADRs inflicts a massive financial burden on the pharmaceutical industry. Consequently, to avoid this situation, pre-development (*in silico*) prediction and/or in-development identification of compounds likely to elicit ADRs in humans is essential. Off-target ADRs, however, as a consequence of their low incidence and unpredictable nature are currently infrequently detected before the drug is exposed to large clinical populations (Park et al., 2000), after large financial investment has already been made in the compound.

Covalent binding of drugs or their CRMs to proteins is widely recognised as a potential risk factor in the pathogenesis of ADRs (Kalgutkar and Soglia, 2005; Park et al., 2005), with many drugs associated with idiosyncratic liabilities undergoing covalent binding *in vitro* and *in vivo* (Nakayama et al., 2009; Park et al., 2011a). This recognition has resulted in the incorporation of screens in pre-clinical drug development to assess covalent binding, and consequential decision trees into how drugs should be managed if they are found to bind extensively (Evans et al., 2004; Park et al., 2011a; Sakatis et al., 2012).

Whilst the covalent binding of drugs or their CRMs to proteins critical for cellular function has been suggested to have a role in idiosyncratic reactions towards drugs

(Park et al., 2005), including the pathogenesis of immune-allergic ADRs, such as anaphylaxis and hepatotoxicity associated with carboxylate compounds, it is the covalent binding of drugs or their CRMs with proteins and their subsequent presentation to the immune system by MHC molecules that is thought to be associated with their pathogenesis (Pichler, 2003; Park et al., 2011b), although direct non-covalent interaction of drugs at T-cell receptors has been suggested for some compounds (Pichler, 2002) including sulfamethoxazole (Schnyder et al., 1997).

With 14% of the drug withdrawn from the market between 1960-1999 containing a carboxylic acid group (Fung et al., 2001), attention has been drawn to the possible consequences of drug bioactivation at this site (Skonberg et al., 2008). Further to this background situation, many carboxylate drugs remaining on the market do so with black-box warnings to their safety (Skonberg et al., 2008; Stepan et al., 2011), usually as a consequence of their association with off-target anaphylaxis or hepatotoxicity.

A common metabolic pathway of carboxylate compounds is their direct and often extensive glucuronidation at the carboxylic acid group (Table 2.1), forming acyl glucuronides (AG). AGs have been shown to be chemically unstable and protein reactive *in vitro* (Benet et al., 1993; Stachulski et al., 2006). With covalent binding of carboxylate compounds to serum albumin in humans and animals correlating to plasma AG exposure (Smith et al., 1986; Hynneck et al., 1988; Smith et al., 1990b; Benet et al., 1993; Castillo et al., 1995; Sallustio and Foster, 1995; Liu et al., 1996), it has been proposed that CRM's derived from carboxylic acids can elicit ADRs. Consequently, this perceived association has resulted in anxiety in the pharmaceutical industry over the progression of compounds found to be metabolised to AGs, and incorporation of the phrase:

‘Phase II conjugation reactions usually 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 testing may be needed (Faed, 1984)’

into the FDA’s guidance for industry in the safety testing of drug metabolites (FDA, 2008).

However, to date, no direct and compelling evidence has shown covalent binding of AG metabolites to proteins *in vivo*. Furthermore, with the expected rapid clearance of phase II metabolites (Caldwell, 1982), whether AGs actually are responsible for the covalent binding of carboxylate compounds is unclear, and certainly undefined. Consequently, further investigations into and potential cessation of development of compounds extensively metabolised into AGs, as advised by the FDA may be unnecessary.

To address this, the central aim of this thesis was to investigate whether AG metabolites could directly form detectable covalent adducts to serum albumin *in vivo*.

6.2 RISK ASSESSMENTS OF ACYL GLUCURONIDE REACTIVITY

The first focus of this thesis was to investigate diclofenac-AG as a suitable tool for the investigation of AG reactivity *in vivo* for the studies undertaken in consequence chapters of the thesis. The criteria set-out for the suitability of an AG was that concerns made over its acyl glucuronidation would be made if the drug was developed as a NCE today. In accordance with the FDA's guidance for industry, the extensive (75%) acyl glucuronidation of diclofenac-AG in isolated liver microsomes supplemented with UDPGA (Kumar et al., 2002) and detection in pre-clinical species (Stierlin and Faigle, 1979; Sparidans et al., 2008) suggests that 'additional safety testing may be needed' (FDA, 2001).

Safety testing of AGs are focussed on the investigating the protein reactivity of the molecule. The most commonly used mechanism in pharmaceutical studies for the investigation of AG protein reactivity is through investigation of their rate of degradation in phosphate buffer, pH 7.4 at 37°C (Ebner et al., 1999; Wang et al., 2004; Walker et al., 2007; Potter et al., 2011; Stopfer et al., 2011). This follows the finding of excellent correlation ($r^2=0.995$) between the rate of degradation and protein binding of 9 AGs following *in vitro* incubation under physiological conditions (Benet et al., 1993), and allows correlation of the reactivity of different AG molecules to be assessed and compared (Shipkova et al., 2003; Stachulski et al., 2006).

Incubations in Chapter 2 revealed diclofenac-AG to spontaneously degrade under these conditions, with a $t_{1/2}$ of degradation of 0.79 hours. Whilst some discrepancy was found between this value of degradation and the value previously reported by (Ebner et al., 1999), increased time-points at the early portions of the degradation curve and homology with the other reported degradation $t_{1/2}$ of 0.7 hours (Sawamura et al., 2010)

provides increased confidence in this data. This rapid degradation of diclofenac-AG places it amongst the most reactive AGs reported. Further studies were undertaken to confirm that this intrinsic reactivity also represented protein reactivity. Following incubations with human serum albumin, covalent binding of diclofenac-AG was shown using both an established alkaline hydrolysis technique as well as direct tandem mass spectrometric analysis of tryptic peptides of the modified protein. This confirmation of the chemical instability and protein reactivity of diclofenac-AG, combined with its extensive acyl glucuronidation does suggest that if diclofenac was developed as a NCE today, it is most likely that concerns would be raised about potential risk over its acyl glucuronidation.

Correlation of the rate of degradation of the 1- β AG as to the consequential perception of safety of the compound however is less assured. A study correlating the marketable fate of 21 carboxylate drugs with the degradation $t_{1/2}$ of their 1- β AGs did show a good separation between compounds classified as safe. No separation however, could be achieved to separate drugs with warnings attached to their safety and those withdrawn due to safety concerns (Sawamura et al., 2010). Drugs in this warning category include naproxen, probenecid, ibuprofen and diclofenac. With NSAIDs some of the drugs most commonly exposed to man, (Aithal, 2011). 6% of the US population using a prescription NSAID and 24 % of the US population exposed to ibuprofen in a month (Paulose-Ram et al., 2003), and with diclofenac, ibuprofen and naproxen the most commonly prescribed NSAIDs by general practitioners in the UK (Langman et al., 2001), potentially useful compounds whose risk is not particularly high may not be progressing through pre-clinical drug development as a consequence of their acyl glucuronidation.

6.3 CHEMICAL INTERACTIONS OF ACYL GLUCURONIDE METABOLITES WITH PROTEINS

Secondary investigations in the thesis were undertaken in Chapter 2 to utilise methods developed in the validation of diclofenac-AG as a suitable AG for study to explore the chemistry of interaction between AGs and protein, and potentially to allow some prediction as to how the AG might behave *in vivo*.

Incubations in phosphate buffer revealed diclofenac-AG to degrade primarily by acyl migration, with little hydrolysis detected. The preferential degradation of by acyl migration rather than hydrolysis has also been reported for many other AGs (Watt and Dickinson, 1990; Iwaki et al., 1999; Corcoran et al., 2001; Akira et al., 2002; Berry et al., 2009; Johnson et al., 2010; Karlsson et al., 2010). Increased hydrolysis of diclofenac-AG was found following incubation with HSA at reduced (400 μ M) AG concentrations, with an absence of noticeable hydrolysis at higher concentrations of (2mM) AG in the incubation mixture. This was hypothesised to reveal the weak esterase potential of AGs, as has previously been reported for other AGs (Watt and Dickinson, 1990; Sallustio et al., 1997; Iwaki et al., 1999; Mizuma et al., 1999; Akira et al., 2002) as well as other ester drugs (Ma et al., 2005). Furthermore, extensive hydrolysis was revealed following incubation of the drug in isolated human plasma. This was considered to be a reflection of esterases known to reside in the plasma (Fukami and Yokoi, 2012), and again is reflective of the findings with other AGs (Watt and Dickinson, 1990; Dickinson and King, 1991; Dubois et al., 1994). This esterase activity observed following incubation in isolated human plasma is likely to be reflected in *in vivo* studies, and in man may result in reduced plasma exposure to AGs, reducing their potential for covalent modification of protein. This has been proven *in*

vivo where co-administration of zomepirac with the esterase inhibitor PMSF resulted in increased plasma exposure of zomepirac-AG, and a consequent increase in the covalent modification of protein (Smith et al., 1990b).

These findings suggest that as well as the known extensive renal and biliary clearance of AGs in man, plasma hydrolysis of AGs *in vivo*, potentially by both HSA and plasma esterases may enhance the rate of clearance of AGs from the plasma, and consequently result in reduced AG exposure, consequently reducing the potential for AGs to modify plasma *in vivo*.

Further experiments in Chapter 2 were directed to study the covalent modification of HSA by AGs. An alkaline hydrolysis method was set-up to allow quantification of the covalent modification of HSA by protein, as commonly used in AG experiments. This revealed modification to be both time and concentration dependent. It is unclear from both this experiment and the literature as to whether following a maximum covalent modification of HSA by AGs being reached, whether a decline in the detection of binding is seen. This is due to the consistent variation in the assay (Watt and Dickinson, 1990; Dickinson and King, 1991; Dubois et al., 1994). Whilst not conclusive, a reduction in binding would mean that covalent modification of AGs is reversible, and although not extensive, may reduce the rate of covalent adduct accumulation *in vivo*.

Direct tandem mass-spectrometric identification of the covalent modification of peptides was used to provide quantitative analysis of the interactions of diclofenac-AG with HSA. These experiments confirmed that diclofenac-AG formed both glycation and transacylation adducts with HSA following *in vitro* incubation. Covalent binding of the AG was primarily located in or around hydrophobic pockets on the HSA molecule, known to be important in the non-covalent interaction of many drugs with HSA

(Ghuman et al., 2005). Potentially this may suggest the importance of non-covalent interaction of AGs with protein for covalent binding with protein. Further work in the investigation of the chemical interactions of diclofenac-AG with HSA revealed the differential role of the 1- β diclofenac-AG to form transacylation adducts. Combined with the earlier work of (Smith et al., 1990a) who suggested that acyl migration was required for glycation adducts. This may suggest that as a consequence of the expected rapid elimination of AGs from the plasma as expected by their rapid urinary and biliary clearance (Smith et al., 1985; Verbeeck et al., 1988; Castillo et al., 1995; Balani et al., 1997; Hofmann et al., 2005; Wang et al., 2006) and extensive hydrolysis in the plasma Chapter 2 and (Iwaki et al., 1999; Mizuma et al., 1999; Akira et al., 2002), AGs may be expected to be cleared before extensive acyl migration, and consequently if they do form covalent adducts *in vivo*, it may be suggested that these could be expected to be through transacylation.

6.4 APPLICABILITY OF THE RAT TO STUDY THE POTENTIAL OF ACYL GLUCURONIDE METABOLITES TO FORM COVALENT ADDUCTS *IN VIVO*

Following the validation of diclofenac-AG as a useful tool to investigate the protein reactivity of AGs *in vivo*, it was subsequently decided to investigate the applicability of the rat as a model system to investigate AG protein reactivity towards serum albumin. Initial investigations were undertaken to ascertain the covalent modification of RSA by diclofenac-AG following *in vitro* incubation. These experiments revealed a reduction in the detection of the covalent modification of RSA than HSA following incubation with diclofenac-AG. Modification of HSA could also be detected at 10-fold lower AG incubation concentrations than RSA. This suggests that RSA is less susceptible to covalent modification by diclofenac-AG, although, as a consequence of the potential for differential ionisation of tryptic digest peptides of RSA and HSA in the mass spectrometer, assertive assessment of a quantitative reduction of covalent binding cannot be made. However, a 74.7% reduction in the covalent binding of diflunisal-AG to RSA, was shown following the use of an alkaline hydrolysis detection of modification (Watt and Dickinson, 1990). Loss of 60% of the identified diclofenac-AG binding sites on HSA in RSA, as a consequence of the 20% sequence variance in the two proteins (Sargent et al., 1981; Minghetti et al., 1986; Carter et al., 1989; Kosa et al., 1997) is hypothesised to be the attributable for the apparent reduced covalent modification of RSA by diclofenac-AG when compared to HSA.

Despite this apparent reduced modification of RSA by diclofenac-AG, reports in the literature have still associated AGs with the covalent modification of plasma proteins in the rat detected following administration of their parent aglycones (Sallustio and Foster, 1995; Liu et al., 1996), with Western blot analysis identifying RSA as a main

target in the plasma for modification (Bailey and Dickinson, 1996). However, again, as a consequence of the inability of antibody and alkaline hydrolysis methods to identify metabolic species responsible for covalent adducts detected, it remains possible that these adducts are not elicited by AGs, and may be formed through other bioactivation pathways of the drugs tested.

With access to relatively large quantities of synthetic diclofenac-AG, it was decided to investigate whether covalent adducts to plasma proteins could be detected following intravenous bolus administration of the AG to the rat. However, despite selection of a dose estimated to allow similar instantaneous AG exposure to RSA in plasma, as allowed detectable covalent adducts in *in vitro* incubations, no covalent adducts could be detected. This was hypothesised to be a consequence of rapid AG clearance of the rat, preventing sufficient exposure to allow detectable covalent adducts. With the physiological role of glucuronidation to enhance a molecule's polarity and affinity for export transporters, consequently resulting in its rapid clearance from biological systems and organisms (Caldwell, 1982; Miners and Mackenzie, 1991) it was hypothesised that diclofenac-AG would be eliminated rapidly from the rat, as has been shown for other AGs (Watt et al., 1991; Iwaki et al., 1995; Dong et al., 2005), potentially with plasma esterases also acting to hydrolyse the drug *in vivo*, further increasing plasma clearance, as predicted through incubations in Chapter 2. This hypothesised rapid clearance may prevent AG exposure reaching sufficient levels to allow detectable covalent modification of RSA *in vivo* in the rat.

To investigate this hypothesis, the plasma clearance of an intravenous bolus dose of diclofenac-AG was investigated, and compared to that of diclofenac. Plasma clearance of diclofenac-AG was found to be over 460% faster than diclofenac, with a plasma half-

life of the AG of 4.12 minutes, indicating this hypothesis of rapid and extensive clearance of diclofenac-AG from the rat is valid. As predicted by *in vitro* investigations in Chapter 2, extensive hydrolysis of diclofenac-AG was detected in the rat. It could not however be completely defined whether high concentrations of plasma diclofenac following administration of its AG were a consequence of plasma (or tissue) AG hydrolysis, or a consequence of enterohepatic recirculation of the aglycone, as has previously been proved to occur in the rat (Stierlin and Faigle, 1979; Fukuyama et al., 1994). Whilst elimination of AG through plasma esterases may be considered a process resulting in reduced AG exposure, enterohepatic recirculation of diclofenac may act to further increase exposure through subsequent re-glucuronidation of the aglycone.

With the plasma clearance of pharmaceuticals from the rat known to be faster than man (Chiou et al., 1998), combined with suspected reduced affinity of AG modification of HSA, the rat does not represent a suitable *in vivo* tool for the investigation of serum albumin modification by AGs in man. The short $t_{1/2}$ of circulatory rat serum albumin of 2.5 days, in comparison to 21 days for humans (Tornqvist et al., 2002) also suggests that repeat dose studies investigating whether adducts could accumulate would not be useful. The increased biliary clearance of AGs in rats in comparison to man, as a result of a humans preferentially exporting larger molecules across the hepatobiliary plasma membrane, unlike rats (Boelsterli, 2003) may however suggest the rat may be more suitable to investigation of hepatic modification of peptides by AGs.

6.5 THE USE OF CONTINUOUS INTRAVENOUS INFUSION TO INVESTIGATE POTENTIAL MECHANISMS FOR THE PRODUCTION OF DANGER SIGNALS BY DICLOFENAC

Whilst the covalent binding of haptens to proteins is considered critical for their presentation to the immune cell in the hapten hypothesis (Padovan et al., 1997; Park et al., 2001; Pichler, 2003; Posadas and Pichler, 2007; Park et al., 2011b), antigen presentation alone is not considered sufficient for the stimulation of immune responses. Danger signals from apoptotic, necrotic or stressed cells are also considered mandatory (Matzinger, 1994; Pirmohamed et al., 2002; Li and Uetrecht, 2010). Currently, the range of endogenous molecules that may act as danger signals are largely unknown (Li and Uetrecht, 2010), but in theory they may be generated by any mechanism resulting in cell stress, for example cytotoxic action of a drug. With the apparently critical role of danger signals in off-target immune mediated ADRs, identification of potential mechanisms for their provision would be useful in providing better understanding of the mechanisms involved in idiosyncratic ADRs, and potentially helping towards a better understanding of the risk of NCEs. To address this, investigations were undertaken with diclofenac, a model drug used to study off-target hepatotoxicity (Boelsterli, 2003; Aithal, 2004), to investigate whether hepatotoxicity could be elicited in the rat following its continuous intravenous infusion.

Dose-limiting gastro-intestinal toxicity of diclofenac (Menasse et al., 1978) and its rapid clearance following intravenous bolus administration as shown by Chapter 3 and (Chiou et al., 1998; Peris-Ribera et al., 1991) represent a major challenge in the study of the potential for diclofenac to elicit hepatocellular stress *in vivo*. In an attempt to

overcome these obstacles, a continuous intravenous infusion drug delivery system, hypothesised to allow continuous drug exposure throughout the 48 hour study was used. This would negate the drug's rapid clearance, whilst the intravenous route of administration allows reduced drug exposure to the gastro-intestinal tract, reducing risk for the development gastro-intestinal toxicity in rats. Whilst the use of continuous intravenous administration of drugs allowing continuous exposure is well established and used clinically (Howell et al., 1997; Leder et al., 1999; Howden and Richards, 2001; Recchia et al., 2001), few reports of its use in a toxicology setting have been reported. However, despite infusion of a maximum tolerated dose of diclofenac to the rat for 48 hours, no evidence of hepatotoxicity could be detected. Instead, plasma measurements of diclofenac concentration revealed an adaptive response to diclofenac infusion in animals resulting in enhanced plasma clearance of the drug. Further to this, increased expression of the ROS scavenging enzymes, catalase and superoxide dismutase were detected. Whilst time points of toxicological investigation were not ideal, due to Home Office license constraints on blood volume sampling, these findings suggest that absence of hepatotoxicity may be a consequence of these adaptive responses to drug exposure.

Whilst the adaptive mechanisms responsible for enhanced plasma diclofenac clearance could not be revealed, it is possible that this increased clearance may reduce the potential for drug metabolite or CRM accumulation. Cellular accumulation of drugs or their metabolites has been associated with the pathogenesis of many idiosyncratic ADRs (Park et al., 2011a). With no idiosyncratic ADRs reported for drugs administered at a daily dose of below 10 mg per day (Utrecht, 2003; Stepan et al., 2011), clearly a threshold of drug exposure is required before a risk of idiosyncratic liability is reached.

Cellular accumulation of drugs or their CRMs is likely to increase the threshold to these ADRs. Consequently, if this adaptive response, resulting in increased drug clearance translates to patients receiving chronic drug therapy, failure of a patient to adapt leading to reduced efficiency for drug or CRM elimination compared with the rest of the population may represent an increased risk of the individual for development of an idiosyncratic reaction towards the drug. Genetic polymorphis studies in man, associating MRP2, CYP2C8 and UGT2B7 with increased incidence of hepatotoxicity in patients receiving diclofenac (Daly et al., 2007) does suggest that accumulation of CRMs may have a role in the development of diclofenac-induced hepatotoxicity in man specifically. Before these hypotheses can be made confidently, the absence of hepatotoxicity and mechanisms of increased clearance need to be confirmed. This could be undertaken through repeating the infusion study, allowing consistent assessment of plasma biomarkers of hepatotoxicity throughout the time course. Livers should be isolated from rats immediately following cessation of infusion for histopathological assessment of hepatotoxicity and export transporter induction. Western blot analysis investigating the hepatic induction of metabolising enzymes and export transporters, as well as metabolising enzyme function assays using microsomes or hepatocytes isolated from rats in the study may be used to confirm any histopathological findings from immunohistochemical staining.

6.6 COVALENT MODIFICATION OF HSA ISOLATED FROM VOLUNTEER PATIENTS BY DICLOFENAC ACYL GLUCURONIDE

6.6.1. Identification of the modification of HSA by diclofenac acyl glucuronide in patients receiving therapeutic doses of diclofenac

With the inapplicability of the rat model to investigate covalent modification of serum albumin by AGs as outlined in section 6.4, it was decided to investigate if covalent modifications could be detected in patients receiving therapeutic doses of the drug. Collaboration with the Department of Rheumatology and the Nottingham Digestive Diseases Centre at the Queens Medical Centre in Nottingham provided access to plasma samples isolated from patients receiving therapeutic administration of diclofenac for at least a year. Albumin was isolated from these plasma samples, and covalent binding was assessed using direct mass-spectrometric analysis of tryptic digests of isolated albumin.

With the retention of glucuronic acid in the adduct, the identification of glycation adducts to serum albumin isolated from three out of the six patients tested showed for the first time, definitive evidence for the covalent binding of AG metabolites to HSA *in vivo*. With the well-established associations of covalent binding with immunological ADRs, largely shown through investigations into penicillin hypersensitivity (Park et al., 2011a), this identification of AG covalent binding *in vivo* clearly represents an area of toxicological interest (Faed, 1984; Spahn-Langguth and Benet, 1992; FDA, 2008).

However, it must also be noted that glycation adducts could only be detected to HSA isolated from 50% of the patients analysed. Whilst it is assured that transacylation adducts are elicited as a consequence of direct bioactivation at the carboxylic acid group, due to the covalent linkage with protein at this site, metabolites other than AGs

may also be responsible. Coenzyme A thioester conjugates of carboxylate compounds have also been shown undergo transacylation reactions with protein (Li et al., 2002; Olsen et al., 2003), and glutathione (Grillo and Benet, 2002; Sidenius et al., 2004). Consequently, they have the potential to also transacylate proteins *in vivo* (Skonberg et al., 2008; Grillo, 2011). However, as suggested in section 6.3, with the 1- β isomer primarily responsible for the formation of transacylation adducts (Chapter 2), and potentially increased 1- β AG isomer exposure when compared to the acyl migration isomers, as a consequence of rapid AG clearance, potentially before extensive acyl migration *in vivo*, detection of transacylation adducts *in vivo* may also be a consequence of acyl glucuronidation.

However, it must be remembered that to date, unlike the β -lactam antibiotics, immunogenicity of AG derived covalent adducts, has not yet been shown. The only real evidence in the literature for the immunogenicity of AG adducts suggested by the generation of antibodies in rats exposed to RSA modified *in vitro* by diflunisal-AG through incubation (Worrall and Dickinson, 1995). The requirement for co-administration of Freund's adjuvant to elicit antibody production, however, hardly provides good evidence for the immunogenicity of AGs. With many drugs found to covalently bind to proteins without associations to toxicity, clearly an important next step in the research into the risk of acyl glucuronidation would be to investigate the immunogenicity of covalent adducts generated by AGs. Consequently, it is clear that as well as confirming the covalent binding of AGs to serum albumin with other carboxylate compounds, investigations into the resulting immunogenicity is important. Approaches similar to those used in the investigation of β -lactam immunogenicity may be useful, where identification of circulating antibodies able to recognise the drug

isolated from drug-hypersensitive patients (Christie et al., 1988; Torres et al., 1997), and proliferation of lymphocytes isolated from hypersensitive patients following drug exposure or exposure to the drug target (Meng et al., 2011; El-Ghaiesh et al., 2012). Importantly, investigations into the covalent modification of proteins, and immunogenicity of these proteins should be undertaken, as outlined below, in combination with modified proteins also being tested for their immunogenicity.

6.6.2. Considerations of disposition in the covalent modification of serum proteins by carboxylate compounds

The disposition of AGs may provide further evidence for their role in hypersensitivity off-target ADRs associated with carboxylate compounds. Generalised hypersensitivity reactions are usually considered to be a consequence extracellular covalent modification of proteins, resulting in the drug-hapten presentation by MHC-II molecules, and stimulation of CD4+ T-cells and B-cells (Pichler, 2003). Consequently, due to the well-defined systemic circulation of AGs (Volland et al., 1990; Benet et al., 1993; Mayer et al., 1993; Zia-Amirhosseini et al., 1994; Stangier et al., 2000; Zhou et al., 2001; Wang et al., 2006) acyl glucuronidation may be suggested to be the bioactivation pathway responsible for hypersensitivity ADRs to carboxylate compounds. However, it is also possible that intrahepatically modified peptides may be exported outside the cell, and result in the stimulation of MHC-II responses. Suggested reactivity of oxidative metabolites of some carboxylate metabolites and their subsequently formed quinone-imines (Tang et al., 1999; Chen et al., 2006) may also have a role to play on the extracellular covalent binding of carboxylate compounds.

On the other hand, tissue-specific delayed hypersensitivity ADRs, as observed with diclofenac-induced hepatotoxicity, are thought to be associated with hapten presentation following the modification of intracellular proteins, with antigen presentation through MHC-I molecules, resulting in CD8⁺ T-cell responses (Pichler, 2003). This suggests modification of intracellular proteins is required for the development of hepatotoxic ADRs towards carboxylate compounds. The intracellular nature of protein modification suggests that CRMs of carboxylate compounds unable to escape the hepatocyte also have a role to play in the pathogenesis of these ADRs.

Immunohistochemical studies have been used to visualise and identify covalently modified hepatic proteins in the rat. These studies have shown covalent modification of hepatic proteins to be located primarily on the canalicular membrane of hepatocytes. AGs, as a consequence of their high canalicular concentrations, estimated to be between 50 to 5000 times higher than in the plasma (Sallustio et al., 1996; Sallustio et al., 2000), have been hypothesised to be responsible for covalent adducts located across the canalicular membrane. The high concentration of AGs at the canalicular membrane is considered to be a consequence of their high affinity for transport across the basolateral membrane into the hepatocyte by MRP3, and subsequently across the canalicular membrane by MRP2 and BCRP, as has been shown for diclofenac-AG through the selective transgenic knockout of mouse transporters (Lagas et al., 2010) and rats (Seitz et al., 1998). Extensive reduction in the covalent binding of diclofenac through inhibition of glucuronidation (Kretz-Rommel and Boelsterli, 1993) and disruption of metabolite export by MRP2 (Seitz et al., 1998) also further suggests the potential of these metabolites to form covalent adducts to hepatic proteins *in vivo*. Whilst these studies do provide strong evidence for the role of AGs to

form covalent adducts in the liver, they may still arise as a consequence of the bioactivation of compounds into several metabolites other than AGs. Until the physiological consequences of covalent binding are ascertained, questions will remain as to its role in toxicology.

6.7 SUMMARY

Through this thesis, *in vitro* experiments revealed diclofenac-AG to be chemically unstable and protein reactive following *in vitro* incubation. This revealed it was a suitable AG for use throughout the rest of the thesis to investigate AG protein reactivity *in vivo*. Further *in vitro* experiments revealed that plasma hydrolysis may act alongside drug elimination to enhance the clearance of AGs from organisms. Experiments in the rat revealed it is not a suitable tool for investigating AG protein reactivity towards serum albumin, potentially as a consequence to species differences in the primary sequence between HSA and RSA. The rapid plasma clearance of AGs was confirmed in the rat, with continuous infusion of diclofenac revealing an adaptive change resulting in an enhanced rate of plasma clearance of diclofenac, alongside induction of the ROS scavenger enzymes catalase and superoxide dismutase. The absence of any signal of hepatotoxicity in these animals suggests these adaptive changes act to protect the animal from any deleterious consequences of drug-exposure. The final experiments in the thesis were focused on the identification of whether the AG of diclofenac could be identified covalently bound to serum albumin isolated from patients receiving therapeutic doses of the drug. The identification of glycation adducts to HSA isolated from 50% of patients tested identify for the first time that AGs can form covalent adducts *in vivo*. The definitive identification of transacylation adducts *in vivo*, which is also novel, also further confirms that bioactivation at the carboxylic acid of site of carboxylate drugs results in the formation of CRMs capable of forming covalent adducts *in vivo*, however identification of CRM(s) responsible for these transacylation adducts was not possible. These findings re-affirm concerns over the safety of the carboxylic acid functional group in pharmaceuticals,

and reaffirms anxiety over acyl glucuronidation as a potential toxification pathway. To assess whether these covalent adducts actually are a toxicological concern, physiological consequences of these covalent modifications needs to be assessed.

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SUPPLEMENTARY INFORMATION

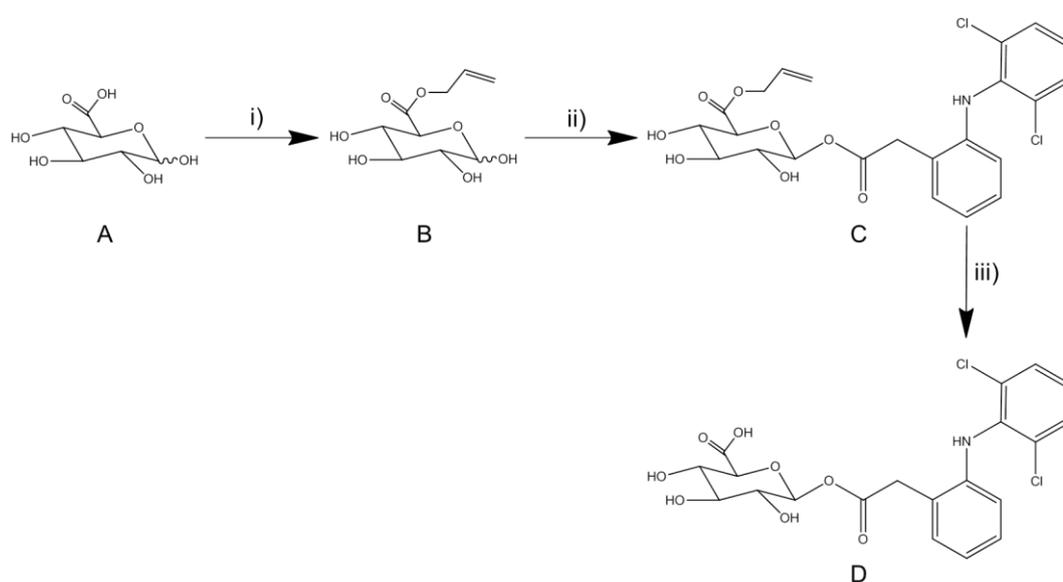
Supplementary 1.1 Chemical synthesis of diclofenac-AG

The first stage describes the synthesis of allyl α , β -D-glucuronate (Supplementary Figure 1B). D-glucuronic acid (Supplementary Figure 1A) (4 g, 20 mmol) was stirred with polymer-bound fluoride (6.7 g, 20 mmol) in 30 ml of dry dimethylformamide at 20°C for 3 hours. Following this, 1.9 ml of 22 mmol allyl bromide was added and the reaction mixture was stirred at 40°C for 20 hours. The reaction mixture was filtered away, and the resin washed twice with 10 ml dimethylformamide. The combined filtrate was concentrated under high vacuum to provide the crude products as a pale yellow oil. Flash chromatographic separation using ethyl acetate, isopropyl alcohol and dH₂O at ratios of 5, 3, 1 (v/v) provided the ester at 75% yield.

The second stage describes the conversion of allyl α , β -D-glucuronate to diclofenac allyl glucuronate (Supplementary Figure 1C). Diisopropyl azodicarboxylate (0.1 ml, 0.5 mmol) was slowly added over 10 mins to a solution of diclofenac (0.170 g, 0.5 mmol), triphenylphosphine (0.135 g, 0.5 mmol) and allyl α , β -D-glucuronate (0.12 g, 0.5 mmol) in 3 ml anhydrous tetrahydrofuran stirred under nitrogen at 0°C. After 1 hour the solution was evaporated to dryness and purified using silica chromatography, eluting with 5% MeOH in dichloromethane. Appropriate fractions were pooled and evaporated to give the product as an α/β mixture. A second chromatography, eluting with 5% ethanol in diethyl ether provided the pure β product as a white solid with a yield of 30%.

The final stage describes the conversion of 1- β diclofenac allyl glucuronate to 1- β diclofenac-AG (Supplementary Figure 1D). A solution of 1- β diclofenac allyl glucuronate (0.3 mmol) and tetrakis(triphenylphosphine)palladium (0.03 mmol) made in dry tetrahydrofuran and cooled to 0°C. To this mixture morpholine (0.3 mmol) was

added, with the reaction mixture stirred under nitrogen for 1 hour, and was subsequently evaporated to dryness. The crude product was subsequently purified by chromatography, eluting with 20% ethanol-dichloromethane. Appropriate fractions were concentrated to provide the desired 1- β diclofenac-AG product. Occasionally, reversed-phase column chromatography (acetonitrile, dH₂O, 10-40%) or recrystallization was required to remove residual traces of palladium. Pure 1- β diclofenac-AG was obtained with a 75% yield in this step as a white solid.



Supplementary figure 1: Schematic representation of the chemical synthesis of diclofenac-AG. Reaction intermediates are (A) D-glucuronic acid (B) Allyl α,β -D-glucuronate (C) Diclofenac ally glucuronate (D) Diclofenac-AG. Reaction steps are (i) Stirring of (A) with polymer bound fluoride in dimethylformate at 20°C followed by addition of allyl bromide at 40°C. (ii) Slow addition of diisopropyl azodicarboxylate to a solution of diclofenac, triphenylphosphine and (B) in anhydrous tetrahydrofuran at 0°C. (iii) Addition of morpholine to a solution of (C) tetrakis(triphenylphosphine)palladium at 0°C.

Purity of synthetic 1- β diclofenac-AG was assessed using a combination of LC-UV, tandem mass-spectrometric and NMR assessment. For acceptable use in experiments, purity was above 98%. LC-UV conditions as described in section 2.2.4 were used to assess diclofenac purity.

Supplementary Table 1: MRM transitions for peptides modified by diclofenac-AG identified in either in vitro incubations in chapter 2, or in clinical samples in chapter 5

Peptide	Charge	Q1		Q3
		Transacylation	Glycation	
¹³⁷ KYLYEIAR ¹⁴⁴	2+	667.3	754.8	215.1
¹³⁷ KYLYEIAR ¹⁴⁴	2+	667.3	754.8	250.1
¹³⁷ KYLYEIAR ¹⁴⁴	3+	445.2	503.5	215.1
¹³⁷ KYLYEIAR ¹⁴⁴	3+	445.2	503.5	250.1
¹⁸¹ LDELRDEGKASSAK ¹⁹⁵	3+	599.6	657.9	215.1
¹⁸¹ LDELRDEGKASSAK ¹⁹⁵	3+	599.6	657.9	250.1
¹⁸¹ LDELRDEGKASSAK ¹⁹⁵	4+	449.9	493.7	215.1
¹⁸¹ LDELRDEGKASSAK ¹⁹⁵	4+	449.9	493.7	250.1
¹⁹¹ ASSAKQR ¹⁹⁷	2+	513.2	600.7	215.1
¹⁹¹ ASSAKQR ¹⁹⁷	2+	513.2	600.7	250.1
¹⁹¹ ASSAKQR ¹⁹⁷	3+	342.5	400.8	215.1
¹⁹¹ ASSAKQR ¹⁹⁷	3+	342.5	400.8	250.1
¹⁹⁸ LKCASLQK ²⁰⁵	2+	613.3	700.7	215.1
¹⁹⁸ LKCASLQK ²⁰⁵	2+	613.3	700.7	250.1
¹⁹⁸ LKCASLQK ²⁰⁵	3+	409.2	467.5	215.1
¹⁹⁸ LKCASLQK ²⁰⁵	3+	409.2	467.5	250.1
³⁴⁹ LAKTYETTLK ³⁵⁹	2+	787.8	875.3	215.1
³⁴⁹ LAKTYETTLK ³⁵⁹	2+	787.8	875.3	250.1
³⁴⁹ LAKTYETTLK ³⁵⁹	3+	525.5	583.9	215.1

Supplementary Table 2: MRM transitions for peptides modified by diclofenac-AG identified in either *in vitro* incubations in chapter 2, or in clinical samples in chapter 5

Peptide	Charge	Q1		Q3
		Transacylation	Glycation	
³⁴⁹ LAKTYETLEK ³⁵⁹	3+	525.5	583.9	250.1
³⁴⁹ LAKTYETLEK ³⁵⁹	4+	394.4	438.2	215.1
³⁴⁹ LAKTYETLEK ³⁵⁹	4+	394.4	438.2	250.1
⁴²⁹ NLGKVGSK ⁴³⁶	2+	540.8	628.3	215.1
⁴²⁹ NLGKVGSK ⁴³⁶	2+	540.8	628.3	250.1
⁴²⁹ NLGKVGSK ⁴³⁶	3+	360.8	419.2	215.1
⁴²⁹ NLGKVGSK ⁴³⁶	3+	360.8	419.2	250.1
⁴³³ VGSKCCK ⁴³⁹	2+	558.7	646.2	215.1
⁴³³ VGSKCCK ⁴³⁹	2+	558.7	646.2	250.1
⁴³³ VGSKCCK ⁴³⁹	3+	372.8	431.1	215.1
⁴³³ VGSKCCK ⁴³⁹	3+	372.8	431.1	250.1
⁵²⁵ KQTALVELVK ⁵³⁴	2+	703.8	791.4	215.1
⁵²⁵ KQTALVELVK ⁵³⁴	2+	703.8	791.4	250.1
⁵²⁵ KQTALVELVK ⁵³⁴	3+	469.5	527.9	215.1
⁵²⁵ KQTALVELVK ⁵³⁴	3+	469.5	527.9	250.1
⁵³⁹ ATKEQLK ⁵⁴⁵	2+	548.3	635.7	215.1
⁵³⁹ ATKEQLK ⁵⁴⁵	2+	548.3	635.7	250.1
⁵³⁹ ATKEQLK ⁵⁴⁵	3+	365.8	424.1	215.1
⁵³⁹ ATKEQLK ⁵⁴⁵	3+	365.8	424.1	250.1

Supplementary Table 3: Summary of HSA peptides identified as modified in clinical samples, showing cation exchange fractions modified peptides were detected and their representative m/z values

Patient	Cation exchange fraction	Peptide	Transacylation	Glycation	Ion
N01	39-40	¹⁹⁸ LKCASLQK ²⁰⁵		G	700.8 2+
	41-42	¹⁹⁸ LKCASLQK ²⁰⁵	T		612.8 2+
	41-42	⁴²⁹ NLGKVGSK ⁴³⁶	T		540.3 2+
	41-42	⁵³⁹ ATKEQLK ⁵⁴⁵	T		547.7 2+
	43-44	¹⁹¹ ASSAKQR ¹⁹⁷	T		512.7 2+
	51-52	¹⁸¹ LDEL RDEGKASSAK ¹⁹⁵	T		599.3 3+
N02	41-42	¹⁹⁸ LKCASLQK ²⁰⁵	T		612.8 2+
	41-42	⁴²⁹ NLGKVGSK ⁴³⁶	T		540.3 2+
	41-42	⁵³⁹ ATKEQLK ⁵⁴⁵	T		547.7 2+
	43-44	¹⁹¹ ASSAKQR ¹⁹⁷	T		512.7 2+
	51-52	¹⁸¹ LDEL RDEGKASSAK ¹⁹⁵	T		599.3 3+
N03	41-42	¹⁹⁸ LKCASLQK ²⁰⁵	T		612.8 2+
	41-42	⁵³⁹ ATKEQLK ⁵⁴⁵	T		547.7 2+
	43-44	¹⁹¹ ASSAKQR ¹⁹⁷	T		512.7 2+
N08	39-40	¹⁹⁸ LKCASLQK ²⁰⁵		G	700.8 2+
	39-40	¹⁹⁸ LKCASLQK ²⁰⁵		G (-H ₂ O)	691.9 2+
	39-40	⁵²⁵ KQTALVELVK ⁵³⁴	T		469.2 3+
	41-42	¹⁹⁸ LKCASLQK ²⁰⁵	T		612.8 2+
	41-42	⁴²⁹ NLGKVGSK ⁴³⁶		G	628.3 2+
	41-42	⁵³⁹ ATKEQLK ⁵⁴⁵	T		547.7 2+
	43-44	¹⁹¹ ASSAKQR ¹⁹⁷		G	600.7 2+
	43-44	¹⁹¹ ASSAKQR ¹⁹⁷	T		512.7 2+
	43-44	⁴³³ VGSKCCK ⁴³⁹	T		559.2 2+
	51-52	⁵²⁵ KQTALVELVK ⁵³⁴		G	435.6 3+
N09	43-44	¹⁹¹ ASSAKQR ¹⁹⁷		G	600.7 2+
	43-44	¹⁹¹ ASSAKQR ¹⁹⁷	T		512.7 2+
N10	43-44	¹⁹¹ ASSAKQR ¹⁹⁷	T		512.7 2+

Supplementary Table 4: MRM transitions run for detection of peptides modified by diclofenac-AG. None of the peptides represented in this table were identified as modified following either in vitro incubations of diclofenac-AG with HSA (Chapter 2) or HSA isolated from patients receiving therapeutic doses of diclofenac (Chapter 5).

Peptide	Charge	Q1		Q3
		Transacylation	Glycation	
¹ DAHKSEVAHR ¹⁰	2+	714.3	801.8	215.1
¹ DAHKSEVAHR ¹⁰	2+	714.3	801.8	250.1
¹ DAHKSEVAHR ¹⁰	3+	476.5	534.9	215.1
¹ DAHKSEVAHR ¹⁰	3+	476.5	534.9	250.1
¹¹ FKDLGEENFK ²⁰	2+	752.8	840.3	215.1
¹¹ FKDLGEENFK ²⁰	2+	752.8	840.3	250.1
¹¹ FKDLGEENFK ²⁰	3+	502.2	560.3	215.1
¹¹ FKDLGEENFK ²⁰	3+	502.2	560.3	250.1
¹⁴⁶ HPYFYAPELLFFAKR ¹⁶⁰	3+	726.3	784.6	215.1
¹⁴⁶ HPYFYAPELLFFAKR ¹⁶⁰	3+	726.3	784.6	250.1
¹⁴⁶ HPYFYAPELLFFAKR ¹⁶⁰	4+	544.9	588.7	215.1
¹⁴⁶ HPYFYAPELLFFAKR ¹⁶⁰	4+	544.9	588.7	250.1
¹⁶¹ YKAAFTECQAADK ¹⁷⁴	2+	970.8	NA	215.1
¹⁶¹ YKAAFTECQAADK ¹⁷⁴	2+	970.8	NA	250.1
¹⁶¹ YKAAFTECQAADK ¹⁷⁴	3+	647.5	705.9	215.1
¹⁶¹ YKAAFTECQAADK ¹⁷⁴	3+	647.5	705.9	250.1
¹⁶¹ YKAAFTECQAADK ¹⁷⁴	4+	485.9	529.7	215.1
¹⁶¹ YKAAFTECQAADK ¹⁷⁴	4+	485.9	529.7	250.1
²¹⁰ AFKAWAVAR ²¹⁸	2+	649.3	736.8	215.1
²¹⁰ AFKAWAVAR ²¹⁸	2+	649.3	736.8	250.1
²¹⁰ AFKAWAVAR ²¹⁸	3+	433.2	491.5	215.1
²¹⁰ AFKAWAVAR ²¹⁸	3+	433.2	491.5	250.1

Supplementary Table 5: MRM transitions run for detection of peptides modified by diclofenac-AG. None of the peptides represented in this table were identified as modified following either *in vitro* incubations of diclofenac-AG with HSA (Chapter 2) or HSA isolated from patients receiving therapeutic doses of diclofenac (Chapter 5).

Peptide	Charge	Q1		Q3
		Transacylation	Glycation	
²⁷⁵ LKECCEKPLLEK ²⁸⁶	2+	912.9	NA	215.1
²⁷⁵ LKECCEKPLLEK ²⁸⁶	2+	912.9	NA	250.1
²⁷⁵ LKECCEKPLLEK ²⁸⁶	3+	608.9	667.2	215.1
²⁷⁵ LKECCEKPLLEK ²⁸⁶	3+	608.9	667.2	250.1
²⁷⁵ LKECCEKPLLEK ²⁸⁶	4+	456.9	500.6	215.1
²⁷⁵ LKECCEKPLLEK ²⁸⁶	4+	456.9	500.6	250.1
³⁶⁷ VFDEFKPLVEEPQNLIK ³⁸³	3+	775.1	833.4	215.1
³⁶⁷ VFDEFKPLVEEPQNLIK ³⁸³	3+	775.1	833.4	250.1
³⁶⁷ VFDEFKPLVEEPQNLIK ³⁸³	4+	581.6	625.3	215.1
³⁶⁷ VFDEFKPLVEEPQNLIK ³⁸³	4+	581.6	625.3	250.1
⁴⁷³ VTKCCTESLVNR ⁴⁸⁴	2+	872.8	960.4	215.1
⁴⁷³ VTKCCTESLVNR ⁴⁸⁴	2+	872.8	960.4	250.1
⁴⁷³ VTKCCTESLVNR ⁴⁸⁴	3+	582.2	640.6	215.1
⁴⁷³ VTKCCTESLVNR ⁴⁸⁴	3+	582.2	640.6	250.1
⁴⁷³ VTKCCTESLVNR ⁴⁸⁴	4+	436.9	480.7	215.1
⁴⁷³ VTKCCTESLVNR ⁴⁸⁴	4+	436.9	480.7	250.1
⁵⁴² EQLKAVMDDFAAFVEK ⁵⁵⁷	3+	706.9	765.3	215.1
⁵⁴² EQLKAVMDDFAAFVEK ⁵⁵⁷	3+	706.9	765.3	250.1
⁵⁴² EQLKAVMDDFAAFVEK ⁵⁵⁷	4+	530.4	574.2	215.1
⁵⁴² EQLKAVMDDFAAFVEK ⁵⁵⁷	4+	530.4	574.2	250.1

Supplementary Table 6: Concomittant medications and patient characteristics of patients N01-N03 analysed for covalent binding of diclofenac-AG to human serum albumin, and plasma concentrations of diclofenac and diclofenac-AG

	N01	N02	N03
Sex	Male	Female	Female
Date of birth (dd/mm/yy)	07/11/1968	05/02/1945	06/07/1959
Diclofenac dose	50mg bid EC	50mg bid EC	50mg am 75mg pm EC MR
Diagnosis	Rheumatoid arthritis (2010)	Rheumatoid arthritis (1998)	Rheumatoid arthritis (1990)
Other medical issues	Sciatica	Osteoporosis fracture L2 Erosive antral gastritis	Carcinoma Breast (10/2010)
Concomittant medications	Methotrexate 25mg/wk (Oct 2010)	Methotrexate 10mg/wk (02/2010)	Methotrexate 20mg/wk (1999)
	Folic Acid	Folic Acid (02/2010)	Folic acid 5mg /wk
	Leflunomide (02/2011)	Prednisolone	Calcichew D3 Forte bid
		Alendronate	Prednisolone 5mg daily (12/2010)
		Calcichew D3 Forte	Omeprazole
		Dermovate	Tamoxifen 20mg daily (Dec 2010)
		Etanercept (12/2009)	Zoladex iv monthly (Dec 2010)
		Omeprazole (04/2011)	
		Citalopram (04/2011)	

Supplementary Table 7: Concomittant medications and patient characteristics of patients N08-N10 analysed for covalent binding of diclofenac-AG to human serum albumin, and plasma concentrations of diclofenac and diclofenac-AG

	N08	N09	N10
Sex	Male	Male	Female
Date of birth (dd/mm/yy)	16/12/1947	17/12/1962	09/04/1934
Diclofenac dose	50mg tds EC	50mg tds	50mg bid
Diagnosis	Rheumatoid arthritis (approx. 1980s)	Ankylosing Spondylitis 1977	Massive swelling right knee Jan2011, working diagnosis OA.
Other medical issues	Insulin dependent diabetes mellitus Coronary artery bypass graft	Wegener's Granulomatosis with left ICA occlusion associated with left hemisphere Infarction (2006)	Generalised nodal osteoarthritis; migraine since childhood; hypertension;
Concomittant medications	Sulfasalazine	Azathioprine 150mg/day	Co-codamol prn
	Hydroxychloroquine	Simvastatin 40mg/day	Lansoprazole 15mg OD
	Insulin	Lansoprazole 15mg/day	
	Aspirin	Paracetamol prn	
	Atenolol		
	Paracetamol		
	Alendronate		
	Beclomethasone		
	Rosuvastatin		
	Adcal		
	Thyroxine		
	Ramipril		
Tadalafil			