UTILITY OF THE HRN™ (HEPATIC CYP REDUCTASE NULL) MICE FOR INVESTIGATING MECHANISMS OF LIVER TOXICITY OF CARBOXYLIC-ACID-CONTAINING DRUGS

BY

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ABSTRACT

Many carboxylic-acid-containing drugs cause liver injury in humans. Examples include fenclozic acid (FA), which was withdrawn due to jaundice observed in clinical trials, and diclofenac (DFC) which remains widely prescribed despite being associated with liver damage. To explore whether these toxicities could be due to metabolic bioactivation mediated by cytochrome P450 (CYP) or conjugative enzymes, covalent binding (CVB) assays were done using liver microsomal incubations from wild-type and hepatic cytochrome P450 reductase null (HRN™) mice, which are deficient in CYP activity. High levels of CYP-mediated CVB of [^{14}C]-FA and [^{14}C]-DFC were observed in wild-type microsomes, but not in HRN™ microsomes. No UDPGA-mediated CVB was detected in microsomes incubated with [^{14}C]-FA. Wild-type and HRN™ mice were orally administered DFC or FA orally for 7days. At 100 mg/kg, FA caused a significant ($p<0.05$) time-dependent increase in plasma alanine amino transferase (ALT) in wild-type but not HRN™ mice. Aberrant liver histopathology and liver clinical chemistry were evident in HRN^{TM} mice and treatment with DFC and FA "normalised" the elevated ALT levels. These data demonstrate that FA undergoes CYP mediated bioactivation and that HRN^{TM} mice are well suited to investigations of metabolism, but not of liver toxicity, due to impaired liver function.

DEDICATION

This project is dedicated to the ONE WHO CAUSES TO BECOME, **JEHOVAH GOD,** who has seen me through all these challenging years. I also dedicate it to my family, my loving mum and siblings who are supportive and who keep their confidence in me, and who do not relent in their prayers. Thank you for your love and support.

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ABBREVIATIONS

ABT	1-amino benzotriazole
AG	acyl glucuronide
ALP	Alkaline phosphatase
ALT	alanine aminotransferase
AST	Aspartate aminotransferase
ATP	adenosine triphosphate
BCA	bicinchoninic acid
COX	Cyclo-oxygenase
CPM	counts per minute
CVB	covalent binding
CYP	cytochrome P450
DFC	diclofenac
DNA	deoxyribonucleic acid
DRF	dose-range-finding
EDTA	Ethylenediaminetetraacetic acid
EM	electron microscopy
FA	fenclozic acid
FMO	flavin-containing mono-oxygenase
GLDH	glutamate dehydrogenase
GSH	glutathione
hCYP	human cytochrome P450
HM	Heavy mitochondrial pellets
HPLC-MS	High performance liquid chromatography-Mass spectrometry
HPLC-RAD-MS	High performance liquid chromatography-Radiochemical-Mass Spectrometry
HRN™	hepatic cytochrome P450 reductase null
IADR	idiosyncratic adverse reaction

LM	light mitochondrial pellets
MPT	membrane permeability transition
MRP2	multi-drug resistance protein type 2
NADH	Nicotinamide adenine dinucleotide
NADPH	Nicotinamide adenine dinucleotide phosphate
NSAID	non-steroidal anti-inflammatory drugs
OATP	organic anionic transporting peptides
OD	optical density (absorbance)
POR	Cytochrome P450 oxidoreductase
SD	standard deviation
SDH	Succinate Dehydrogenase
SDS	sodium dodecyl sulphate
SEM	standard error of the mean
SER	smooth endoplasmic reticulum
SOD	superoxide dismutase
UDP	uridine diphosphate
UDPGA	Uridine diphosphate glucuronic acid
UGT	UDP-glucuronosyltransferase
WT	wild-type

CHAPTER ONE

1.0 INTRODUCTION

1.1 CARBOXYLIC-ACID AND DRUG TOXICITY

The carboxylic acid moiety occurs in many drug classes including anti-convulsants, hypolipidaemics, non-steroidal anti-inflammatory drugs (NSAIDs), quinolone antibiotics (e.g. levofloxacin), angiotensin receptor inhibitors (e.g. telmisartan), leukotriene receptor antagonists (e.g. montelukast) and some diuretics (e.g. furosemide) (Skoberg *et al.*, 2008; Sawamura *et al.*, 2010). Some drugs like celecoxib are also known to be oxidatively metabolised to carboxylic acid. In addition, many endogenous compounds like bile acids, keto-acid, amino acids, and triglycerides also contain the carboxylic acid moiety. Because these endogenous compounds have a clearly-defined metabolic pathway, xenobiotics with similar structural features can be metabolised in a highly predictable manner.

Many of the drugs containing a carboxylic acid functional group have been associated with idiosyncratic and other predictable forms of toxicity in experimental animals or in humans. It was reported that over 14% of drugs withdrawn from the market in the closing years of the last century were those containing carboxylic acid (Fung *et al.*, 2001), and non-steroidal anti-inflammatory drugs (NSAID) topped the list. Literature search shows that severe forms of toxicity and adverse drug reaction of some NSAIDs are partly responsible for either failure to progress from development to the market or withdrawal from clinical use.

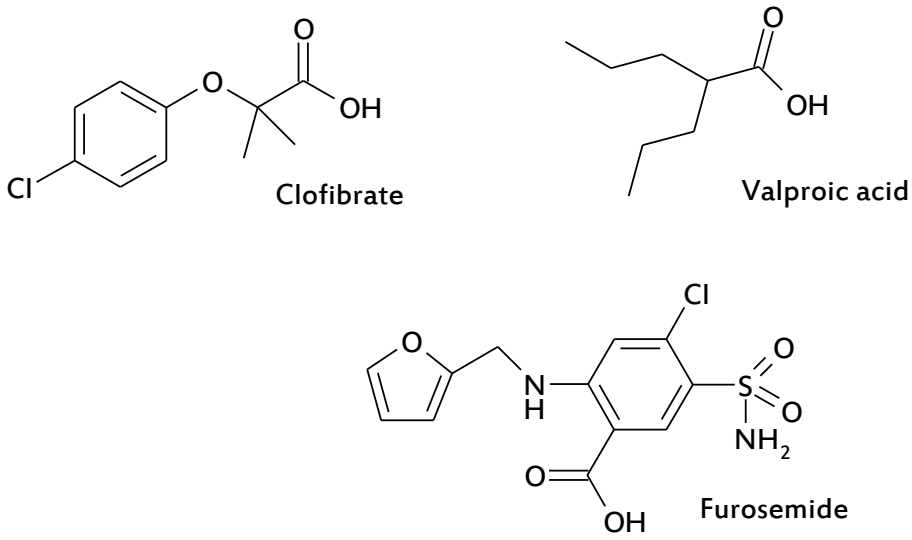


Fig 1.1: Examples of carboxylic-acid-containing drugs

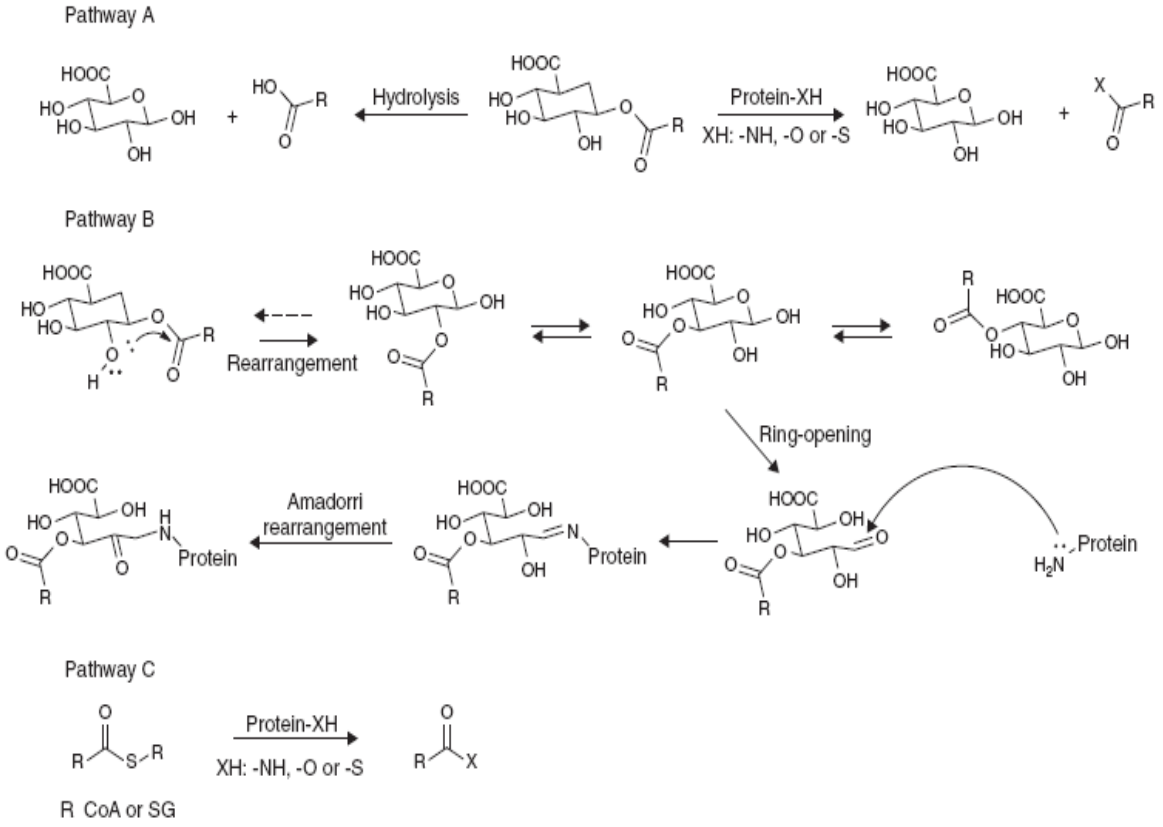
A review by Goldkind and Laine (2006) showed that bromfenac, ibufenac, and benoxaprofen were withdrawn principally due to hepatic injuries.

What is responsible for this trend among this group of NSAIDs? It is possible that this could be due the one thing they have in common i.e. the carboxylic acid moiety.

The carboxylic acid portion of these molecules is a substrate of UDP-glucuronosyltransferases (UGTs), which catalyse the conjugation of the drug to endogenous glucuronic acid to produce acyl glucuronides and/ or acyl iso-glucuronides. Carboxylic acids may also be metabolised by acyl-CoA-synthetase, resulting in the formation of acyl-CoA thioesters. These two products are electrophilic and highly reactive (Skoberg *et al.*, 2008). In addition, there is the possibility to form acyl glutathione thioesters which may also be chemically reactive.

1.1.1 Acyl glucuronides

Acyl glucuronides are formed by glucuronidation reaction catalysed by UGTs, the most relevant isoform in humans being the UGT2B7 expressed mainly in the liver, where a bulk of biotransformation takes place. Consequently, high levels of glucuronides are found in hepatocytes. This is further aided by hepatic uptake, which is mediated by transport proteins (e.g. the organic anionic transporting peptides, OATP) present on the sinusoidal plasma membranes of the liver. Acyl glucuronides of many compounds are excreted into the bile via active hepatobiliary transport, which in many instances is mediated by multi-drug resistance protein type 2 (MRP2). Biliary concentration of acyl glucuronides has been observed to be about 5000 fold higher than in the circulating plasma (Seitz *et al.*, 1998; Boelsterli, 2002). A commonly proposed mechanism of toxicity induced by acyl glucuronides is as a consequence of covalent binding with cellular proteins. Some of the initial findings on covalent binding of acyl glucuronides to proteins were undertaken by Smith *et al.*, (1986) who observed that zomepirac acyl glucuronide irreversibly bound to human plasma protein *in vivo* and *in vitro*. It is understood that adduct formation with protein occurs by either a direct attack of the nucleophilic group of the protein with a resultant displacement of the glucuronic acid from the parent drug, or a base-catalysed reaction in which there is a series of positional isomerism culminating in ring opening on the glucuronide acid portion of the conjugate and exposing its keto group to nucleophilic attack by the amino group of the protein (Figure 1.2). The second mechanism is thought to be the main route of acyl glucuronide-protein adducts formation (Skoberg *et al.*, 2008).



Source: Skoberg *et al.*, 2008

Fig 1.2: Proposed mechanisms of protein adduct formation by acyl glucuronides and acyl-coA thioesters

Mechanisms by which acyl glucuronides might cause toxicity have been proposed. The first is via direct activation of neutrophils and macrophages, resulting in the release of proinflammatory mediators and cytokines. In an *in vitro* study, Wieland *et al.* (2000) produced acyl glucuronides of mycophenolic acid by incubating the parent drug with human liver microsomes. When the resulting acyl glucuronides were incubated with human mononuclear leukocytes, a time- and dose-dependent release of cytokines was observed, as were increased transcription and release of interleukin-6 and tumour-necrosis factor. If these events were to

occur *in vivo*, they could result in an acyl-glucuronide-mediated immune response with its attendant toxicological implication. In addition to this, evidence that acyl glucuronides covalently bind with DNA to form adducts has been presented. Sallustio *et al*. (1997) demonstrated that the acyl glucuronide of clofibrate and gemfibrozyl could cause adducts with DNA resulting in strand breakage, which is a classical mechanism of genotoxicity. A third potential mode of acyl-glucuronide-induced toxicity is, as mentioned earlier in this section, covalent modification of cellular proteins with an attendant alteration of signal transduction, triggering of immune response against modified peptides and a likely loss of function of the affected protein (Boelsterli, 2002). There have been sketchy but relevant evidence that acyl glucuronide can result in loss of protein function. Such was the case with the acyl glucuronide of zomepirac which, when incubated with tubulin *in vitro*, caused covalent modification and impaired assembly of tubulin in a dose-dependent manner (Bailey *et al*., 1998). Tubulin is the main component of microtubules and is involved in key cellular processes which include segregation of genetic material, intracellular transport, and maintenance of cell shape and positioning of cell organelles (Unger *et al*., 1990). Therefore, were impairment of microtubule assembly to occur *in vivo,* this could be expected to result in an elimination of microtubule with cell death as a consequence (Unger *et al*., 1990). Another direct evidence of effect of acyl glucuronide of carboxylic acid-containing drugs on protein function was seen with suprofen, which is an NSAID withdrawn due to its renal effect, and was also seen to cause liver injury. Upon covalent binding, the acyl glucuronide of this NSAID was reported to produce remarkable loss in superoxide dismutase (SOD) activity (Chiou *et al*., 1999). Since SOD is an endogenous antioxidant, its depletion may result in oxidative stress and potentially cell death.

1.1.2 Acyl-CoA Thioesters

Acyl-CoA thioesters are important for a number of cellular reactions which include biosynthesis of complex lipids, conjugation of endogenous and exogenous carboxylic acids to amino acids and the enzyme-catalysed S-acylation of proteins, which is a vital step in the regulation of activities of proteins involved in signal transduction (Hall and Quan, 1994). Endogenously, their formation is a *sine qua non* for fatty acid metabolism, elongation and formation of lipids. This reaction is catalysed by fatty acid CoA ligases. Unlike many acyl glucuronide metabolites, acyl CoA thioesters of drugs are highly unstable molecules which are not excreted from cells *in vivo* or *in vitro* in significant quantities. Their main fate is conjugation with amino acids such as glycine, taurine and carnitine esters, which predominantly takes place in the mitochondrial matrix. The most common carboxylic acid conjugates are those with glycine and taurine (Sarda *et al.*, 2012). Drugs that have been associated with metabolism to this class of conjugates include tolmetin, zomepirac, pivalic acid and valproate (Brass, 2002, Olsen *et al.*, 2003; Olsen *et al*, 2005; Li *et al*, 2003). Experimentally, an indicator of the formation of acyl-CoA intermediates is detection of acyl-CoA-dependent metabolites in hepatocyte incubations, in bile or urine extracts (Skoberg *et al.*, 2008). The chemical reactivity of acyl-CoA thioesters of carboxylic acid-containing drugs is due to the electrophilic carbonyl carbon, which enables them to readily attack the nucleophilic centres (thiol-, amino-, or hydroxyl- groups) in proteins and other components of the cell. It has been proposed that acyl-CoA thioesters could exert toxic effects by competition with CoA-activated intermediate fatty acids, which are formed during fatty acid synthesis. In addition, acyl-CoA thioesters have been found to bind covalently to proteins and can also react with glutathione via s-transacylation to form unstable glutathione conjugates, which could perhaps mediate toxicity (as has been proposed for acyl glucuronides).

This research project has focus on and examined two carboxylic-acid-containing drugs, one with a well-reported toxicity profile and the other with scant information in the literature due to its withdrawal from development as a potential anti-rheumatic therapy following liver injury observed in patients. The two drugs are diclofenac and fenclozic acid, respectively.

1.2 DICLOFENAC

Diclofenac 2-(2, 6-dichloranilino) phenylacetic acid is a non-selective Cyclo-oxygenase (COX) inhibitor developed in 1976 by CIBA Geigy, now Novartis, in an attempt to synthesise an NSAID with high efficacy and tolerability (Sallmann, 1986). A potent anti-inflammatory agent, diclofenac blocks the oxidation step in the conversion of arachidonic acid to prostaglandins, notably prostaglandin E_2, which is a well-known mediator of inflammatory processes (Gan, 2010) Inhibition of this pathway results in the alleviation of the cardinal manifestations of inflammation, namely redness, pain, swelling and fever. This has been exploited in management of such conditions as musculoskeletal complaints, especially arthritis, rheumatoid- and osteoarthritis, polymyositis, gout, and dental pain.

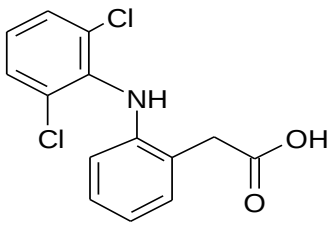


Fig 1.3: Diclofenac

1.2.1 Metabolism of Diclofenac

In humans, diclofenac is mainly oxidatively metabolised by CYP2C9, giving rise to 4′-hydroxydiclofenac (Tang *et al.*, 1999). The 4′-hydroxy metabolite of diclofenac can also be produced by CYP2C8 (Sanjeev *et al.* 2002).

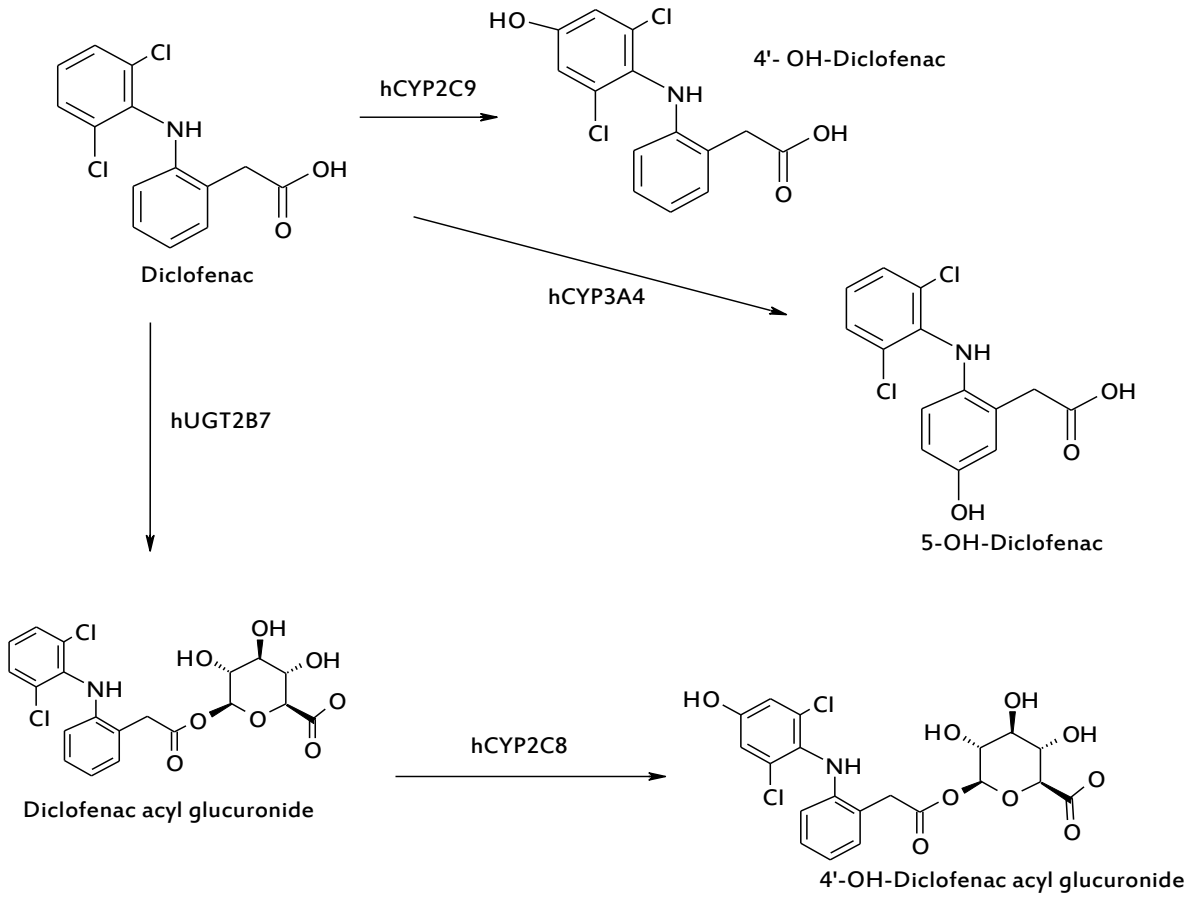


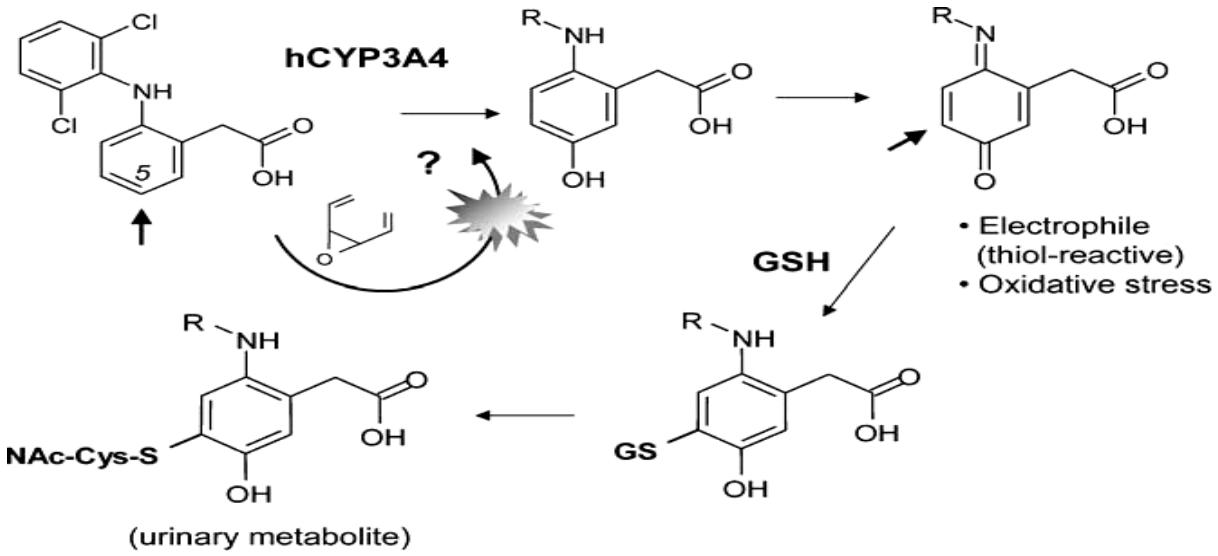
Fig 1.4: Metabolic fate of diclofenac in humans. As the main hepatic phase I enzyme for diclofenac metabolism in humans CYP2C9 biotransforms diclofenac to its 4′-OH metabolite. UGT2B7 is known to conjugatively metabolise diclofenac to an acyl glucuronide that can thereafter be metabolised to its 4′-OH metabolite. All these metabolites are known to form reactive species

Furthermore, another important route for diclofenac metabolism in humans is 5-hydroxylation predominantly catalysed by CYP3A4. Studies with human liver microsomes reveal that diclofenac is glucuronidated by the hepatic UGT2B7 isoenzyme (King *et al.*, 2001).

This is considered to be the predominant metabolic route of this drug in humans since it has been estimated that 75% of its hepatic clearance is by acyl glucuronidation (Sanjeev *et al*. 2002). In addition, both glucuronidation and 4′-hydroxylation may occur, resulting in formation of 4′-hydroxy acyl glucuronide. *In vivo*, metabolism and disposition of diclofenac is complex due to hepatobiliary metabolite excretion mediated by MRP2, hydrolysis of acyl glucuronides by the enteric β-glucuronidase enzyme and enterohepatic circulation. It has been proposed that these processes have toxicological implications (Tang, 2003), which are discussed in details below. In other species, a variety of conjugative metabolites of diclofenac have been elucidated. The dog was observed to principally metabolise diclofenac by taurine conjugation, while in rat acyl glucuronidation followed by biliary excretion is a major metabolic route. In the rainbow trout, multiply-substituted glucuronides of diclofenac have been identified which include ether glucuronides and hydroxyl-substituted acyl glucuronides, as have hydroxysulphates (Kallio *et al*., 2010, Stierlin *et al*., 1979). Sarda and co-workers (2012) discovered some further novel metabolites in the mouse. From mouse urine, they observed that a taurine conjugate was the major metabolite and also identified a 4′-hydroxylated taurine conjugate. In addition they saw 4′- and 5-hydroxy ether glucuronide as well as a hitherto unrevealed conjugate which might be with glucose. There were also indolinone conjugates, which might have been formed due to a dehydration of the carboxylic acid moiety with a consequent ring closing forming a lactam ring. These coexisted with an ether glucuronide of a hydroxyl metabolite.

1.2.2 Role of Diclofenac Metabolism in Hepatotoxicity

Diclofenac remains in clinical use despite its well-reported incidences of rare forms of idiosyncratic liver toxicity (Tang, 2003). As early as 1981, there were reported cases of hepatitis in patients managed with diclofenac. In one case reported by Dunk *et al.*, (1982), liver biopsy specimens showed a moderately severe acute hepatitis with canalicular cholestasis and Kupffer-cell proliferation, and plasma cells and eosinophils in the inflammatory infiltrate. These were reversed when the diclofenac treatment was withdrawn. A review by Boelsterli *et al.* (1995) linked NSAID-mediated hepatotoxicity (including diclofenac) with immunological reactions. A variety of investigative studies have indicated an association between diclofenac-induced liver toxicity and biotransformation of the drug. The phenylamine portion of the structure is the principal target of oxidative metabolism, which is catalysed by CYPs and mainly involves hydroxylation to excreted metabolites (see above).

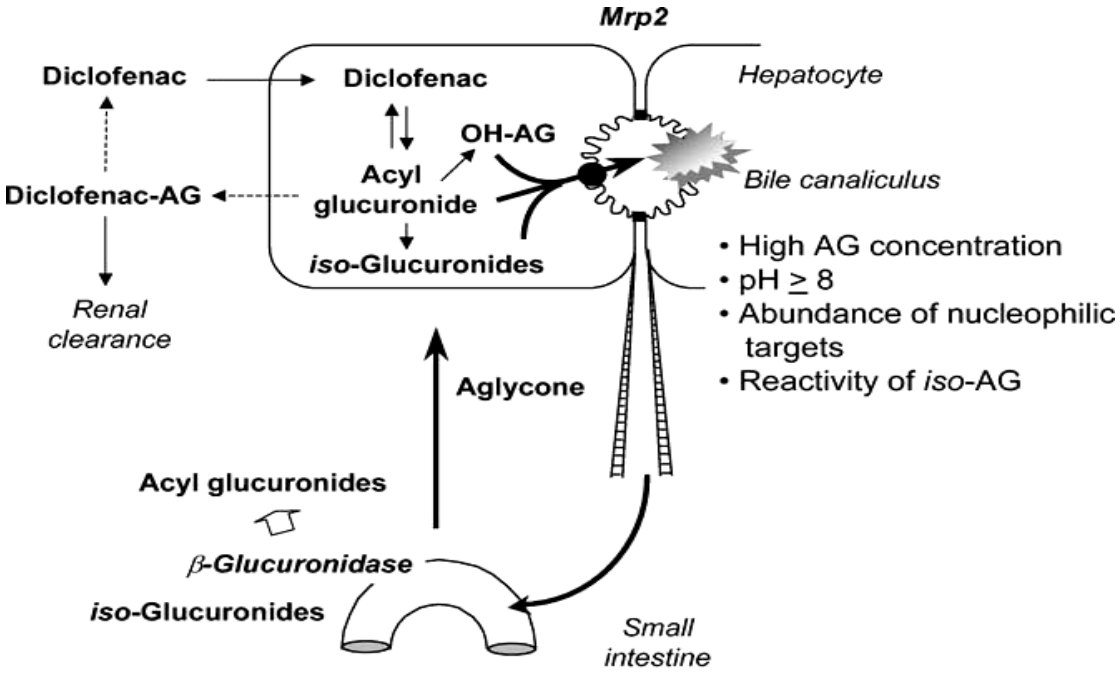


Source: Boelsterli, 2003

Fig. 1.5: Formation of potentially reactive metabolites of diclofenac. P-benzoquinone imine formed from 5-OH-diclofenac is followed by S-glutathionylation and is excreted as urinary mercapturic acid in humans. CYP3A4 can also form a potentially reactive epoxide which at a high concentration can bind to and inactivate the enzyme *in vitro*.

However, diclofenac oxidation may also produce highly reactive epoxide (arene oxide) intermediates and quinone imines at the 5- or 4'- positions of the benzene rings of the molecule. *In-vitro* studies have demonstrated that epoxide formation from diclofenac is catalyzed by CYP3A4 and results in enzyme inactivation arising from protein adduct formation. Quinone imines are highly reactive electrophiles which react chemically with thiol groups on proteins as well as other endogenous thiol-containing cellular components. They also readily bind with glutathione (GSH) and at a high cellular concentration may result in GSH depletion and hence oxidative stress. In a study involving the use of rat hepatocytes, induction of the cytochrome P450 system was shown to result in an increased potency of cytotoxicity of diclofenac (Schmitz *et al*. 1992). This cellular event was associated with an uncoupling of the mitochondrial oxidative phosphorylation, oxidative stress, and induction of immune responses. As earlier mentioned, the carboxylic acid moiety of diclofenac is a substrate for UGTs and product is the chemically reactive acyl glucuronides (diclofenac 1-o-β acyl glucuronide) and/or iso-glucuronides. The latter are formed via a series of isomerisation steps that culminate in ring opening and exposure of the keto group of the glucuronic acid, which is highly electrophilic and is resistant to the β-glucuronidase enzyme. Acyl glucuronides (AG) of diclofenac are substrates for the active transporter MRP2, which concentrates these metabolites in the bile from where they are emptied into the gastro-intestinal tract, thereby setting stage for entero-hepatic circulation. Within the gastro-intestinal tract, enteric microflora release β-glucuronidase which cleaves glucuronide conjugates thereby releasing the unconjugated drug for further absorption and metabolism by the liver (Boelsterli, 2003). Hydrolysis of acyl glucuronides can also be achieved by non-specific esterases or a simple aqueous hydrolysis of the metabolite which occurs at alkaline pH, enabling recirculation of diclofenac from bile into the general circulation. These products are transported by the MRP2 into the biliary tree as

illustrated below (Figure 1.6). Adducts can be formed between the AGs and proteins in the bile canaliculus, while the enteric β-glucuronidase can effect a cleavage of the AG thereby releasing the parent compound, starting enterohepatic cycling. On the other hand, *iso*-glucuronides, which are not glucuronidase-labile, do not enter the cycle. This process is known to increase the exposure time of the liver to diclofenac and its metabolites, hence providing higher chances for cellular insults arising from formation of reactive intermediates.



Source: Boelsterli, 2003

Fig. 1.6: Disposition of diclofenac acyl glucuronide (AG), hydroxydiclofenac acyl glucuronide, and *iso*-glucuronides in the liver.

Boelsterli (2003) has pointed out that diclofenac-induced liver injury in humans typically is characterised by elevated plasma amino transferase activities alongside jaundice and in some

instances cholestatic hepatocellular injury, that hypersensitivity reactions may also arise and that there is a delayed onset of manifestation. These features indicate that liver injury is a gradual and cumulative process in susceptible patients which ultimately can result in massive insult affecting the hepatobiliary excretory system. Reactive metabolites of diclofenac have high affinity for sulphydryl groups and amino groups on protein and non-protein peptides, enabling them to interact chemically with cellular and plasma proteins and resulting in adduct formation. One of the consequences of this adduct formation is the down-regulation of the activity of CYPs. Shen *et al.* (1997) reported that adducts formed *in vivo* caused a 72% decrease in cyp2c11 activity, which is a male-specific enzyme, in rats. Yasumori and co-workers (1987) had earlier shown that rat cyp2c11 has 76% identity and 85% homology to human CYP2C9, which metabolises the drug in humans. However, Masubuchi *et al* (2001) reported that metabolites of diclofenac did not inhibit CYP2C9 activity in human liver microsomes, although evidence of metabolite-mediated inactivation of human CYP3A4 was obtained, with epoxides suspected to be responsible. One consequence of covalent modification of proteins is haptenation, which has the potential to trigger drug-specific adaptive immunological reactions. Protein adduct formation might also result in inactivation of crucial protein functions such as cell signalling, resulting in a disregulation of cellular functions and tissue injury (Boelsterli, 2002). In addition to raising the possibility that covalent modification of CYP3A4 could play a role in the mechanism of diclofenac-induced liver injury, these results also indicate a potential for drug-drug interactions, in view of the fact that over 50% of drugs used clinically are metabolised by this enzyme. As mentioned in the previous section, there is evidence that reactive acyl glucuronide of diclofenac can form adducts with liver proteins. Kretz-Rommel and Boelsterli (1994) demonstrated the potential of diclofenac acyl glucuronides for covalent adduct with rat liver microsomal protein via the imine mechanism

described above which has been linked with immune-mediated toxic response. It has also been shown that diclofenac exposure of mice causes haemolytic anaemia. This might be linked to the reported cases in which metabolites of diclofenac covalently bind with erythrocyte membrane proteins, thus linking covalent modification of membrane protein with haemolysis (Ware *et al.,* 2001). Genetic polymorphism has been reported in the expression of the CYP2C9 gene with the main ones being CYP2C9*1, CYP2C9*2, CYP2C9*3. However, none of these polymorphic forms predisposes to diclofenac-induced liver injury (Aithal *et al.*, 2000, Pachkoria *et al.*, 2007)

1.3 FENCLOZIC ACID

Fenclozic acid (FA) was developed in the late 1960's by Imperial Chemicals Industry (ICI), which is a predecessor company of AstraZeneca, as a potent anti-inflammatory, antipyretic and analgesic agent (Hepworth *et al.*, 1969).

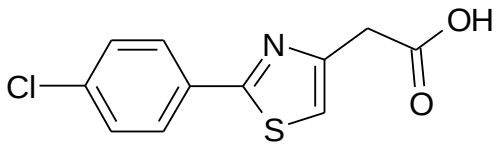


Fig 1.7: Fenclozic acid

Patented as 'Myalex®', and internally coded as 'ICI 54, 450', fenclozic acid was remarkably potent at oral doses of 2.5-100mg/kg as anti-inflammatory agent in studies undertaken in animals. These involved evaluation of carrageenin-induced oedema in rats, adjuvant-induced inflammation in mice and ultraviolet-light-induced erythema in guinea pigs. The toxicity of the drug was evaluated in numerous species and a good overall safety profile was observed. In rats,

LD_{50} were 850 mg/kg (oral) and 300 mg/kg (iv), while in mice it was 1000mg/ kg (oral) and 250 mg/kg (iv). A three-month chronic toxicity study at a maximum dose of 80 mg/kg in rats caused death due to duodenal ulceration and peritonitis. With a half life of over 24 hours, FA had a high serum concentration and binding (Hepworth *et al.*, 1969; Newbould, 1969).

1.3.1 Pharmacology and Toxicology of Fenclozic acid in human volunteers

Pharmacokinetic studies were carried out in healthy human volunteers and arthritic patients treated with FA at doses of 25-200 mg/kg twelve hourly for up to one week (Chalmers *et al.*, 1969a). There was a longer serum half life in arthritic patients than in healthy volunteers, a high volume of distribution in all volunteers and a generally similar pharmacokinetic profile when compared with preclinical animal species. This was followed by an efficacy study which used a double-blind cross-over trial in in-patients with rheumatoid arthritis. When compared with acetyl salicylic acid, FA exhibited anti-rheumatic effects at 200-, 300- or 400 mg daily dose as compared with 3.6g of aspirin, accompanied with fewer side effects than aspirin. However, half life was seen to be greater than 40 hours (Chalmers *et al.*, 1969b). However, further investigations carried out at different facilities in the UK revealed that FA induced an unacceptable increase in liver transaminase enzyme levels with a simultaneous development of jaundice after a minimum of fourteen days of treatment (Hart *et al.*, 1970). This resulted in the withdrawal of FA from clinical trial and suspension of its registration as a potential antirheumatic drug.

1.3.2 Metabolism of Fenclozic Acid

Bradbury *et al.* (1981) reported the presence of hydroxylated metabolites and taurine conjugate of [^{14}C]-FA after administration of oral doses of 2- and100 mg/kg in urine of free range rats.

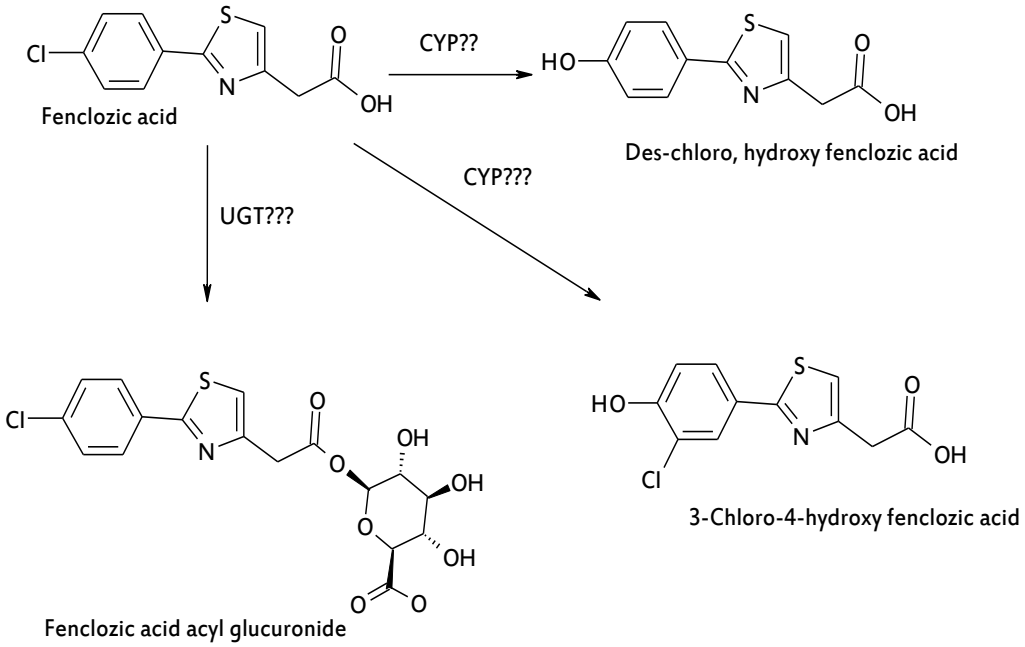


Fig. 1.8: Metabolism of Fenclozic acid: Some *in vivo* metabolites of fenclozic acid as described by Foulkes *et al.* (1970). Question marks on the CYP and UGT show that the specific enzymes involved are as yet uncharacterised.

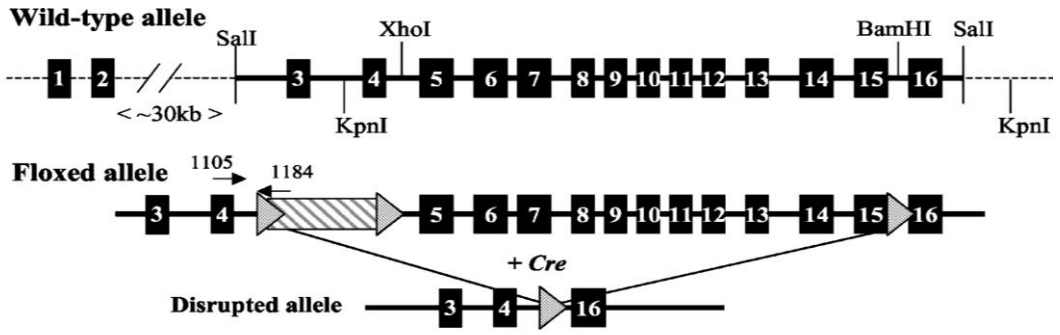
Similar doses administered intraduodenally to anaesthesised male rats yielded taurine and glucuronide conjugates as well as hydroxylated and decarboxylated FA which were excreted in the urine and the decarboxylated metabolite which was detected in urine and bile. Foulkes (1970) had previously reported that a dilute aqueous or chloroform solution of FA underwent photochemical transformation resulting in decarboxylation, which was reduced to 3.5% when

the solution was prepared in pH 7.0 phosphate buffer and stored in the dark. In view of this, it was then postulated that the observed decarboxylated metabolite detected in rat, dog and monkey urine might be a result of an initial chemical reaction that took place on the dose solution prior to administration. Foulkes (1970) also reported detection of a 4-phenyl hydroxyl metabolite of FA in the rat and dog urine. Monkey, rat and dog urine also yielded a glucuronide of FA, while rat bile was found to contain the hydroxyl- metabolite and a glucuronide conjugate. Faecal materials were only seen to contain the parent compound and unconjugated metabolites. This study singled out the monkey as a species that extensively metabolised FA to its glucuronide conjugate, with little or no evidence of oxidative metabolism. In addition, analyses undertaken recently within AstraZeneca in C57BL6J and HRN™ mice revealed *in vivo* metabolism of FA to carnitine and glycine conjugates (Pickup *et al.*, unpublished).

1.4 THE HEPATIC CYTOCHROME P450 REDUCTASE NULL (HRN™) MOUSE MODEL

Gene knockout mice are very powerful tools for exploration of the role played by specific enzymes in metabolism and toxicity of chemicals *in vivo* (Gonzalez, 2001). A good illustration of the value of this approach has been provided by Zaher *et al.* (1998), who observed a longer survival time in CYP 1a2/2e1 double knockout mice as against wild-type mice exposed to paracetamol which could be attributed to an absence of metabolic bioactivation in the knockout animals. However, results obtained with knockout mice can be affected by compensatory upregulation of expression of other CYP enzymes. An alternative approach would be to produce mice in which all CYP activity has been impaired. This has been achieved for liver CYPs via the generation of the hepatic Cytochrome P450 reductase null (HRN™)

mouse, which is a genetically-modified strain that lacks the electron donor, NADH-cytochrome P450 oxidoreductase (POR) gene. POR is a 78kDa membrane-bound flavoprotein which catalyses the transfer of electrons from NADPH to CYPs located on the endoplasmic reticulum and to other microsomal enzymes and electron acceptors (Wang, 2005). Its deletion results in the simultaneous inactivation of all the hepatic CYPs resident in the endoplasmic reticulum (Henderson *et al.*, 2003), while the mouse remains essentially healthy, viable and fertile (Wang *et al.*, 2005). The HRN™ mouse model was created in 2003 by insertion of LoxP sites into introns 4 and 15 of the POR gene (Figure 1.9). When these mice were crossed with those that express the Cre recombinase off the albumin promoter, the offspring exhibited lack of the liver-specific POR in hepatocytes.



Modified from: Henderson *et al.,* 2003

Fig. 1.9: Modification of the POR gene to effect a deletion of the CYP reductase (CPR) enzyme. Squares represent the CPR genes while the triangles stand for the *LoxP* sites which cleave off the CPR genes located between exons 5 and 15.

Due to their lack of activity of functional hepatic CYPs, the HRN™ mice have a compromised ability to metabolise drugs and to produce bile acids, which is a CYP-dependent process causing a reduction in concentrations of circulating cholesterol and triglycerides. Livers from HRN™were found to be larger and paler than those of the corresponding wild-type strain and they exhibited elevated serum alanine amino transferase (ALT) activities, which is indicative of

some liver damage and/or altered hepatocyte turnover rate. In addition, there was a profound increase in the expression of xenobiotic-related CYP proteins, although these enzymes were catalytically inactive due to a lack of the POR gene.

Studies have shown the utility of HRN^{TM} mice in pharmacological and toxicological research. These include efficacy and metabolism studies, metabolite profiling (Sarda *et al.*, 2012), toxicokinetic studies and mechanistic studies (Henderson *et al.*, 2003). It is important to note that although deletion of the hepatic POR impairs CYP activity in livers of HRN^{TM} mice, it does not affect extraheptic CYPs. Therefore the model can also be used to assess the contribution of extra-hepatic CYP activity, such as in the lungs, kidneys and gastro-intestinal tract, to *in vivo* drug metabolism.

1.5 AIMS AND OBJECTIVES OF STUDY

The primary goal of the study was to gain novel insight into the role played by hepatic metabolism in the metabolic bioctivation and liver toxicities of diclofenac and fenclozic acid. The work was undertaken *in vitro* and *in vivo*, using mouse models. *In vitro* metabolic profiling and covalent binding studies were undertaken using liver microsomes from normal mice and from HRN^{TM} mice which were incubated with both drugs in the presence of cofactors which supported CYP- or UGT- dependent metabolism. Hence the mechanism of bioactivation of fenclozic acid into reactive metabolites was explored by means of *in vitro* tools. In addition, normal mice and HRN^{TM} mice were dosed *in vivo* with both compounds, following which evidence of liver injury was sought by analysis of serum clinical chemistry and liver histopathology and metabolism of the compounds was explored by metabolite profiling.

CHAPTER TWO

2.0 MATERIALS AND METHODS

2.1 Materials

2.1.1 Chemicals and Reagents

[^{14}C]-fenclozic acid was obtained from Isotope Chemistry (AstraZeneca R&D, Macclesfield, UK) it had a purity of >99% and a specific activity of 60.5 mCi/mmol. [^{14}C]-diclofenac was obtained in two batches. The first batch was obtained from American Radiolabelled Chemicals Inc (St Louis Missouri, USA), it had a purity of 99% and a specific activity of 55mCi/mmol The Second batch was obtained from Isotope Chemistry (AstraZeneca R&D, Mölndal (Mölndal, Sweden)., it had a purity of 98% and a specific activity of 57.24 nCi/nmol. Fenclozic acid was obtained from Compound Management (AstraZeneca, R&D, Macclesfield, UK). Diclofenac was obtained from Sigma-Aldrich Company Ltd (Poole, Dorset, UK). Ultima Gold liquid scintillation cocktail was obtained from Perkin Elmer (Waltham, USA). Pierce™ BCA Protein Assay Kit was obtained from Thermo Scientific (Rockford Illinois, USA). Formic acid was obtained from VWR International Ltd (Poole, Dorset, UK). 10% Sodium dodecyl sulphate solution (Applichem GmbH, Darm Stadt, Germany) EnzyChrom™ Alanine Transaminase Assay Kit was obtained from BioAssay Systems (Hayward, USA). All other reagents were obtained from Sigma-Aldrich Company Ltd (Poole, Dorset, UK). Safety assessment/hazard categorisation was done on each of them and the protocol that involve their use.

2.1.2 Biological Samples

Snap frozen mouse livers from male C57BL6J mice (10-11 week old) or male HRN™ (Hepatic Cytochrome P450 reductase null; 12-27 weeks old) mice were obtained from Biospecimens Unit, AstraZeneca R&D (Macclesfield, UK). Snap frozen beagle dog livers (weeks old) were obtained from Biospecimens Unit, AstraZeneca R&D (Macclesfield, UK).Mouse plasma from male C57BL6J mice (10-11 week old) was obtained from Biospecimens Unit, AstraZeneca R&D, (Macclesfield, UK).

2.1.3 Animals

Thirty male C57BL6J mice (7-8 weeks old) were obtained from Charles River (Margate, UK). Fourteen male HRN™ mice (4-11 weeks old) were obtained from Taconic Farms Inc (German Town, New York, USA). All animals were kept in the Rodent Unit of AstraZeneca R&D facility, Macclesfield, UK. They were individually housed in standard conditions, freely fed with pelleted diets (R&M No.1, E.SQC pelleted diets, supplied by Special Diets Limited, England) and water from the site drinking water. Animals were exposed to a 12-hour light and 12-hour dark cycle. All procedures involving the use of animals were carried out in accordance with the approved licenses and guidelines of the British Home Office (Animals and Scientific Procedures) Act of 1986. They were allowed to acclimatize for at least one week before commencement of study.

2.2 EXPERIMENTAL PROTOCOLS

2.2.1 *IN VITRO* ASSAYS

2.2.1.1 Preparation of subcellular fractions

The procedure was based on the method reported by Graham (2002). Briefly, frozen livers were thawed at room temperature, weighed, placed in 1.15% KCl solution and scissors minced to remove the blood. The KCl solution was drained off and then 9 volumes of ice cold SET buffer pH 7.4 (0.25 M sucrose, 20 mM Tris base, 5 mM EDTA) was added. The samples were then homogenised using a Potter homogeniser (B. Braun Biotech Int'l GmbH, Melbungen Germany) 6 strokes to produce a 10% homogenate. The homogenates were centrifuged at 1,800 g for 10 mins at 4°C using a Sorvall® RT7 bench top centrifuge (Kendro Lab Products , USA). The pellet was discarded and the supernatant (S1) centrifuged at 3,000 g using a Sorvall® Discovery 90-SE ultra centrifuge (Kendro Lab Products , USA) for 10 mins at 4°C. Both the heavy mitochondrial pellet (HM) and supernatant (S2) were kept. The HM pellet was washed by re-suspending in mitochondrial buffer (0.2 M mannitol, 50 mM sucrose, 1 mM EDTA, 10 mM HEPES),and then centrifuging at 3000g using a Sorvall® Discovery 90-SE ultra centrifuge for 10 min at 4°C. The supernatant (S3) was discarded and the HM pellet reconstituted in mitochondrial buffer at 1 ml/g liver. Aliquots (250 µl) were stored frozen at -80°C. The supernatant (S2) was centrifuged at 17,000 g using a Sorvall® Discovery 90-SE ultra centrifuge for 15 min at 4°C. The supernatant (S4) was stored on ice whilst the light mitochondrial pellet (LM) was washed by re-suspending in mitochondrial buffer and then centrifuging at 17,000 g using a Sorvall® Discovery 90-SE ultra centrifuge for 15 min at 4°C. The supernatant was discarded and the LM pellet reconstituted at 1ml/g liver. Aliquots (250 µl) were stored frozen at -80°C.

Supernatant (S4) was centrifuged at 105,000g using a Sorvall® Discovery 90-SE ultra centrifuge for 70 mins at 4°C. The supernatant (S5; cytosol) was stored in 1 ml aliquots at -80°C for possible subsequent analysis. The pellet (microsomal pellet) was washed by re-suspending in KCl/Tris buffer (20 mM Tris base, 1.15% KCl) was and then centrifuged at 105,000 g for 70 mins at 4°C. The supernatant was discarded and the microsomal pellet suspended in SET buffer at 1mL/g liver. Aliquots (250 µl) were stored frozen at - 80°C.

2.2.1.2 Protein Assay

Microsomes and mitochondrial pellets were analysed for protein using the BCA protein assay (Smith, et al. 1985) which is a colourimetric method based on the protein-induced biuret reaction that causes a reduction of Cu (II) to Cu (I) and a subsequent chelation of Cu (I) by two molecules of bicinchoninic acid (BCA) to form a water-soluble purple-coloured complex that absorbs strongly at 562nm, the intensity of which increases with increasing protein concentration.

A 10 point standard curve (0 - 2 mg protein/ml) was produced by serially diluting the 2mg/ml bovine serum albumin protein standard using distilled water. The microsomal and mitochondrial samples were diluted (1:50, 1:100 and 1:200) to ensure the protein values fell within the range of the standard curve. Aliquots (25 µl) of both the protein standards and the sample dilutions were pipetted into a clear-bottom 96-well plate and then 200 µl Working Reagent (WR 50 parts of reagent A (sodium carbonate, Sodium bicarbonate and Sodium tartrate in 0.1 N sodium hydroxide) to 1 part reagent B (4% cupric sulphate)) was added. The plate was then incubated at 37°C for 30 minutes, cooled for 2-3 mins and absorbance

determined using an Envision plate reader (PerkinElmer CA, USA) and a wavelength of 562nm. A standard curve was constructed and protein concentration calculated and expressed as mg protein/ml.

2.2.1.3 Succinate Dehydrogenase (SDH) Assay

Mitochondrial SDH was determined according to the method described by Green *et al.* (1955). It is an assay that measures change in absorbance (at 500nm wavelength) due to reduction of cytochrome c in the biological sample in the presence of cyanide. Briefly, to each of two 1 ml cuvettes 100 µl of 0.1M phosphate buffer, 100 µl of 10 mg/ml bovine serum albumin, 50 µl potassium cyanide, 595 µl of distilled water, 5 µl of the mitochondrial pellets and 100 µl of cytochrome c from equine heart were added. Contents of the cuvette were mixed by inversion and the absorbance 'blanked' in an Agilent 8453 UV-Visible Spectrophotometer (Agilent Technologies, California, USA) at a wavelength of 550nm. The reaction was started by adding sodium succinate (50 µl, 0.1 M). The cuvette was mixed by inversion and the change in absorbance determined kinetically for 3 minutes. Enzyme activity was calculated using the Beer Lambert law (extinction coefficient of $19.7mM^{-1}.cm^{-1}$) and was expressed as nmol reduced cytochrome c/min/mg protein.

2.2.1.4 Cytochrome c Reductase Assay

The assay was carried out using a Cytochrome c Reductase (NADPH) assay kit (Sigma-Aldrich Company Ltd, Poole Dorset, UK) containing the Assay Buffer (300 mM potassium phosphate buffer, 0.1mM EDTA), Enzyme Dilution Buffer (300 mM potassium phosphate buffer, 0.1 mM

EDTA and 0.5 mg/mL bovine serum albumin), cytochrome c from equine heart, NADPH and 0.42 units/mL cytochrome c reductase (NADPH). The working solution was 36 µM of cytochrome c in assay buffer. NADPH stock solution was 40 mg/mL in water. The positive control was prepared by diluting an aliquot of the cytochrome c reductase (NADPH) between 10 and 100 fold with the enzyme dilution buffer just before assaying. Each set of reactions required a total of 75 µl of the diluted positive control. The enzyme preparations (samples and control) were kept on ice. The working solution and the NADPH stock Solution were warmed to 25°C just before use. The spectrophotometer (Agilent 8453 UV-Visible Spectrophotometer, Agilent Technologies, United States, California, USA) was set to 550 nm and kinetic program was run at 25°C. Working solution (950 µl) and 50 µl of microsomal sample or positive control were placed in a 1 ml cuvette and mixed by inversion. The spectrophotometer was blanked and the reaction was started by addition of 100 µl of NADPH solution. The absorbance of the reaction mixture was read by the kinetic programme at 550 nm. The cytochrome c reductase activity in the microsomal samples were calculated and expressed as pmol/min/mg protein.

2.2.1.5 Covalent Binding Assay

Covalent binding assay (CVB) was conducted using the method previously described by Day *et al.* (2005). Dog, C57BL6J or HRN mouse liver microsomes (1 mg/ml protein) were incubated in a 96-well nunc plate (plate 1) containing 0.1M potassium phosphate buffer plus 10mM $MgCl_2$, pH 7.0 pre-warmed to 37°C and either 2 mM NADPH (with or without 1 mM 1-aminobenzotriazole (ABT)), 4mM UDPGA or no co factor in a final volume of 500 µl. The samples were pre-incubated for 9 mins on heater block (Techne, Dri-Block®, DB-2D) set to

38.5°C and then the reaction started by the addition of either 10 µM [^{14}C]-fenclozic acid or [^{14}C]-diclofenac. The plate was then transferred to an incubator and incubated at 37 °C for 1 hour. Covalent binding at T=0 and t=60 mins was determined by taking a 200 µl aliquot from each well of plate 1 and adding it to a 2ml 96-well nunc plate (plate 2) already containing 300µl (1.5 volume) of cold acetone to terminate the reaction and precipitate the protein. A further 500 µl (2.5 volume) of cold acetone was added to fully precipitate the protein and plate was mixed (Heldolph Instruments, Schwabach, Germany). The plate was then stored at 4°C to precipitate the protein. Metabolism at T=0 and t=60 mins was determined. A 50 µl aliquot (from plate 1) was transferred into another 1 ml 96-well plate (plate 3) already containing 50 µl of cold 3% formic acid in acetonitrile. The plate was then stored at -20°C prior to analysis by HPLC-RAD-MS.

Non specific binding was determined by incubating microsomes or mitochondrial samples in the absence of test compound for 1 hour at 37°C and then 'back-adding' 10µM of [^{14}C]-fenclozic acid or [^{14}C]-diclofenac. The samples were then treated as for the T=60 covalent binding samples. The protein pellets in plate 2 was washed with 80% methanol and collected on to Whatman GF/B fired filter paper using the Brandel 96-sample cell harvester (Brandel Inc, Maryland, USA). The protein collected on individual filter discs were transferred into a scintillation vial and 1 ml of 5 % sodium dodecyl sulphate (SDS) solution added to solubilise the protein. The vials were covered and incubated overnight at 55°C. The next day, the vials were swirled gently and returned to the oven for another 1 hour. The protein content was then determined using the Pierce BCA Protein Assay Kit using BSA as a standard and the BioTek® Synergy HT Multidetection Microplate Reader with KC4 software. Ultima Gold scintillation fluid (5 ml) was added to each vial swirl-mixed and bound radioactivity was measured by liquid

scintillation counting on a Packard 1900TR analyser. The level of non-extractable radioactivity covalent binding (CVB) was expressed as: pmole equivalents/ mg protein using the specific activity of the dose solution given and the protein levels of each sample. All incubations were performed in duplicates.

2.1.6 Metabolite profiling by HPLC-RAD

The acetonitrile quenched samples from the CVB assay were transferred to a 500 µl Eppendorf tube, centrifuged (Eppendorf, Stevenage, UK) at 1000 rpm for 10 minutes and the supernatant collected and stored at -20°C for subsequent metabolite identification.

"Samples were injected (50 µl injection volume) onto and separated on a Hypersil Gold C18, 5 µm, 250 × 4.6 mm column (ThermoScientific, Warrington, UK) with a 3 µm (C18) pre-column filter (Hichrom Ltd., Reading, UK), all maintained at 30°C and eluted over 60 min using Agilent 1200 Series pumps (Agilent Technologies, Stockport, UK) at a flow rate of 1 mL/min. The aqueous mobile phase (solvent A) was 10 mM ammonium acetate (unadjusted, ca.pH 6.8) with acetonitrile constituting the organic mobile phase (solvent B). The initial mobile phase consisted of 5% solvent B, which was increased to 14% over a period of 4 min, then further increased to 34% in 41 min, and to 45% over 5 min before finally increasing to 95% over 0.1 min. This high organic concentration was maintained for 5 min before returning to 5% solvent B for column equilibration for 5 min prior to subsequent injections. The post-column eluent was split 1:5 v/v between the mass spectrometer and radiodetector, respectively, using a QuickSplit(tm) binary fixed flow-splitter (Analytical Scientific Instruments, El Sobrante, CA), to

enable simultaneous MS and radiodetection. Radiochemical detection was performed on-line using a LabLogic β-Ram 3 detector (LabLogic Systems Ltd., Sheffield, UK) with a 500 µL liquid flow cell and Ultima Flo-M scintillation cocktail (Packard Instruments, Pangbourne, UK) running at 1.6 mL/min (ca. 2:1 v/v scintillant/eluent). The data were collected and analysed using LAURA integration software version 3.4.1.10e (LabLogic Systems Ltd., Sheffield, UK)."

2.2.2 *IN VIVO* STUDIES

2.2.2.1 Preparation of Dose Formulations

Diclofenac was dissolved in the dose vehicle, 0.1 M sodium phosphate buffer pH 7.7, at room temperature at concentrations of 1, 2 and 3 mg/ml. Fenclozic acid was dissolved in 0.1 M sodium phosphate buffer pH 7.7 with warming to 37 ^{o}C at concentrations of 5 and 10 mg/ml. On cooling to room temperature the 5 mg/ml fenclozic acid dose formulation stayed in solution, however the fenclozic acid in 10 mg/ml formulation came out of solution. As a result this formulation had to be prepared fresh daily for the duration of the studies. Stability studies on the 1, 2, 3 mg/ml diclofenac and 5 mg/ml fenclozic acid dose formulations performed by the Analytical Chemistry Laboratory, (Drug Metabolism and Pharmacokinetics Kinetics (DMPK) Unit, AstraZeneca R&D, Macclesfield, UK) showed that these formulations were stable when stored in amber bottles at room temperature for 10 days. Aliquots of each of the dose formulations were kept for analysis.

2.2.2.2 Dose-range-finding (DRF) study in C57BL6J mice

Thirty male C57BL6J mice were randomised by body weight, 5 animals per group so that the mean weight for each group was within 5% of the control group and allowed to acclimatize for at least 1 week prior to the start of the study. The study was designed such that the control animals (group 1) and the low dose diclofenac (group 2, 10 mg/kg/day) and fenclozic acid (group 5, 50 mg/kg/day) animals were exposed to the compounds for a period of 7 days with close monitoring. Providing the animals tolerated these doses, subsequent animals were exposed to the middle and high doses of diclofenac (20 and 30 mg/kg/day, groups 3 and 4) and high dose fenclozic acid (100 mg/kg/day, group 6).

The body weight (BW), food consumption (FC) and clinical observations (CO) for each animal were monitored daily commencing 3 days prior to the start of the study. The first day of study was designated day 1 while the day prior to dosing was designated day -1. A blood sample (50 μl) was taken from each animal into lithium-heparin tubes by tail bleeding on day –1 for the determination of pre-dose plasma ALT levels, The sample was centrifuged at 3000 g for 10 min at 4°C and the resultant plasma stored frozen at - 80 ^{o}C. In accordance with the Home Office license approved for the study, dose formulations were administered daily for 7 days by oral gavage at a volume of 10 ml/kg body weight for all the dose levels—10, 20 and 30 mg/kg body weight for diclofenac; 50 and 100 mg/kg BW for fenclozic acid —based on the body weight determined immediately prior to dosing. Mice in the control group received dose vehicle alone, sodium phosphate buffer solution (pH 7.7). The animals were dosed between 08:00 and 12:00 hours on each day and closely monitored for the first 3 hours after each dose. Any mouse that demonstrated signs of toxicity and distress that exceeded the mild severity limit of the Home Office license would have been terminated and necropsied pre-terminally. Additional

blood samples for the determination of plasma ALT concentration were taken by tail bleeding the animals prior to dosing on days 2 and 4 of the study. The animals were terminated on day 8, 24 hours following the last dose. Immediately prior to termination urine samples were collected from each animal. The animals were transferred to a clean plastic cage for and any urine in the cage at the end of this time was collected, mixed with an equal volume of deionised water and snap frozen in liquid nitrogen. The samples were stored frozen for possible subsequent metabolite analysis by HPLC-MS

2.2.2.3 HRN™ Study

Fourteen male HRN™ mice were randomised as described above and divided into 3 groups. The control group contained 4 mice and received the dose vehicle alone. The remaining two groups contained 5 mice each and they received the maximum tolerated dose for the 2 compounds determined in the DRF study—30 mg/kg/day diclofenac and 100 mg/kg/day fenclozic acid. The formulations were administered between 08:00 and 12:00 hours on each day for 7 days with close monitoring for the first 3 hours after each dose as for the DRF study.. Blood and urine samples were collected as in the DRF study.

2.2.2.4 Necropsy

Animals were euthanized under halothane anaesthesia. Depending on the volume that could be collected, about 1 ml blood was collected by cardiac puncture via the vena cava and transferred into lithium heparin tubes. An aliquot of the whole blood (20 µl) was diluted with an equal volume of cold water, two volumes cold 3% formic acid in acetonitrile was added and the sample centrifuged at 1,500 g for 10 mins. The supernatant was collected, mixed with an equal volume of cold deionised water, snap-frozen in liquid nitrogen and stored frozen at -80ºC for possible subsequent metabolite analysis by HPLC-MS. The remaining plasma was transferred to clinical pathology laboratory for biochemical analysis. The liver was excised, examined for any abnormalities and weighed intact. The gallbladders from the mice were removed, snap-frozen in liquid nitrogen and stored frozen at -80ºC for possible subsequent metabolite analysis by HPLC-MS. The left lateral lobe of the liver and samples of the intestines, the jejunum, ileum, colon, caecum, rectum, oesophagus and stomach/duodenum, were all fixed and preserved in buffered formalin for subsequent pathological analysis. The cranial, thoracic and abdominal cavities were visually examined for the presence of any abnormalities.

2.2.2.5 Clinical Chemistry

Lithium heparin blood samples obtained on day 8 were centrifuged in a Sorvall® RT7 centrifuge at 3000 rpm at 4°C for 10 minutes. The resultant plasma was collected, transferred into analyser cups and the samples processed for alanine aminotransferase, aspartate aminotransferase, glutamate dehydrogenase, bile acids, alkaline phosphatase, total bilirubin, total protein, calcium, triglycerides, cholesterol, blood glucose, creatinine and urea on the

Roche P Modular Automated Chemistry analyser. Aliquots (about 20µl) of the plasma were taken for the determination of ALT using the manual ALT assay to serve as a comparison between manual and automated methods on day 8 of the study.

2.2.2.6 Manual Alanine Amino transferase (ALT) Assay

Plasma ALT concentration was determined colourimetrically using an EnzyChrom™ Alanine Transaminase Assay Kit (Bioassays Systems, Hayward, CA, USA). Working Reagent for the sample and standard wells was prepared as follows: (200 µl Assay Buffer, 5 µl Co substrate, 1 µl LDH and 4 µl NADH). The reagent was vortex mixed and warmed to 37°C in a water bath. To prepare the blank reagent for the Blank well, all the reagents for the working reagent above with the exception of NADH which was replaced with water.

Plasma (10 µl of 1:2, 1:10 or 1:40 dilutions) was pipetted into separate wells in a 96-well plate, with 20 µl of deionised water pipetted into the NADH standard and blank wells. Then, 200 µl of Working Reagent was added to each sample and standard well and 200 µL of blank reagent to the blank well. The plate was mixed on a plate mixer (Heldolph Instruments, Schwabach, Germany) for about 10 seconds and transferred to the Envision plate reader (PerkinElmer CA, USA) and the rate of NADH consumption determined kinetically at 340 nm and 37°C for 40 minutes. Since NADH consumption in the reaction is proportional to the ALT levels, enzyme level for each sample was calculated by subtracting the absorbance values at 5-minute time interval within which an initial linear plot was obtained. This was also calculated for the NADH Standard and the blank. The ALT levels were calculated using the formula below, assuming time intervals of 5 minutes:

$$ALT = 381 \times \frac{\Delta OD_S - \Delta OD_{NADH}}{OD_{STD} - OD_{BLK}} \quad (U/L)$$

OD_{STD} and OD_{BLK} are the OD_{340nm} values of NADH Standard and Blank at 5 min, respectively. The factor 381 is derived from

$$\text{Factor} = 10 \text{ mM NADH} \times \frac{4 \mu L \text{ Vol.}_{NADH}}{210 \mu L \text{ Vol.}_{WR}} \times \frac{200 \mu L \text{ Vol.}_{WR}}{220 \mu L \text{ Vol.}_{Total}} \times \frac{11 \text{ (sample dilution)}}{5 \text{ min}}$$

$$= 381 \mu M/min$$

2.2.2.7 Histopathology of the Liver

Livers were trimmed and placed into cassettes, processed with a Thermo Excelsior automated tissue processor (Thermo Scientific, Cheshire, UK), and embedded into paraffin wax using Leica EG 1160 Tissue Embedder (Leica Microsystems, Wussloch, Germany). Sections were cut at 4 microns with the Finesse Rotary Microtome (Thermo Shandon, Cheshire, UK), and stained with haematoxylin and eosin using Leica autostainer XL (leica Microsystems, Wussloch, Germany). Sections were examined using Leitz Dialux 20EB light microscope (ENST Leitz GmbH, Wetzlar, Germany). Images were captured at X200 and X400 magnifications using Aperio Scanscope AT (Aperio, California, USA)

2.3 STATISTICAL ANALYSIS

In the *in vivo* study, statisticians worked to allow a 2-fold change in mean from vehicle to be detected with reasonable levels of certainty (80% power). To achieve this, the number of animals per group was 5 except in the control group of the HRN™ mice due to the limited number of animals. For all data, statistical analysis was done using the statistical function on Microsoft® Office Excel. Analysis was carried out using Student t-test and values $p<0.05$ were considered significant.

CHAPTER THREE

3.0 RESULTS

3.1 *IN VITRO* STUDY

3.1.1 NADPH-Cytochrome c Reductase Assay

Figure 3.1 below shows the activity of NADPH-Cytochrome c reductase in liver microsomes from dog, HRN™ and C57BL6J mice. The C57BL6J mouse was shown to manifest the highest level of the functional CYP activity, whilst the lowest activity was found in the HRN™ mouse. Enzyme levels in dog liver microsomes might indicate a species difference or be due to prolonged storage of the dog liver.

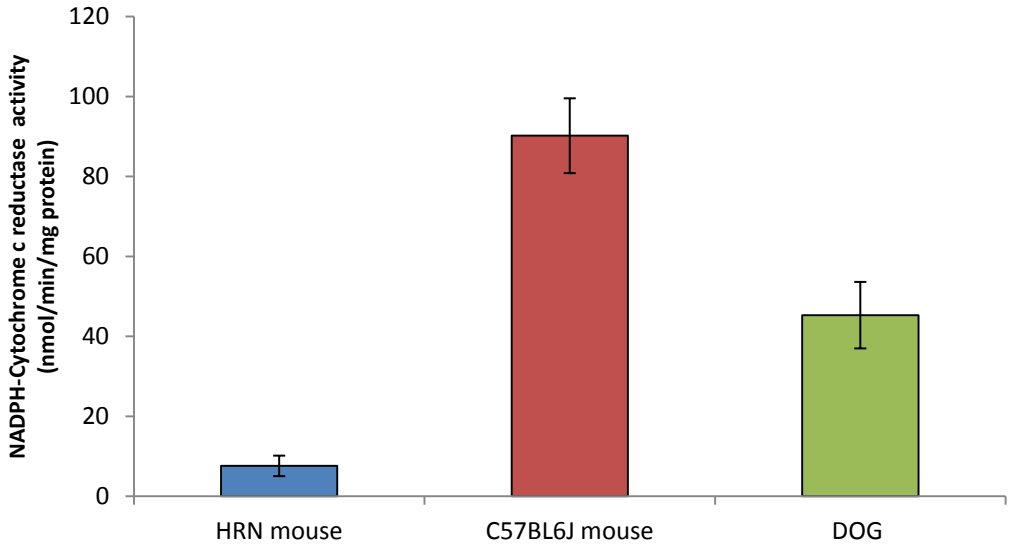


Fig 3.1: Comparison of NADPH-Cytochrome c reductase activity in liver microsomes from HRN™ and C57BL6J mice and the dog.

Data presented are Mean±SD

3.1. 2 Succinate Dehydrogenase (SDH) Assay in Mitochondrial Pellets

SDH is a marker enzyme of mitochondria (Graham 2002). Mitochondria pellets from dog, HRN™ and wild-type (C57BL6J) mice were assayed for SDH activity. Succinate dehydrogenase levels were highest both in the light and heavy mitochondrial pellets of HRN mice while dog and the wild-type mice showed a lower level of this enzyme (Figure 3.2).

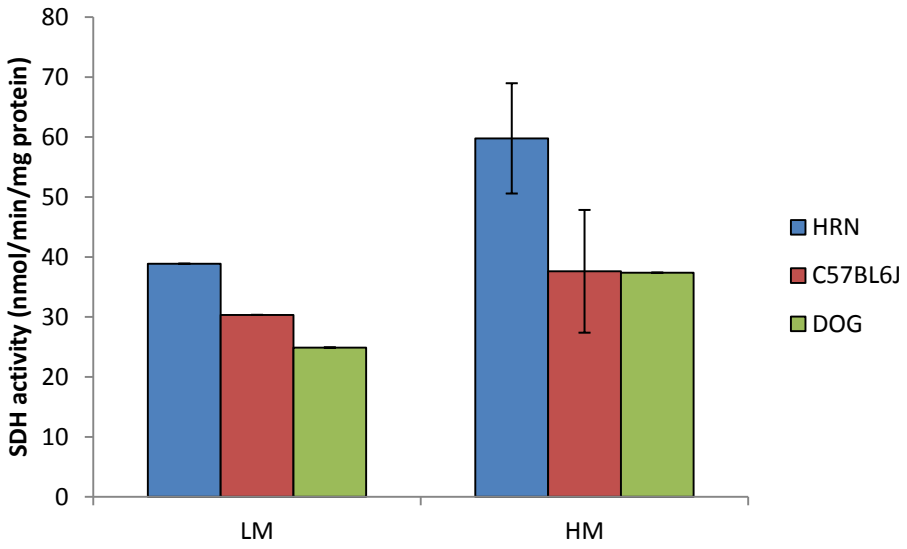


Fig 3.2: Succinate Dehydrogenase activity in light and heavy mitochondrial fractions obtained from HRN™ and C57BL6J mice and the dog livers: It measured change in absorbance (at 500nm wavelength) resulting from reduction of cytochrome c in the biological sample in the presence of cyanide. LM: Light mitochondrial Pellets; HM: heavy Mitochondrial Pellets.

Data presented as Mean±SD, n=4 (HM), n=3 (LM, dog), n=2 (LM, C57BL6J & HRN)

3.1.3 Covalent Binding of [^{14}C]-Diclofenac

This assay measured the covalent binding of reactive metabolites with liver microsomal proteins. NADPH- and UDPGA-dependent metabolism of ^{14}C-diclofenac resulted in the highest covalent binding with dog liver microsomes. When compared with the incubation

without NADPH after 60 minutes, there was a significant ($p<0.05$) increase in covalent binding in the presence of NADPH (figure 3.3). However, in the presence of the CYP inhibitor, ABT (1-amino benzotriazole), covalent binding was significantly ($p<0.05$) decreased. The same trend is true for C57BL6J mouse liver microsomes. On the other hand, HRN™ liver microsomes did not manifest any significant change in the covalent binding in the presence of NADPH.

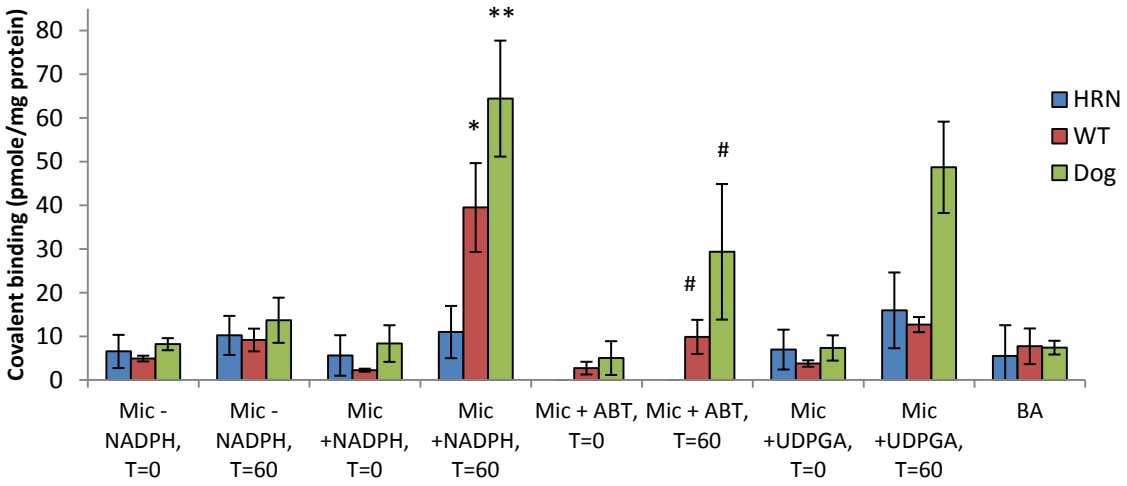


Fig 3.3: Covalent binding in liver microsomes incubated with ^{14}C-diclofenac: HRN, C57BL6J mouse and dog liver microsomes were incubated with ^{14}C-diclofenac in the presence of co-factors (NADPH or UDPGA) for 60 minutes. Proteins were harvested, protein assayed and radioactivity measured.

WT: C57BL6J mouse, Mic=microsomes, Mic-NADPH=microsomal incubation without NADPH. NADPH=nicotinamide adenine dinucleotide phosphate, ABT=1-amino benzotriazole. UDPGA=Uridine diphosphate glucuronic acid. BA: back added. Data was analysed using Student's t-test, followed by one-way analysis of variance. * Significantly different from background (Mic + NADPH, T=0) at $p<0.05$, ** significantly different from background (Mic + NADPH, T=0) at $p<0.01$., # significantly different from CVB in incubations without inhibitor (Mic+NADPH, T=60).

Data are Mean±SD; n=3.

3.1.4 Covalent Binding of [^{14}C]-Fenclozic acid

After 60 minutes of incubation, NADPH-dependent covalent binding was significantly ($p<0.05$) higher in dog and C57BL6J mouse liver microsomal incubations when compared with those without NADPH. ABT significantly ($p<0.05$) reduced the covalent binding in dog and C57BL6J mouse liver microsomal incubations after 60 minutes, with no significant effect on HRN mouse liver microsomes. Although the increase in CVB in the HRN™ mouse liver microsome in the presence of NADPH was significant, the effect could not be said to be NADPH-dependent as it was sustained in the presence of ABT. There was no covalent binding in the presence of UDPGA in all three microsomal incubations as values were lower than those for the back added samples (Figure 3.4).

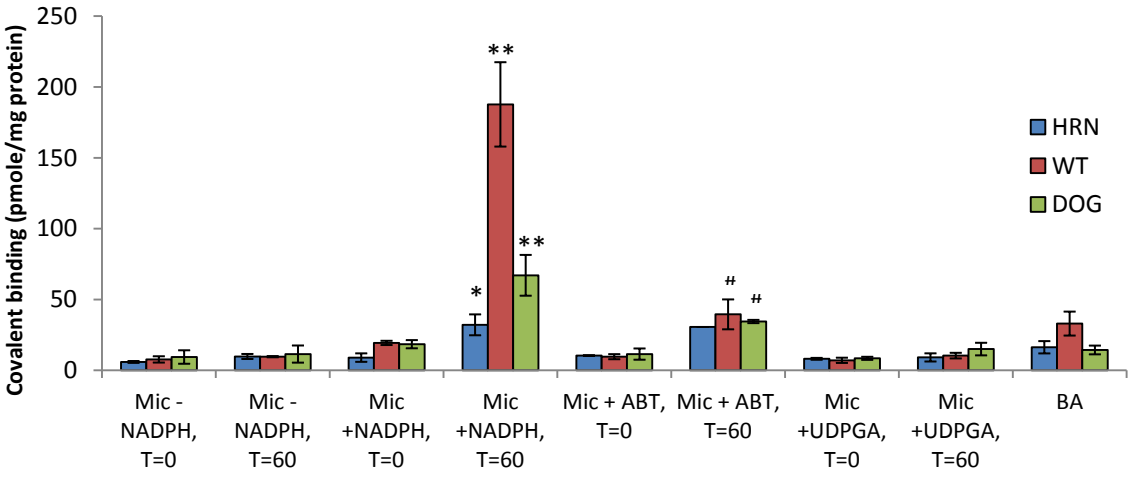


Fig 3.4: Covalent binding in liver microsomes incubated with ^{14}C-fenclozic acid: HRN, C57BL6J mouse and dog liver microsomes were incubated with ^{14}C-fenclozic acid in the presence of co-factors (NADPH or UDPGA) for 60 minutes. Proteins were harvested, protein assayed and radioactivity measured.

WT: C57BL6J mouse, Mic=microsomes, Mic-NADPH=microsomal incubation without NADPH. NADPH=nicotinamide adenine dinucleotide phosphate, ABT=1-amino benzotriazole. UDPGA=Uridine diphosphate glucuronic acid. BA: back added. Data was analysed using Student's t-test, followed by one-way analysis of variance. * Significantly different from background (Mic + NADPH, T=0) at $p<0.05$, ** Significantly different from background (Mic + NADPH, T=0) at $p<0.01$., $^{\#}$ Significantly different from CVB in without inhibitor (Mic+NADPH, T=60).

Data are Mean±SD; n=3.

3.1.5 Metabolism of [^{14}C]-Diclofenac in Microsomal Incubations

Table 3.1, figures 3.5 and 3.6 shows the different metabolic patterns in the HRN™, wild-type and dog liver microsomes. In the presence of NADPH, there was minimal oxidative metabolism produced in HRN™ liver microsomes, indicating negligible CYP activity, whereas in the wild-type mouse, these metabolites (M1, M2 and M3). Dog and HRN™ mouse liver microsomes produced M4. In the presence of UDPGA, a similar metabolite pattern was observed in all three microsomes indicated by UDP-glucuronosyltransferase activity to glucuronides (M5, M6 and M7) (figure 3.6). The peak area of M4 was higher in dog liver than in HRN™ mouse or wild-type mouse liver.

Table 3.1: Summary of mass spectrometric data and assignment of selected [^{14}C]-diclofenac-derived metabolites

Peak ID	t_R (min)	Assignment	$[M-H]^-$ (predominant $^{35}Cl_2/^{14}C_1$ isotope)	Elemental Composition $[M-H]^-$
P	42.4	[^{14}C]-Diclofenac	296.0123	$C_{13}{}^{14}C_1H_{10}O_2N_1Cl_2$
M1	25.8	Mono-hydroxy, ketone oxidation	325.9866	$C_{13}{}^{14}C_1H_8O_4N_1Cl_2$
M2	28.9	4′-hydroxy	312.0073	$C_{13}{}^{14}C_1H_{10}O_3N_1Cl_2$
M3	31.4	5-hydroxy	312.0073	$C_{13}{}^{14}C_1H_{10}O_3N_1Cl_2$
M4	38.6	Ketone oxidation	309.9916	$C_{13}{}^{14}C_1H_8O_3N_1Cl_2$
M5	39.3	1-β-*O*-acyl glucuronide	472.0442	$C_{19}{}^{14}C_1H_{18}O_8N_1Cl_2$
M6	42.2	Transacylated acylglucuronide	472.0441	$C_{19}{}^{14}C_1H_{18}O_8N_1Cl_2$
M7	42.9	Transacylated acylglucuronide	472.0443	$C_{19}{}^{14}C_1H_{18}O_8N_1Cl_2$

t_R – retention time

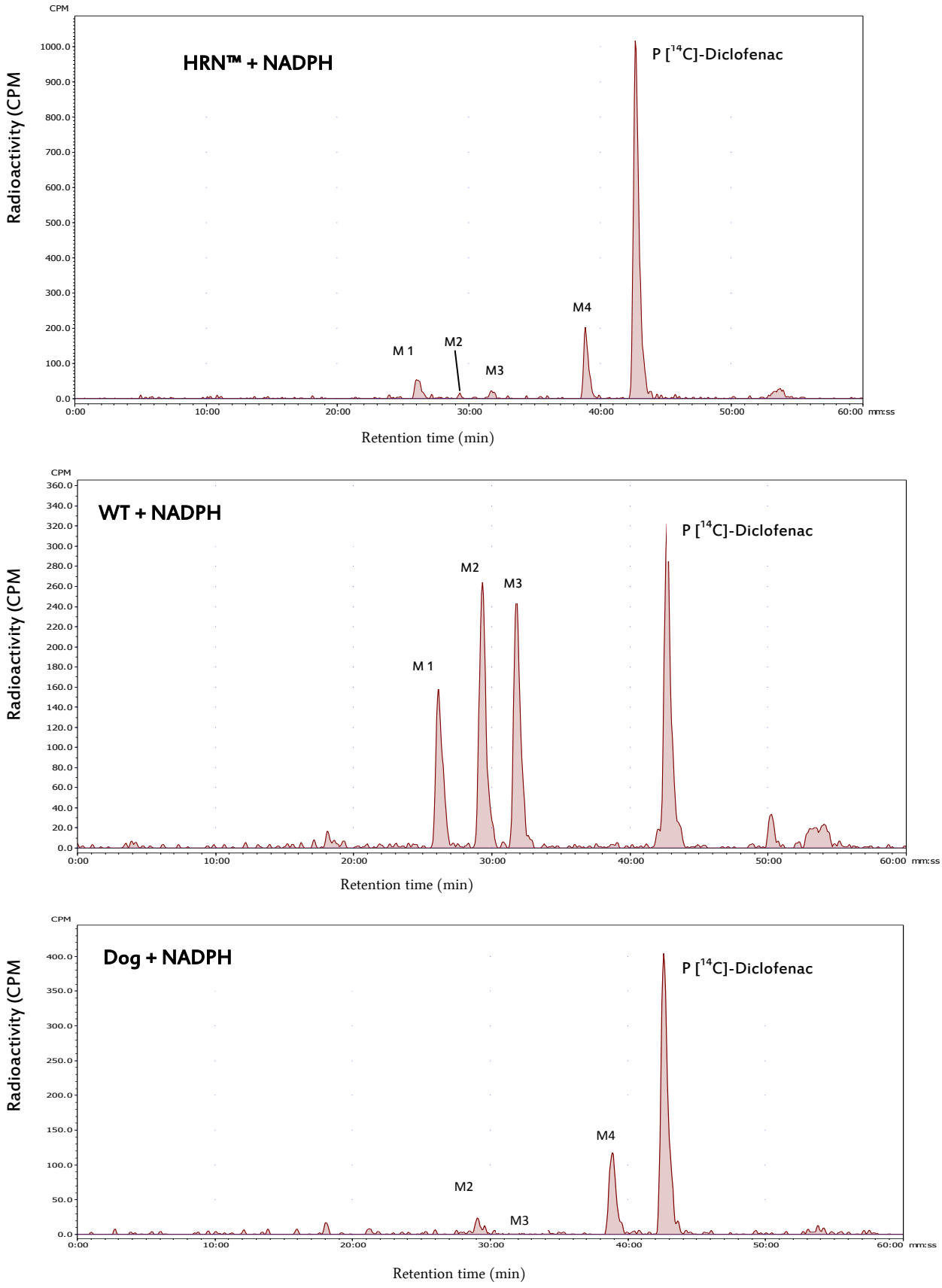


Fig: 3.5: Radio chromatogram of *in vitro* metabolism of [^{14}C]-diclofenac in HRN™, WT or Dog liver microsome in the presence of NADPH.

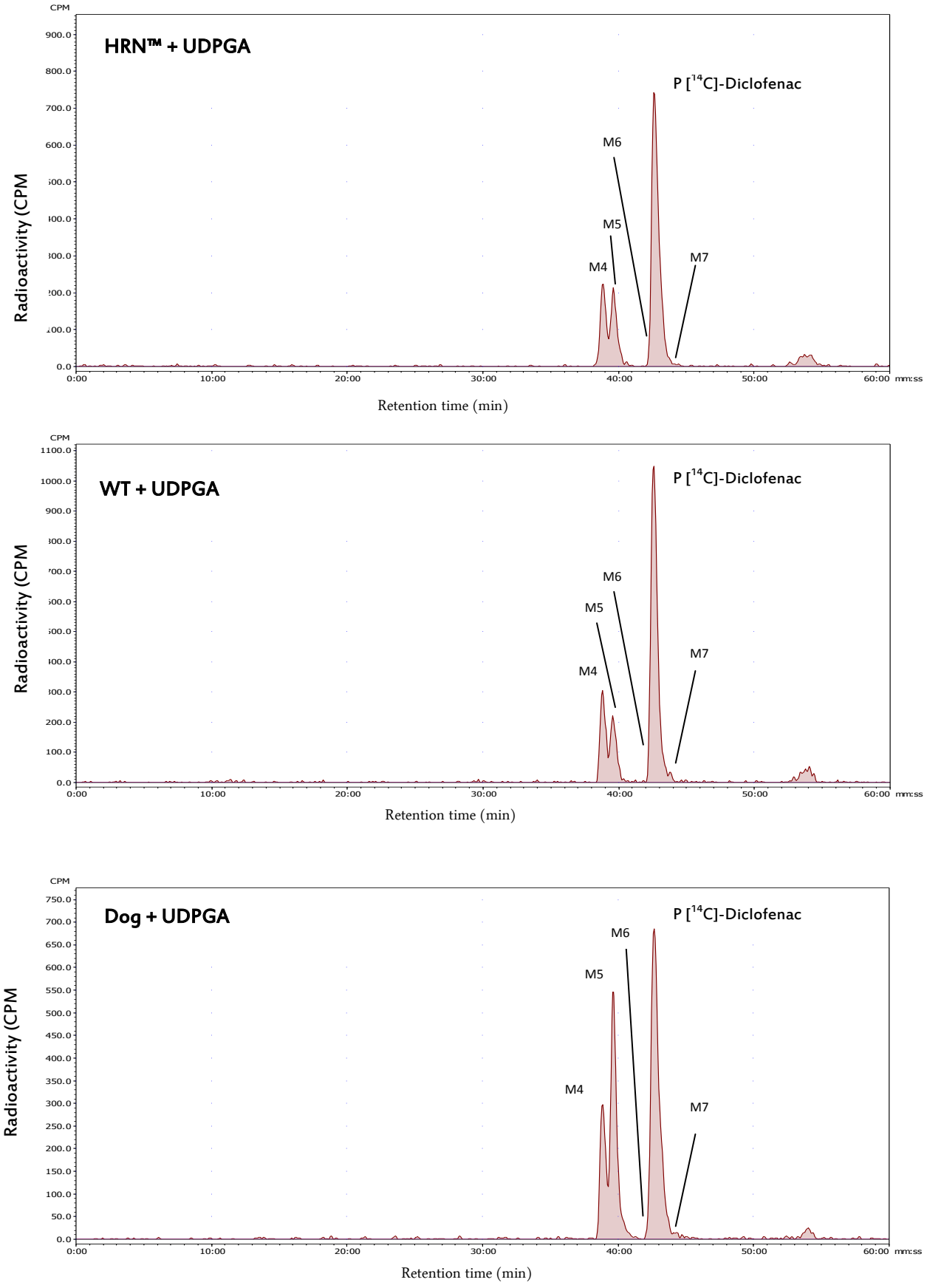


Fig: 3.6: Radio chromatogram of *in vitro* metabolism of [^{14}C]-diclofenac in HRN™, WT or Dog liver microsome in the presence of UDPGA

3.2 *IN VIVO* STUDY

3.2.1 Dose-Range Finding Study in C57BL6J mice

3.2.1.1 Effect of diclofenac or fenclozic acid on body weight of C57BL6J mice

Table 3.2 below shows the percentage change in daily body weight of C57BL6J mice after oral administration of the test drugs. In all the groups, there was a decrease in body weight, with the mice treated with 100 mg/kg fenclozic acid exhibiting the highest decrease. However, at the end of the study, there was a net weight gain in all groups, except the 100 mg/kg fenclozic

Table 3.2: Effect of oral administration of diclofenac or fenclozic acid on daily body weight of C57BL6J mice. Mice were orally administered diclofenac or fenclozic acid and body weight was taken each day of treatment. The table below show percentage change in body weight each day of treatment. DFC=diclofenac, FA=Fenclozic acid. Values in parentheses represent percentage change in body weight relative to day 1 of study.

	Body weight (mg) Percentage change in body weight relative to day 1(%)							
	day 1	**day 2**	**day 3**	**day 4**	**day 5**	**day 6**	**day 7**	**day 8**
Control	22.82 (0)	22.44 (-1.67)	22.92 (-0.44)	22.62 (-0.88)	22.72 (-0.44)	23 (0.79)	23.2 (1.67)	23.68 (3.77)
DFC 10 mg/kg	23.26 (0)	23.06 (0.86)	23.26 (0)	23.5 (1.03)	23.52 (1.12)	23.68 (1.81)	23.7 (1.89)	23.18 (3.96)
DFC 20mg/kg	23.16 (0)	22.98 (-0.78)	23.38 (0.95)	23.38 (0.05)	23.46 (1.30)	23.34 (0.78)	23.42 (1.12)	23.68 (2.25)
DFC 30mg/kg	23.44 (0)	23.32 (-0.51)	23.4 (-0.77)	23.26 (-0.77)	23.6 (0.68)	23.94 (2.13)	23.42 (-0.9)	23.48 (0.17)
FA 50mg/kg	23.6 (0)	22.98 (-2.63)	23.44 (-2.37)	23.04 (-2.37)	23.38 (-0.93)	23.52 (-0.34)	23.52 (-0.34)	23.96 (1.53)
FA 100mg/kg	23.18 (0)	22.34 (-3.62)	22.66 (-2.67)	22.56 (-2.67)	22.86 (-1.38)	23.32 (0.60)	23.04 (-0.60)	23.16 (-0.09)

3.2.1.2 Liver-to-body weight ratio in C57BL6J mice treated with diclofenac or fenclozic acid

There was a significant ($p<0.05$) increase in liver-to-body weight ratio in the C57BL6J mice treated with 20 mg/kg DFC and 100mg/kg FA when compared with control animals (Figure 3.7). All livers appeared normal on visual examination, while the spleen of one of the mice in the group treated with 100 mg/kg FA was slightly discoloured.

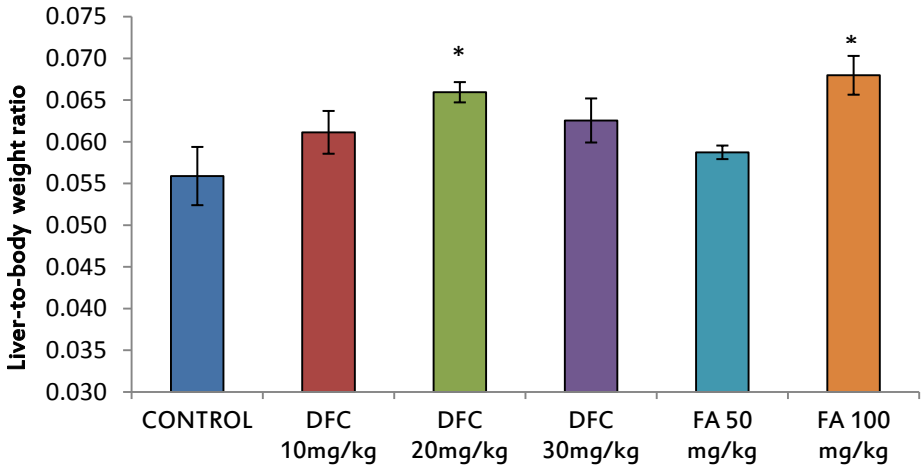


Fig 3.7: Liver-to-body-weight ratio in C57BL6J mice treated with diclofenac or fenclozic acid:

Mice were orally dosed diclofenac or fenclozic acid daily for 7 days. On day 8 mice were necropsied and the liver was visually observed for abnormality. Livers were weighed and the liver-to-body-weight ratio was determined by dividing the weight of the liver by the weight of the individual mouse on termination. Data was analysed using Student's t-test, followed by one-way analysis of variance. n=5; data presented as Mean ± SEM; *significantly different from control at $p<0.05$. DFC: diclofenac; FA=fenclozic acid

3.2.1.3 Time-dependent change in ALT levels in C57BL6J mice treated with diclofenac or fenclozic acid

Figure 3.8 shows time-dependent effect of the oral administration of diclofenac or fenclozic acid on plasma alanine aminotransferase (ALT) in C57BL6J mice. When compared with control

values, ALT levels in mice treated with diclofenac and fenclozic acid at 50 mg/kg exhibited no evidence of alterations. However, elevated ALT values were observed at day 8 after daily administration of fenclozic acid at 100 mg/kg.

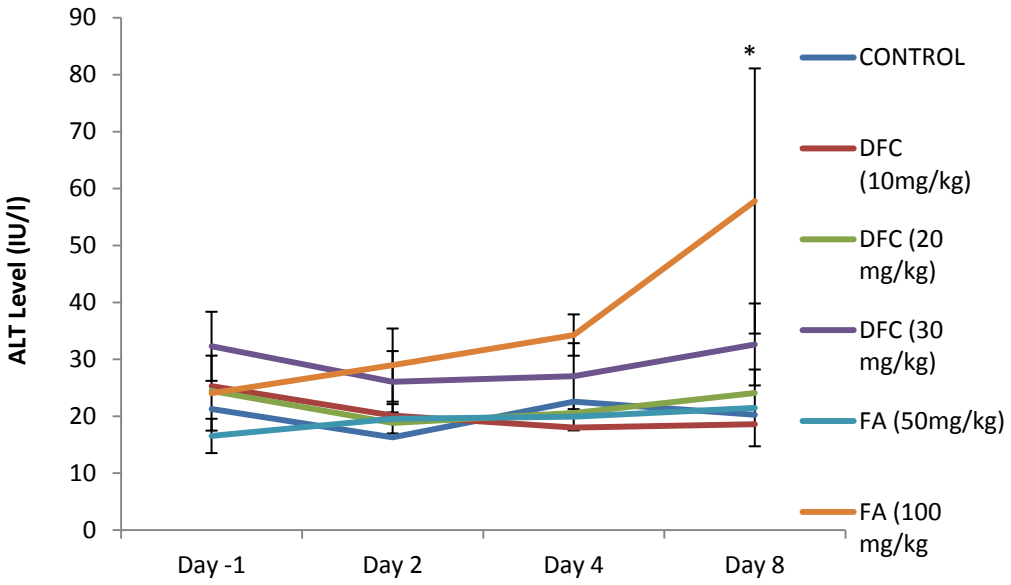


Fig 3.8: Time-dependent change plasma in ALT levels in C57BL6J mice treated with diclofenac or fenclozic acid. Blood was taken from mice by tail veil bleeding on days -1, 2 and 4 of the study. Also, at the end of the study (day 8), blood was obtained by cardiac puncture via the vena cava. In both cases, blood was centrifuged and resultant plasma was assayed for ALT (alanine aminotransferase) as described in methods. Data was analysed using Student's t-test, followed by one-way analysis of variance. n=5; data presented as Mean ± SD; * significantly different from value on day -1 at $p<0.01$. DFC: diclofenac; FA=fenclozic acid

3.2.1.4 Biochemical parameters in C57BL6J mice treated with diclofenac or fenclozic acid

At the end of the dose-range finding study, no significant difference in aspartate aminotransferase (AST) levels were observed between the dosed and control groups. However, there was a significant ($p<0.05$) increase in ALT level in the group that received 100 mg/kg FA when compared with the control. A dose dependent, significant ($p<0.05$) decrease in the level

of plasma alkaline phosphatase was observed at 20- and 30 mg/kg diclofenac, while the activity of plasma glutamate dehydrogenase was significantly decreased at 30mg/kg diclofenac (Figure 3.9).

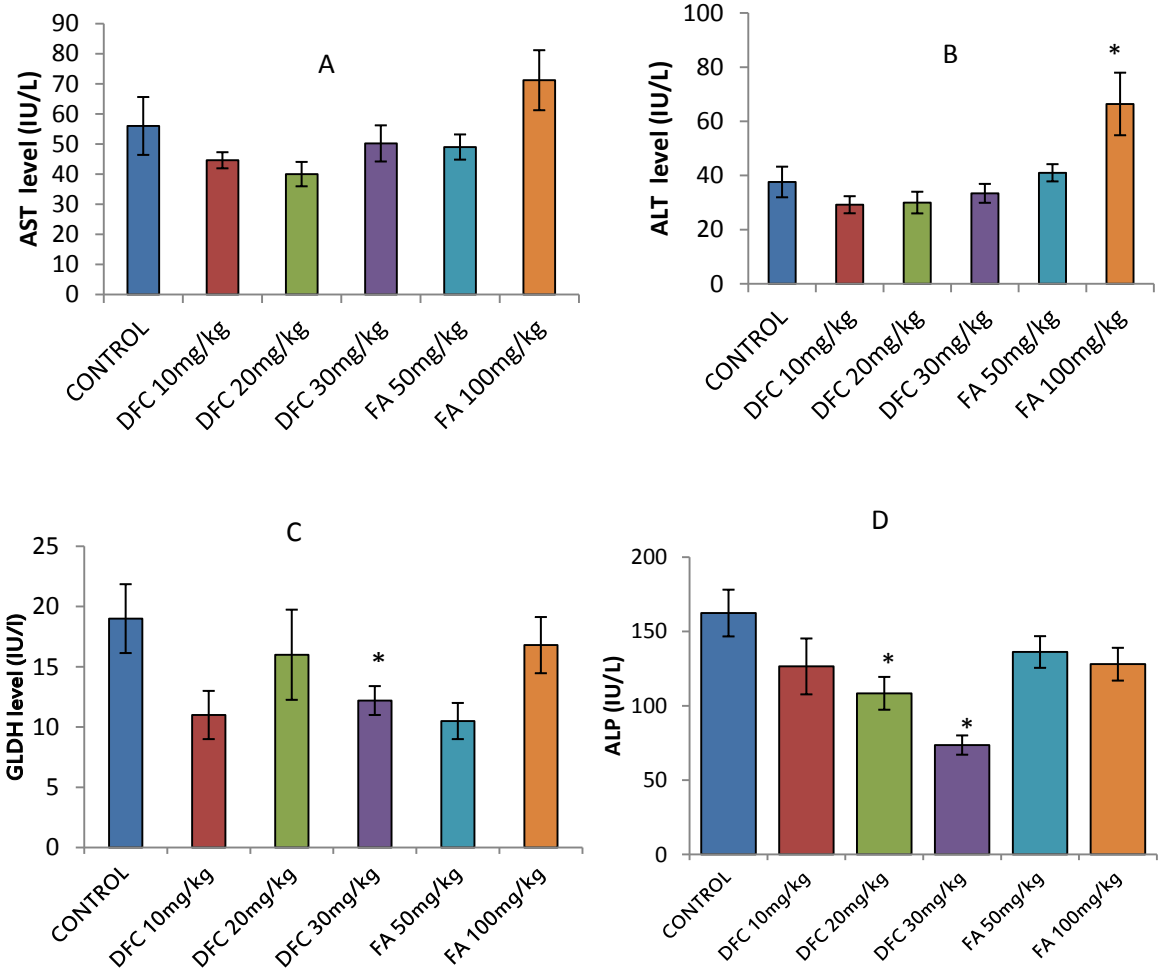


Fig. 3.9: Effect of diclofenac or fenclozic acid on AST, ALT, GLDH and ALP levels in C57BL6J mice

Mice were orally dosed diclofenac or fenclozic acid daily for 7 days. On day 8 mice were necropsied and blood taken via the vena cava, centrifuged and resultant plasma auto analysed for various biochemical parameters shown above. Data was analysed using Student's t-test, followed by one-way analysis of variance.. n=5; data presented as Mean ± SEM; * significantly different from the control group, $p<0.05$. DFC: diclofenac; FA=fenclozic acid

3.2.2 HRN™ STUDY

3.2.2.1 Effect of diclofenac or fenclozic acid on body weight of HRN™ mice

A slight decrease in body weight was observed in the control HRN™ group until day 5 of the study. Thereafter the body weights returned to pre-dose or slightly above pre-dose values (Table 3.2). A small decrease in body weight was also observed in those animals administered with diclofenac and a greater effect in those mice administered with fenclozic acid. In both cases the weight remained below the pre-dose body weight for the duration of the study (Table 3.2). The decrease in body weight did not exceed the severity limit of the licence.

Table 3.3: Effect of oral administration of diclofenac or fenclozic acid on daily body weight of HRN™ mice. Mice were orally administered diclofenac or fenclozic acid and body weight was taken each day of treatment. The table below show percentage change in body weight each day of treatment. DFC=diclofenac, FA=Fenclozic acid. Values in parentheses represent percentage change in body weight relative to day 1 of study.

	Body weight (mg) Percentage change in body weight relative to day 1(%)							
	day 1	day 2	day 3	day 4	day 5	day 6	day 7	day 8
Control	22.6 (0)	22.1 (-0.44)	22.5 (-0.44)	22.28 (-1.44)	22.25 (-1.55)	22.6 (0)	22.88 (1.22)	22.7 (0.44)
DFC 30mg/kg	23.12 (0)	22.74 (-1.64)	22.74 (-1.12)	22.56 (-2.42)	22.94 (-0.78)	22.8 (-1.38)	22.78 (-1.47)	22.7 (-1.82)
FA 100mg/kg	23.18 (0)	22.2 (-4.32)	22.14 (-4.49)	21.92 (-5.44)	22.28 (-3.88)	22.54 (-2.76)	22.68 (-2.16)	22.68 (-2.16)

3.2.2.2 The effect of diclofenac and fenclozic acid on the liver-to-body weight ratio in HRN™ mice

No significant changes in the liver-to-body weight ratio were observed in the HRN™ mice dosed with 30 mg/kg DCF for 7 days when compared with the control animals. An apparent increase in liver-to-body-weight ratio was observed in the group treated with 100mg/kg FA, although this did not achieve statistical significance (Figure 3.10).

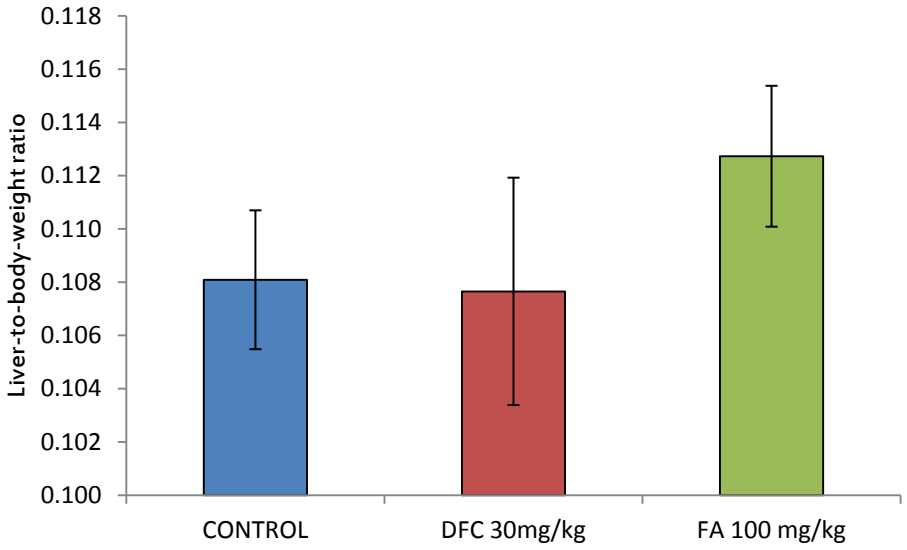


Fig 3.10: Liver-to-body-weight ratio in HRN™ mice treated with diclofenac or fenclozic acid:

Mice were orally dosed diclofenac or fenclozic acid daily for 7 days. On day 8 mice were necropsied and the liver was visually observed for abnormality. It was weighed and the liver-to-body-weight ratio was determined by dividing the weight of the liver by the weight of the individual mouse on termination. Data was analysed using Student's t-test, followed by one-way analysis of variance. n=5 for DFC and FA groups, while n=4 in the control group; data presented as Mean ± SEM; * significantly different from control at $p<0.05$

3.2.2.3 Time-dependent change in ALT levels in HRN™ mice treated with diclofenac or fenclozic acid

In all animals, the mean plasma ALT values prior to dosing were high, when compared with wild-type mice. The mean plasma ALT values for the control group increased throughout the

duration of the study, while in the group receiving 100 mg/kg FA or 30 mg/kg DFC, ALT values were very similar throughout the study (Figure 3.11).

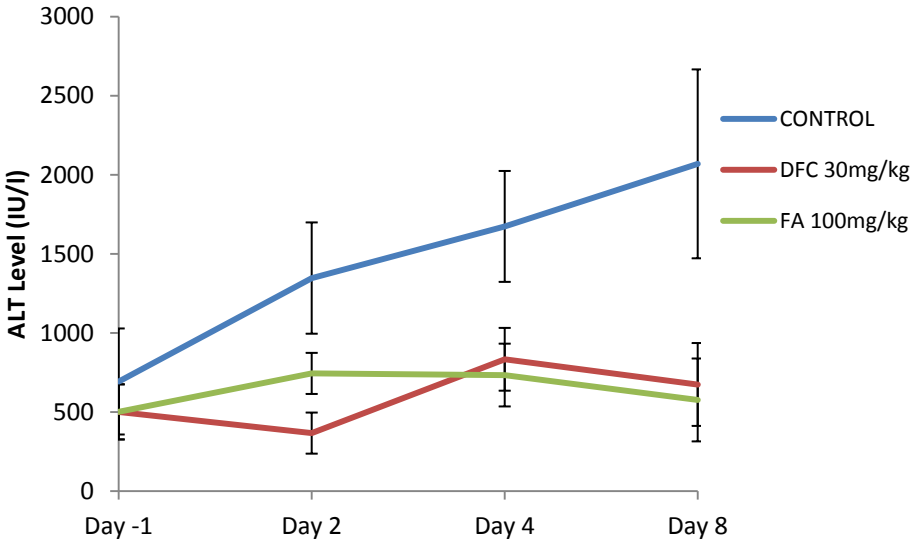


Fig 3.11: Time-dependent change in ALT levels in HRN™ mice exposed to diclofenac or fenclozic acid for 7 days. Blood was taken from the mice by tail veil bleeding on days -1, 2 and 4 of the study. Also, at the end of the study (day 8), blood was obtained by cardiac puncture via the vena cava. In both cases, blood was centrifuged and resultant plasma was assayed for ALT (alanine aminotransferase) as described in the methods. Data was analysed using Student's t-test, followed by one-way analysis of variance. Data are mean values; n=5 for DFC and FA groups, while n=4 in the control group. DFC: diclofenac; FA=fenclozic acid

3.2.2.4 The effect of diclofenac or fenclozic acid on clinical chemistry parameters in HRN™ mice

Markedly significant decreases in the mean levels of bilirubin ($p<0.01$), ALP ($p<0.01$), bile acid ($p<0.05$) and GLDH ($p<0.05$) levels were observed in HRN™ mice administered with 30 mg/kg DFC and 100 mg/kg FA for 7 days. In addition, highly significant ($p<0.01$) reductions in mean ALT levels and a significant ($p<0.05$) decrease in total protein, albumin and AST levels were observed in the group dosed with 30 mg/kg DFC. There were also non-statistically significant decreases in the level of cholesterol in the treated groups in comparison with the control (Figures 3.12 a & b). The ALT, AST, ALP and GLDH values were high when compared with the wild-type mice.

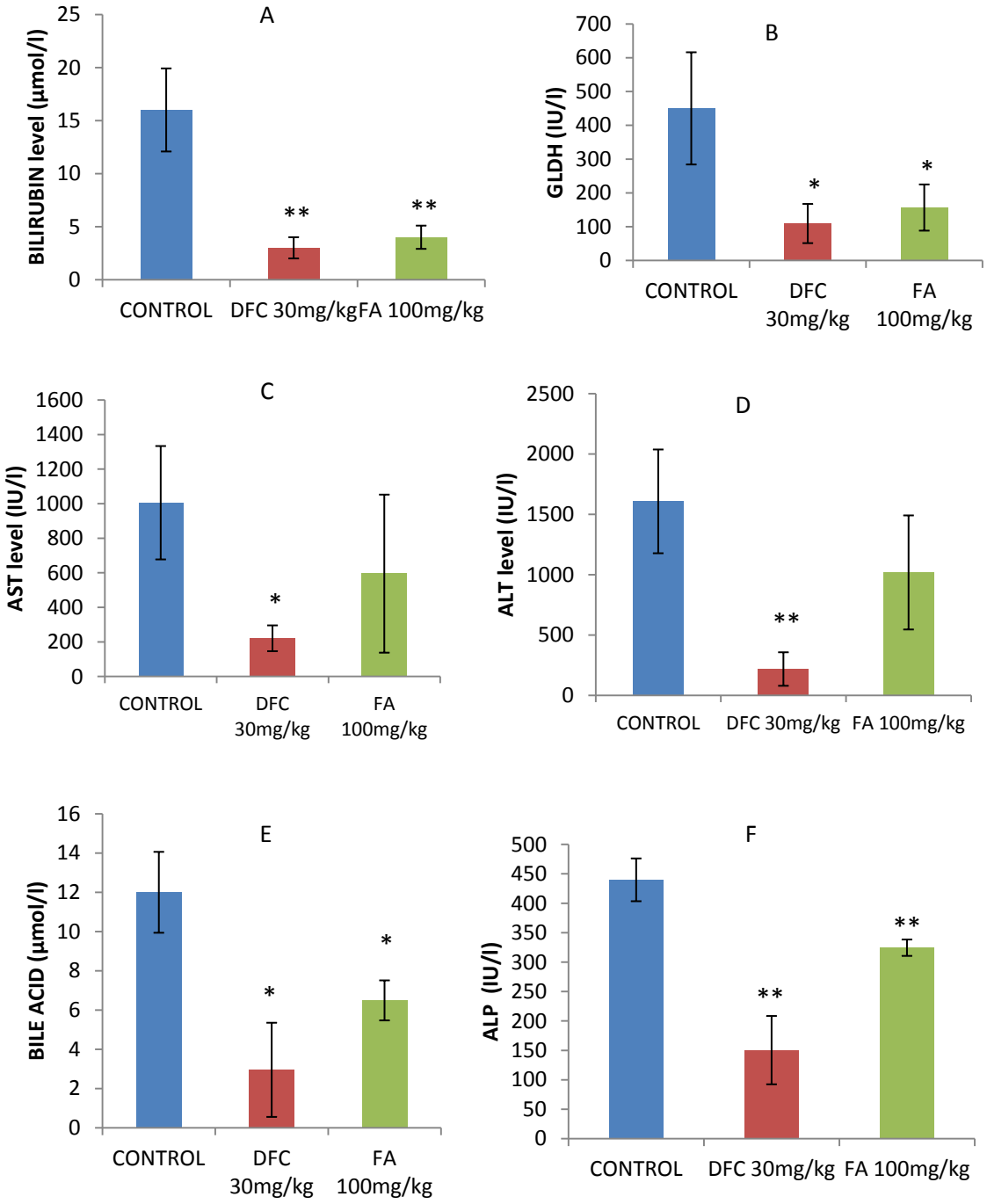


Fig 3.12a: Some biochemical parameters in HRN™ mice treated with diclofenac or fenclozic acid:

Mice were orally dosed diclofenac or fenclozic acid daily for 7 days. On day 8, mice were necropsied and blood taken via the vena cava, centrifuged and resultant plasma auto analysed for various biochemical parameters shown above.. Data was analysed using Student's t-test, followed by one-way analysis of variance. n=5 for DFC and FA groups, while n=4 in the control group; data presented as Mean ± SEM; * significantly different from control at $p<0.05$. ** Significantly different from control at $p<0.01$. DFC: diclofenac; FA=fenclozic acid

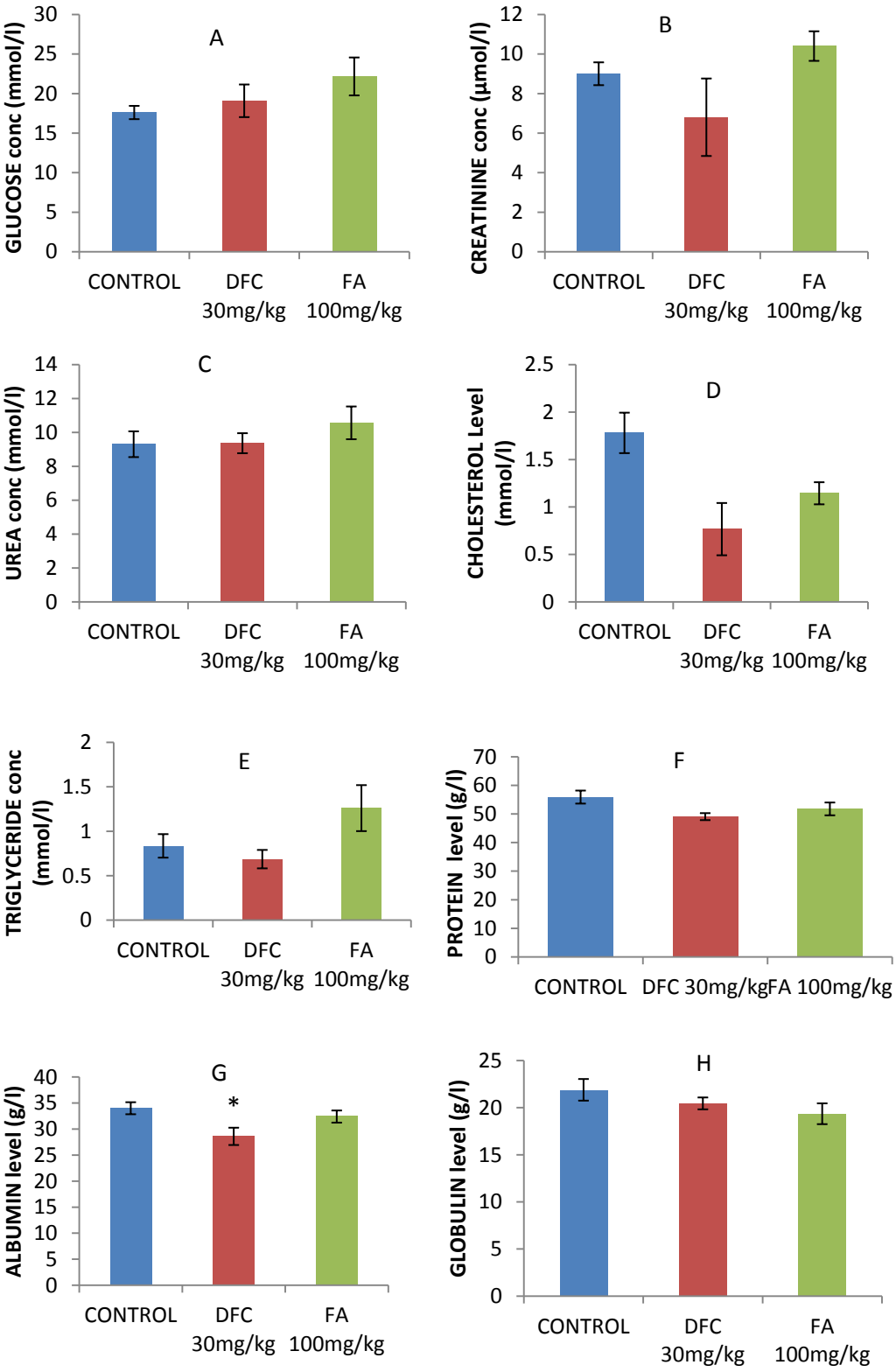


Fig 3.12b: Some biochemical parameters in HRN™ mice treated with diclofenac or fenclozic acid:

Mice were orally dosed diclofenac or fenclozic acid daily for 7 days. On day 8, mice were necropsied and blood taken via the vena cava, centrifuged and resultant plasma auto analysed for various biochemical parameters shown above Data was analysed using Student's t-test, followed by one-way analysis of variance. n=5 for DFC and FA groups, while n=4 in the control group; data presented as Mean ± SEM; *significantly different from control at $p<0.05$. DFC: diclofenac; FA=fenclozic acid

3.3 Histopathology of livers of C57BL6J and HRN™ mice treated with diclofenac and fenclozic acid

Table 3.3 and figure 3.13 report the histological changes in livers of C57BL6J and HRN™ mice treated with diclofenac and fenclozic acid. In figure 3.13, plate A shows mild hepatocyte necrosis with inflammatory cell infiltration and inflammatory cell aggregates in vehicle control-treated C57BL6J mice, while treatment with diclofenac resulted in reduced hepatocyte vacuolation, mild hepatocyte necrosis, mild hepatic necrosis and mixed inflammatory cell aggregation. However, fenclozic acid (E) caused a reduced centrilobular hepatic vacuolation and mixed inflammatory cell aggregates. Vehicle-control-treated HRN™ mice (B) showed a mild hepatocellular fat vacuolation, severe bile duct proliferation, periportal hepatocyte necrosis and mild hepatocyte necrosis with inflammatory cell infiltration. In the diclofenac and fenclozic-acid-treated HRN™ mice (D&F), there was a reduction in the incidence and severity of hepatocellular fat vacuolation and centrilobular hepatic vacuolation, while only diclofenac (D) showed a reduced periportal hepatocyte necrosis and inflammatory cell infiltration. On the other hand, diclofenac and fenclozic acid (D&F) resulted in an increase in incidence and severity of hepatocyte necrosis with inflammatory cell infiltration.

Table 3.4: Pathology report on the effect of oral administration of Diclofenac and fenclozic acid on livers of C57BL6J and HRN mice: Mice were orally administered diclofenac or fenclozic acid for 7 days. On day 8, animals were necropsied, liver sections taken, stained with haematoxylin and eosin, and viewed under a light microscope

Dose group		1	2	3	4	5	6	7	8	9
no of animals		5	5	5	5	5	5	4	5	5
No examined		5	5	5	5	5	5	4	5	5
Finding N.A.D.	:	2	1	1	2	2	1	-	-	-
- hepatocellular fat vacuolation.	:	-	-	-	-	-	-	4	5	5
Grade 2.....	:	-	-	-	-	-	-	-	4	-
Grade 3.....	:	-	-	-	-	-	-	4	1	4
Grade 4.....	:	-	-	-	-	-	-	-	-	1
- multifocal hepatocellular necrosis	:	-	-	-	-	-	-	1	1	1
Grade 2.....	:	-	-	-	-	-	-	1	1	1
- bile duct proliferation	:	-	-	-	-	-	-	4	5	5
Grade 1.....	:	-	-	-	-	-	-	-	-	2
Grade 2.....	:	-	-	-	-	-	-	1	4	1
Grade 3.....	:	-	-	-	-	-	-	3	1	2
- periportal hepatocyte necrosis/inflamm cell infiltration	:	-	-	-	-	-	-	4	5	4
Grade 1.....	:	-	-	-	-	-	-	-	4	3
Grade 2.....	:	-	-	-	-	-	-	4	1	1
- reduced centrilobular hepatocyte vacuolation .	:	-	-	-	3	1	1	-	-	-
Grade 1.....	:	-	-	-	-	1	-	-	-	-
Grade 2.....	:	-	-	-	3	-	1	-	-	-
- increased hepatocyte vacuolation	:	-	2	4	-	-	-	-	-	-
Grade 2.....	:	-	1	4	-	-	-	-	-	-
Grade 3.....	:	-	1	-	-	-	-	-	-	-
- hepatocyte necrosis with inflammatory cell infiltration	:	1	1	-	-	-	-	2	3	5
Grade 1.....	:	1	1	-	-	-	-	2	3	4
Grade 2.....	:	-	-	-	-	-	-	-	-	1
- mixed inflammatory cell aggregates	:	2	3	1	1	3	3	-	-	-
Grade 1.....	:	2	3	1	1	3	3	-	-	-

Group 1: CONTROL; **Group 2:** 10 mg/kg/day Diclofenac; **Group 3**: 20 mg/kg/day Diclofenac; **Group 4:** 30 mg/kg/day Diclofenac;
Group 5: 50 mg/kg/day Fenclozic acid; **Group 6:** 100 mg/kg/day Fenclozic acid (C57BL6J mice)
Group 7: CONTROL; **Group 8:** 30 mg/kg/day Diclofenac; **Group 9:** 100 mg/kg/day Fenclozic acid (HRN mice)

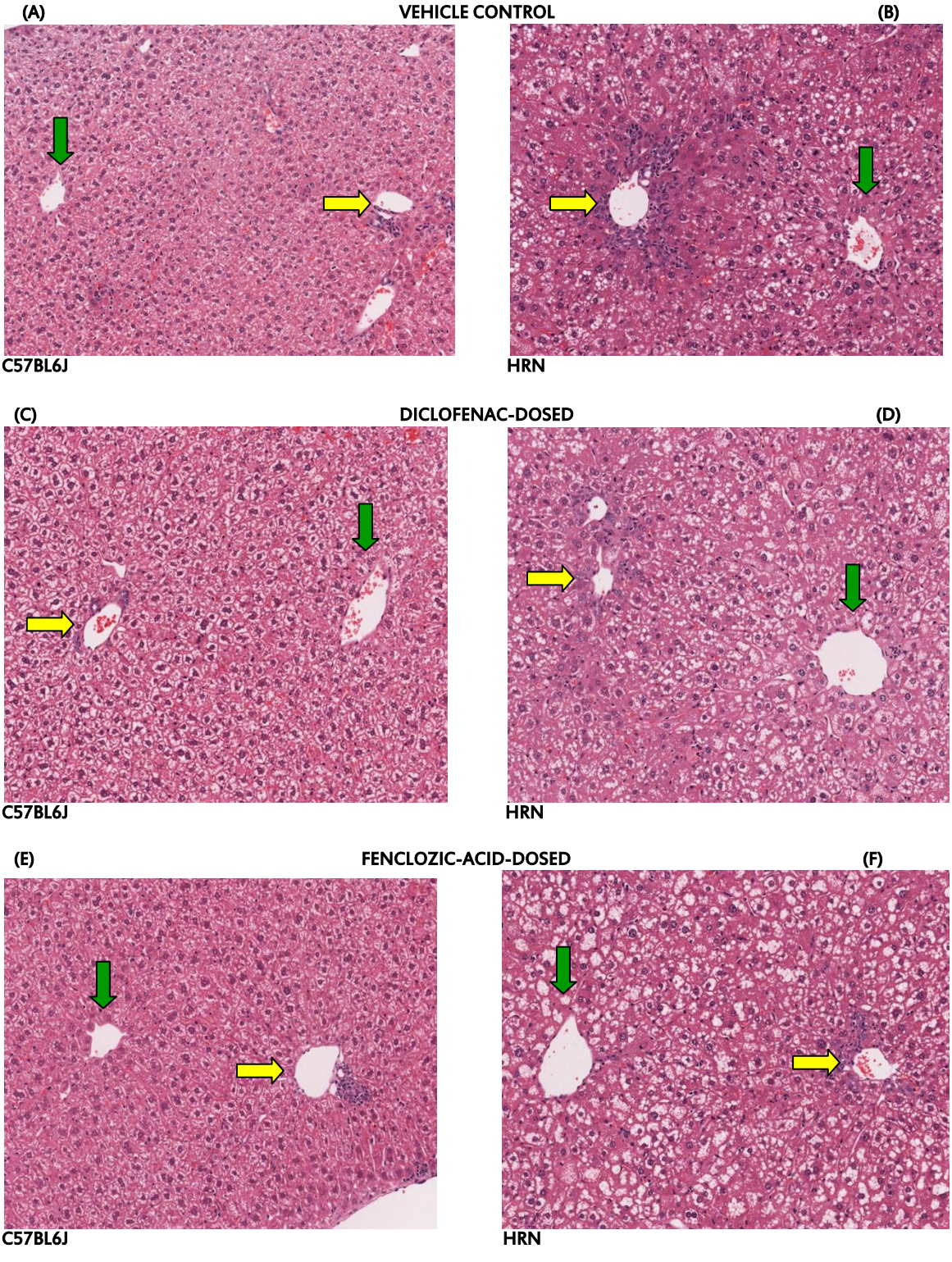


Fig 3.13: Representative photomicrographs of livers from C57BL6J mice dosed with diclofenac or fenclozic acid. Mice were necropsied, liver sections taken and stained with haematoxylin and eosin. Slides were viewed under original objective lens of X20 magnification. Images illustrate hepatocellular appearances in centrilobular (**green arrows**) to periportal regions (**yellow arrows**).

CHAPTER FOUR

4.0 DISCUSSION

4.1 Succinate dehydrogenase and NADPH-Cytochrome c Reductase Assays

When organelles are isolated, there is need for sensitive and robust assays to detect their presence. The usual approach is to test for the activity of an enzyme (marker enzyme), known to be solely localised in the organelle. For example, succinate dehydrogenase (SDH) is found in the mitochondrion, while cytochrome c reductase is localised on the smooth endoplasmic reticulum (Graham, 2002). Marker enzymes are very useful in monitoring the cell fractionation process. In addition, biochemical purity of the isolated organelles can be determined using the marker enzyme, such that the presence of an undesired marker enzyme in a fraction indicates impurity. On the other hand, specific activity of the marker enzyme shows the level of enrichment of the isolate with the organelle of interest (Padh, 1992). Otherwise known as complex II, succinate dehydrogenase (SDH) is an inner membrane-bound enzyme complex. It is the only mitochondrial enzyme involved both in Krebs cycle and oxidative phosphorylation (Cimen *et al*. 2010). It is central to complex II of the electron transport chain, a process crucial to mitochondrial energy generation. In addition to this, SDH, in conjunction with ubiquinone, has been reported to be an important antioxidant enzyme in the mitochondria that moderates superoxide scavenging activity of the respiratory chain (Hajjawi, 2011). Hence, determining its activity in the mitochondrial pellets helps to know the state of the mitochondrion and hence the liver from which it was obtained. The light mitochondrial pellets contain the mitochondrion and some contaminants, including lysosomes, peroxisomes, Golgi membranes, and some

endoplasmic reticulum. The heavy mitochondrial pellets predominantly contain the mitochondria and very few contaminants which are removed by repeated washing. It is the main source of mitochondria used for respiratory studies (Graham, 2002). This explains the relatively higher SDH value in the heavy mitochondrial pellets (Figure 3.2). NADPH-cytochrome c reductase is a flavoprotein localised to the smooth endoplasmic reticulum (SER). It transfers electrons from NADPH to several oxygenases, the most important of which is the cytochrome P450 family of enzymes. Induction of cytochrome c reductase, similar to CYPs, causes proliferation of the SER (Thaler *et al.*, 1972). Hence, its activity is an indirect estimation of the state of the SER, CYPs and hence the metabolic competence of the microsomal preparation it is assayed in. In this experiment, its value in the microsomal preparation from the C57BL6J mouse was within the range previously reported in literature (Williams and Woodhouse, 1995). The HRN™ mouse has been genetically modified to be deficient in the POR gene. Markedly low NADPH-Cytochrome c reductase activity is expected in microsomes prepared from HRN™ mice when compared to those from the C57BL6J mouse liver microsomes. The level in the dog liver microsome was lower than that in the C57BL6J mouse (Figure 3.1). An explanation for this can be seen in a study where preservation of human liver microsomes at -80°C was reported to cause about 40% reduction in CYP and cytochrome c reductase activities (Pearce *et al.*, 1996). Similarly, the relatively low value of this enzyme in the dog liver microsome might be attributed to the duration of the storage of the liver. Despite the slightly low values recorded for the values of the enzyme, there is strong evidence that the livers were viable, as observed in subsequent assays carried out on them (figures 3.3 and 3.4).

4.2 Covalent Binding (CVB) assay in Dog, C57BL6J and HRN™ Mouse Liver Microsomes

The covalent binding assay is an *in vitro* assessment of the potential of a xenobiotic to be bio-activated to chemically reactive metabolites, which form adducts with essential cellular macromolecules (Obach *et al.*, 2008). The pharmaceutical industry has employed this assay as one of the battery of tests carried out on candidate drugs in early prediction of toxicity and idiosyncratic adverse drug reactions (IADR). Data obtained with [^{14}C]-Diclofenac demonstrated significantly increased NADPH-dependent CVB in dog liver microsomes ($p<0.01$) and wild-type mouse (C57BL6J) liver microsomes ($p<0.05$) after 60 minutes of incubation. On the other hand, NADPH-dependent CVB in the HRN™ mouse liver microsomes was at background levels. This supports literature data that HRN™ mice, which are deficient in functional CYP activity, do not participate in phase I drug metabolism (Henderson *et al.*, 2003; Pickup *et al.*, 2012). NADPH-dependent CVB indicates the formation of CYP-mediated reactive metabolites that covalently interact with cellular contents (Obach *et al.*, 2008). Diclofenac is oxidatively metabolised by CYPs 2C8, -2C9 and -3A4. CYP3A4 biotransforms diclofenac to its 5-OH metabolite and can be further metabolised to a *p*-benzoquinone imine which is thiol-reactive, and which can also be involved in redox cycling, culminating in oxidative stress. Alternatively, the phenylamine ring can be oxidised to form an epoxide. Both metabolites are electrophilic and can react with protein and non-protein thiol components of the cell. On one hand, it can result in the depletion of glutathione with consequent oxidative stress and potential cell damage. The epoxide formed can also covalently bind with and inactivate enzymes (Boelsterli, 2003), further strengthening the involvement of CYP in the CVB of diclofenac and its metabolite. UDPGA-dependent covalent binding is an indication of UGT-mediated metabolism. As mentioned in the introduction, the resulting acyl glucuronides can

potentially react with thiol group on cellular protein. Results after 60-minute incubation time showed that the CVB value in the HRN™ mouse liver microsomes was slightly higher than in the wild-type. Dog liver microsome recorded the highest CVB values. This observation might support studies that showed dogs to have a higher (ten-fold) capacity for glucuronidation than humans (Soars *et al.*, 2001).

Similar to the results obtained for [^{14}C]-Diclofenac, the NADPH-dependent covalent binding of [^{14}C]-fenclozic acid to HRN™ mouse liver protein were at background levels and was much lower than what was obtained for dog or wild-type liver microsomes. Once again, this is an indication of a low CYP-mediated metabolism in the HRN™ mice. It also shows that in the presence of NADPH, fenclozic acid strongly forms a highly reactive metabolite in dog and wild-type mice (about 5- and 2 fold higher than in HRN™). In the presence of 1–aminobenzotriazole (ABT), a CYP inhibitor, this CVB was highly reduced to near background levels. CVB in HRN™ mouse liver microsome remained unchanged in the presence of ABT. On the other hand there was no CVB in the presence of UDPGA in all the groups. These fenclozic acid data are in agreement with previous studies carried out in AstraZeneca (unpublished), using human, dog and rat liver microsomes. One study showed a high CVB in the presence of NADPH after 60 minutes but near background levels in the presence of UDPGA over the same time period. When the microsomal preparations were further incubated with recombinant flavin monooxygenases (FMO3 and FMO 5), covalent binding remained at background level. An *in vivo* metabolism study on fenclozic acid carried out in AstraZeneca (unpublished) lends support to this as *in vivo* metabolites in B57BL6J mice constituted mainly of epoxides of the parents compounds. As earlier mentioned, epoxides are highly-reactive metabolites. These data and results from the current experiments might indicate that the reported fenclozic acid toxicity in humans could be related to CYP-mediated metabolism (Hart *et al.*, 1970). Also, the possession of a carboxylic acid moiety does not automatically indicate a compound would

produce a reactive acyl glucuronide. However, this functional group only serves as a structural alert for potential toxicity (Skoberg *et al.*, 2008). Further, *in vitro* CVB does not always translate to toxicity *in vivo*. This was illustrated in a study by Obach *et al.* (2008), in which nine known hepatotoxic and nine known non-hepatotoxic drugs were assessed for CVB in human liver microsomes. There was CVB in seven of each group of drugs, with paroxetine, a non-hepatotoxic drug showing the highest amount of CVB. Therefore, while CVB to certain cellular proteins might be indicative of toxicity, CVB to others might not. It has been reported that binding to certain proteins could be protective as that might be involved in the inactivation of reactive electrophiles (Moro *et al.*, 2012). The protein target involved is very crucial. This emphasises the value of other investigative studies including *in vivo* toxicological studies to fully confirm observations made.

4.3 *In vitro* Metabolism of [^{14}C]-Diclofenac in dog, wild-type and HRN™ mouse liver microsomes

The data shown in the Table 3.1, figures 3.5 and 3.6 are mass spectrometric data and radiochromatograms indicating the formation of metabolites in the presence of the respective co-factors. In the HRN liver microsomes supplemented with NADPH, the formation of very low peaks for 4'- and 5-hydroxy diclofenac indicates little or no CYP-mediated metabolism taking place. This further verifies that the HRN liver lacks functional CYP activity. This is in contrast to the wild-type mouse liver microsomes, in which there were high levels of hydroxylated metabolites. UDPGA-dependent metabolites were detected in all three microsomal incubations, with the dog having the strongest peak value for the 1-β-*O*-acyl glucuronide. This correlates well with the highest UDPGA-dependent CVB in dog liver microsomal incubations. Also, the peak values for 1-β-*O*-acyl glucuronide in both mouse strains had comparable peak

values on the radiochromatograms. It also parallels the CVB values for both strains in which there was no observable difference. These data show that CVB of diclofenac in the liver microsomal incubations studied were related to the metabolism of the parent compound. In addition to the formation of protein adducts, reactive metabolites of diclofenac have potential to induce mitochondrial injury, ATP depletion resulting in necrosis or apoptosis. Gómez-Lechón *et al*. (2003a and 2003b) demonstrated cytotoxicity of diclofenac and its 5OH and 4'OH metabolites on rat and human liver hepatocytes, and reported an induction of apoptotic cell damage via opening of the membrane permeability transition (MPT) pore and activation of caspases 3, 8 and 9. This further confirms the role played by metabolism of diclofenac in its hepatotoxicity.

4.4 *In vivo* Studies in C57BL6J and HRN™ Mice

These studies were designed to investigate the utility of the HRN™ mice in understating the role of conjugative metabolism of carboxylic-acid-containing drugs, with diclofenac and fenclozic acid as a case study. It was a follow up on previous studies carried out at AstraZeneca R&D, UK, which revealed that the HRN™ mouse had clearly different pharmacokinetic profile from its wild-type counterpart (Pickup *et al*, 2012; Sarda *et al*., 2012). The dose-range-finding (DRF) study was a pilot study designed to test the tolerable limit of the drug in the wild-type strain, while the highest tolerable dose would subsequently be used in the HRN™ mouse study. The highest doses in the study (30 mg/kg/day diclofenac and 100 mg/kg/day fenclozic acid) produced no adverse effect up to the severity limit of the approved Home Office licence for the study. There was also no mortality in any groups throughout the study and no clinical signs of concerning levels of overt compound toxicity.

4.4.1 Effect of diclofenac or fenclozic acid on body weight of C57BL6J and HRN™ mice

In the DRF and HRN™ studies, there was loss in body weight among all the groups. This might not appear to be compound-related as the control groups lost 1.67% and 2.21% body weight in the DRF and HRN™ studies respectively (Tables 3.1 and 3.2). In some cases, the weight losses in the control groups were higher than in the groups of mice dosed with the test drugs. There was recovery from day 6 of study in the control groups in both studies. While this effect might be due to either animal handling or effect of blood collection, higher doses of fenclozic acid resulted in the highest loss in body weight in both studies. This observation might be due to fenclozic-acid-related toxicity.

4.4.2 Effect of diclofenac or fenclozic acid on Liver-to-body-weight ratio in C57BL6J and HRN™ mice

Changes in organ weight and organ-to-body-weight ratio are often associated with compound related effects. In multidose toxicity study, organ weight measurement gives insight into the likely toxicodynamics and toxicokinetics of the test compound (Sellers *et al.*, 2007). The organ-to-body weight ratio the DRF study increased in a compound-related manner and was statistically significant ($p<0.05$) at 20 mg/kg diclofenac and 100 mg/kg fenclozic acid (Figure 3.7) On the other hand, in the HRN™ study there were increases, but which were not statistically significant in the fenclozic-acid-treated group (Figure 3.10). While not statistically significant and might not be compound-related, a comparison with the values obtained in the wild-type mice gives an indication of strain-related changes in the liver weight. For example, while the control values of liver–to-body-weight ratios of the wild-type control and 100 mg/kg

fenclozic acid were 0.055 and 0.065 respectively, it was 0.108 and 0.112 in the corresponding HRN™ groups. A similar observation was made in a study by Henderson *et al.* (2003) which revealed that HRN™ mouse livers were enlarged, with liver-to-body-weight ratio being almost twice that in the wild-type mice, in addition to a pale appearance. This indicates the possibility of a background hepatomegaly in the HRN™ mice. Other results, to be discussed in other sections will further give detailed functional differences in HRN™ mice.

4.4.3 Effect of Diclofenac or Fenclozic Acid on Clinical Chemistry Parameters of C57BL6J and HRN™ Mice

Another aim of the DRF is to detect at least a two-fold increase in alanine aminotransferase (ALT), as that would be a basis for determining the occurrence of liver injury in the wild-type mice (Bénichou, 1990). Therefore, ALT was assayed on plasma obtained on days -1, 2, 4 and 8. While values remained unchanged for the control and most other treatment groups, there was a three-fold change in ALT values in the 100 mg/kg fenclozic acid group on day 8 of the study when compared to the value before the first dose. In addition, the ALT value for 100 mg/kg fenclozic acid at the end of the study was significantly ($p<0.05$) higher than in the control C57BL6J mice. This was accompanied by an increase in aspartate aminotransferase (AST), though not statistically significant. Those observations confirmed that highest doses of fenclozic could potentially induce liver damage. Glutamate dehydrogenase (GLDH) levels remained unchanged except in the 50 mg/kg fenclozic acid group in which a significant ($p<0.05$) decrease was seen. Located in the mitochondrial matrix, GLDH is an important enzyme involved in the production of urea and in amino acid oxidation. It is liver-specific and has features that make it a good biomarker of drug-induced hepatocellular toxicity. It is a

specific marker of necrosis with its activity higher in the centrilobular region than in the periportal zone, making the release of GLDH a prominent feature of conditions associated with centrilobular necrosis (O'Brien *et al.*, 2002). There was a dose-dependent decrease in ALP in the diclofenac-treated C57BL6J mice. Increase in ALP is a feature of hepatobiliary injury. However, this study indicates an opposite trend. Ramirez-Alcantara *et al.* (2009) reported a dose-dependent decrease in ALP values in diclofenac-treated C57BL6J mice after an intraperitoneal dose of 60 mg/kg. While not fully understood, it is thought to be linked to the enteropathy caused by an exposure to the NSAID because the decreases observed paralleled ulceration. However, it had been earlier believed that ALP, in protecting the gut against lipopolysaccarides, get leaked into the blood stream during ulceration and hence disrupt mucosal damage (Geddes and Philpot, 2008). A decrease in this implies a compromise of this protective mechanism. Hence, rather than give information on the state of the liver, the decrease observed in ALP in the DRF may relate to the effect of diclofenac on the gut.

In the HRN™ phase of the study, a different trend was observed. There was a time-dependent increase in ALT levels in the control group to a maximum of 2000 IU/l, while the dosed animals did experience mild increases followed by a decrease on the last day of study (Figure 3.8). At the end of the study, levels of other enzyme were high (Figure 3.9a). The values were much higher than what was recorded for their wild-type counterpart in the DRF study and in the literature (Fernández *et al.*, 2010). This strongly indicates background liver dysfunction in the HRN™ mice which might have been previously unexplored or underreported in the literature. However, there are clues to the possibilities of background liver insufficiency as found in early articles that described the HRN™ mice. In the HRN™ mice, Henderson *et al.* (2003) reported hyperlipidaemia along with a 90% decrease in bile acid in the gall bladder, 65% and 50% decrease in serum cholesterol and triglycerides in comparison with the wild-type mice. Also, as

earlier mentioned in the introduction, they reported a significant elevation in serum ALT level. These findings are consistent with the results obtained in this study. In addition, there was a background high alkaline phosphatase (ALP) level in the HRN™ mice, which is an index of cholestatic liver disease and this could be linked with the low value of bile acids. HRN™ mice lack functional CYPs because of their lack of the P450-oxidoreductase enzyme which donates electron to all endoplasmic reticulum CYPs. Aside the CYPs identified in xenobiotic metabolism, other families and isoforms are involved in cell physiology and homeostasis. For example, CYPs51, -7A, 8B, -27A and -46A are involved in cholesterol and bile acid synthesis. Cholesterol is a precursor in the synthesis of vitamin D, bile acids (BAs) and steroid hormones, all of which play important roles in normal cell function and maintenance of the integrity of the cell and health of the whole organism (Lorbek *et al.*, 2012). Hence, a lack of functional hepatic CYPs is associated with impaired liver which makes the HRN™ strain not so suitable to study hepatotoxic effect of drugs.

4.4.4 Effect of Diclofenac or Fenclozic Acid on Liver Histology of C57BL6J and HRN™ Mice

The liver histology of the wild-type mice revealed no drastic compound-related changes. Hepatocyte vacuolation were observed in the groups treated with 10- and 20 mg/kg DFC. Rather than being a sign of toxicity, hepatocyte vacuolation has been reported to be a cellular adaption, which is beneficial to the host (Nayak *et al.*, 1996). Background mild hepatocyte necrosis with inflammatory cell infiltration and mixed inflammatory cell aggregates were also observed the wild-type mice. On the other hand, background hepatocellular fat vacuolation, bile duct proliferation, periportal hepatocyte necrosis, reduced centrilobular hepatocyte vacuolation were seen in the HRN™ mice (Table 3.4 and Figure 3.13). Hepatocellular fat

vacuolation is a sign of fat accumulation in the hepatocytes. As noted earlier, the lack of functional CYPs in the HRN™ mice makes lipid metabolism inadequate and may result in high levels of hepatic free fatty acid. This has been reported to be associated with significant increase in hepatic inflammation, oxidative stress, hepatocellular apoptosis and liver injury (Alkhouri *et al.*, 2009). Other features seen in the liver histology slides confirm inflammation and some hepatocyte necrosis. This harmonises with a previous study in which background single cell necrosis and inflammation were observed in vehicle-treated HRN™ mice (Pickup *et al.*, 2012). This backs up the clinical chemistry data that gives evidence of severe background liver damage in the HRN™ mice. A rather interesting finding from the study is that administration of FA and DFC reduced the incidence and severity of hepatocellular fat vacuolation, bile duct proliferation and inflammatory cell infiltration. Could this imply that NSAIDs are protective against the background liver damage in the HRN™ mice? It is understood that in non-alcoholic fatty liver disease, fatty acids can initiate a cascade of events that causes activation and nuclear translocation of NF-κB and production of other pro-inflammatory cytokines. Studies have shown that suppression of this signalling pathway resulted in a partial protection against liver injury (Alkhouri *et al.*, 2009). Further, other studies have suggested that NSAIDs can inhibit the NF-κB signalling pathway so as to down-regulate pro-inflammatory cytokines, with a consequent inhibition of inflammation (Vaish and Sanyal, 2011). Whether this happened in the HRN™ mice is uncertain. However, evidence from the clinical chemistry data—significant reduction in ALT, AST, GLDH and ALP in the DFC- and FA-treated HRN™ mice—strongly suggest these NSAIDs may offer some anti-inflammatory protection to the 'diseased' HRN™ mouse liver.

CONCLUSION AND FUTURE DIRECTION

The *in vitro* metabolism and CVB data have demonstrated generation of reactive metabolites of diclofenac and of fenclozic acid and, which bind covalently to liver proteins. Bioactivation of diclofenac was supported by CYP and UGT co-factors, while for fenclozic acid only CYP-dependent bioactivation could be demonstrated. The HRN™ mouse provided valuable confirmation of the role of CYP metabolism in the bioactivation of fenclozic acid. The studies in wild-type mice revealed no evidence of liver pathology following treatment with either drug. The data obtained with HRN™ mice revealed evidence of overt liver injury in untreated animals. This precluded use of the animals for investigation of liver toxicity caused by diclofenac or fenclozic acid. An intriguing normalisation of plasma liver parameters was observed in HRN™ mice treated with FA and diclofenac, which raises the possibility that the drugs were protective.

In the future, it will be valuable to identify and map the target proteins of FA and DFC metabolites by administering the radio-labelled compound to the study animal. Bound liver proteins can be characterised by electrophoresis, fluorography and mass spectrometry. Using bioinformatics, the cellular function of such proteins could be determined by searching such target proteins in databases e.g http://tpdb.medchem.ku.edu:8080/protein_database/ (Hanzlik *et al.*, 2007; Moro *et al.*, 2012).

In addition, it will be useful to apply electron microscopy (EM), glutathione (GSH) cytochemistry and immunohistochemistry in the understanding of the precise nature of liver damage in the HRN™ mice. Also, further mechanistic studies are required to elucidate potential mechanisms by which NSAIDs might protect against the background hepatic lesion in

the HRN™ mice. It will be informative to know the specific CYP isoforms responsible for metabolism of fenclozic acid. Since high doses of fenclozic acid caused ALT elevations on day 8 of treatment, raising the possibility of mild liver injury, evaluation of livers from these animals at the electron microscopy (EM) level might be informative and is planned in the future. The mechanism of toxicity of fenclozic acid will also be an interesting area to investigate using *in vitro* systems.

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APPENDIX

APPENDIX 1

Calculations

A. Derivation of formula for SDH and Cytochrome c reductase activities

The aim is to express enzyme activity in nmol/min/mg protein.

1. Estimate the dilution factor to obtain the actual amount of microsomal protein present in the reaction mix in the cuvette.

   In this particular case (cytochrome c reductase), a 1:10 dilution of microsome was made up to 50 µl and prepared in a total of 1100 µl reaction mix.
   Hence, total volume of reaction mix= 1100 µl,
   Volume of microsomes in reaction mix=1/10 X 50 µl=5 µl.

   Dilution factor= volume of microsomes in reaction mix/total volume of reaction mix

   =5/1100 = 1/220

   Amount of protein in the cuvette = dilution factor X protein concentration (mg/ml)

   = 1/220 X protein concentration (mg/ml)-------(i)

2. Calculate activity of cytochrome c reductase in nmol/min

Deriving from the standard equation of cytochrome c reductase activity:

$$\frac{\triangle \text{A500nm/min X dilution factor X total volume of reaction mix}}{\text{extinction coefficient X enzyme volume}}$$

Where $\Delta A_{500nm/min}$= change in absorbance per minute

Reckoning that the enzyme volume, dilution factor and total volume of reaction mix have been accounted for by equation (i), then the remainder of the equation becomes

$$\frac{\triangle \text{A500nm/min}}{\text{extinction coefficient (1/mM)}},$$

and the unit becomes mM X ΔA/time (minutes)= ΔA /time (minutes) X mmol/ 1000 ml.

Hence, equation becomes ΔA/time (min) X1/21.1($mmole^{-1}$)= ΔA/time X 1/0.0211 nmol/ml,

3. Hence Cytochrome c reductase in nmol/min/mg protein becomes

$$\frac{\text{Cytochrome c reductase in nmol/min/ml}}{\text{amount of protein } (\frac{\text{mg}}{\text{ml}})}$$

and the unit derived becomes $\frac{nmol}{\min X\ ml}$ X $\frac{ml}{mg}$

Cytochrome c redustase working formula becomes $\frac{(\Delta A\ X\ 60)}{0.0211}$ ÷ protein concentration X dilution factor,

Were dilution factor = $\frac{\text{volume of microsome added into reaction mix (µl)}}{\text{total volume of reaction mix (µl)}}$

Protein concentration were derived from BCA assay

SDH

This derived formula holds true for the SDH activity except that the extinction factor = 19.7mM^{-1} and dilution factor 1/200.

B. Molar radioactivity for radio isotopes used in CVB assays.

[^{14}C]-diclofenac

Specific activity = 2.12 Kbq /nmol
1 KBq=27 nCi
2.12 KBq=27 X 2.12 nCi
= 57.24 nCi/nmol, or
57.24 mCi/ mmol

1 Ci = 2.22 X 10^{12} dpm

1 mol= 57.24 Ci
1 mol= 57.24 X 2.22 X 10^{12} dpm.

Hence molar radioactivity (mol/dpm) = 7.8 X 10^{-15} mol/dpm

The same calculation applies for second batch of [^{14}C]-diclofenac, specific activity of 55 mCi/mol, and [^{14}C]-fenclozic acid, specific activity 60.5 mCi/mmol.

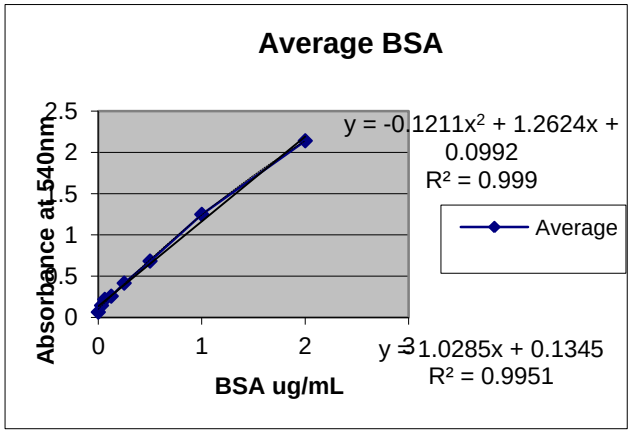
APPENDIX 2

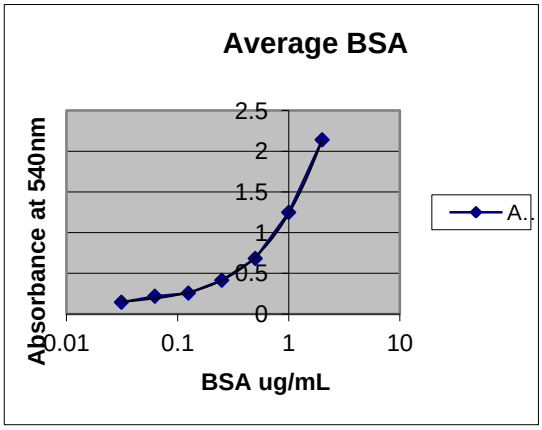
BCA PROTEIN ASSAY ON MICROSOMES AND MITOCHONDRIAL PELLETS.

Plate layout.

	1	2	3	4	5	6	7	8	9	10	11	12
A	0.389	0.269	0.198	3.011	0.685	0.377	1.545	0.31	0.197	0.33	0.216	0.171
B	0.359	0.244	0.177	3.04	0.628	0.345	1.525	0.287	0.177	3.247	0.964	0.532
C	0.383	0.228	0.167	3.03	0.641	0.35	1.507	0.286	0.178	3.099	0.668	0.372
D	0.424	0.266	0.192	3.247	0.82	0.428	3.187	0.877	0.441	2.12	0.268	0.425
E	0.425	0.27	0.186	3.239	0.811	0.433	3.16	0.837	0.445	1.224	0.166	0.262
F	0.418	0.268	0.179	3.18	0.79	0.41	3.272	0.83	0.434	0.681	0.061	0.166
G	0.346	0.225	0.157	3.247	0.965	0.494	3.066	0.64	0.343	0.402	2.158	0.162
H	0.343	0.216	0.155	3.289	0.984	0.535	3.122	0.659	0.341	0.249	1.27	0.063

BSA (mg/mL)			0	0.03125	0.0625	0.125	0.25	0.5	1	2		
Stds Rep 1			0.061	0.166	0.268	0.249	0.402	0.681	1.224	2.12		
Stds Rep 2			0.063	0.12	0.166	0.262	0.425		1.27	2.158		
Stds Rep 3												
Stds Rep 4												
Average			0.062	0.1435	0.217	0.2555	0.4135	0.681	1.247	2.139		
SD			0.001414	0.032527	0.072125	0.009192	0.016263	#DIV/0!	0.032527	0.02687		





Protein Standard plot

Final protein concentration calculations

Raw Data	Microsomes				LM			HM	
	0.02	0.01	0.005	1	0.1	0.04	1	0.1	0.04
Dog	0.389	0.269	0.198	3.011	0.685	0.377	1.545	0.31	0.197
	0.359	0.244	0.177	3.04	0.628	0.345	1.525	0.287	0.177
	0.383	0.228	0.167	3.03	0.641	0.35	1.507	0.286	0.178
Average	0.377	0.247	0.1806667	3.027	0.651333	0.3573333	1.525667	0.294333	0.184
SEM	0.0091652	0.0119304	0.0091348	0.008505	0.017247	0.0099387	0.010975	0.007839	0.006506
HRN	0.424	0.266	0.192	3.247	0.82	0.428	3.187	0.877	0.441
Mouse	0.425	0.27	0.186	3.239	0.811	0.433	3.16	0.837	0.445
	0.418	0.268	0.179	3.18	0.79	0.41	3.272	0.83	0.434
Average	0.4223333	0.268	0.1856667	3.222	0.807	0.4236667	3.206333	0.848	0.44
SEM	0.0021858	0.0011547	0.0037565	0.021127	0.008888	0.0069841	0.033746	0.01464	0.003215
Mouse	0.346	0.225	0.157	3.247	0.965	0.494	3.066	0.64	0.343
	0.343	0.216	0.155	3.289	0.984	0.535	3.122	0.659	0.341
	0.33	0.216	0.171	3.247	0.964	0.532	3.099	0.668	0.372
Average	0.3396667	0.219	0.161	3.261	0.971	0.5203333	3.095667	0.655667	0.352
SEM	0.0049103	0.003	0.0050332	0.014	0.006506	0.0131951	0.016251	0.008253	0.010017

Protein (mg/mL)	Microsomes				LM			HM	
	0.02	0.01	0.005	1	0.1	0.04	1	0.1	0.04
Dog	0.23	0.14	0.08	3.45	0.49	0.22	1.31	0.17	0.08
	0.21	0.12	0.06	3.51	0.44	0.20	1.29	0.15	0.06
	0.23	0.10	0.05	3.49	0.45	0.20	1.27	0.15	0.06
Average	0.22	0.12	0.06	3.48	0.46	0.21	1.29	0.16	0.07
SEM	0.01	0.01	0.01	0.02	0.01	0.01	0.01	0.01	0.01
HRN	0.26	0.13	0.07	4.13	0.61	0.27	3.92	0.66	0.28
Mouse	0.26	0.14	0.07	4.10	0.60	0.27	3.84	0.62	0.28
	0.26	0.14	0.06	3.90	0.58	0.25	4.23	0.62	0.27
Average	0.26	0.14	0.07	4.04	0.59	0.26	4.00	0.63	0.28
SEM	0.00	0.00	0.00	0.07	0.01	0.01	0.12	0.01	0.00
Mouse	0.20	0.10	0.05	4.13	0.74	0.32	3.58	0.45	0.20
	0.20	0.09	0.04	4.30	0.76	0.36	3.73	0.46	0.20
	0.19	0.09	0.06	4.13	0.74	0.35	3.66	0.47	0.22
Average	0.19	0.10	0.05	4.19	0.74	0.35	3.66	0.46	0.20
SEM	0.00	0.00	0.00	0.06	0.01	0.01	0.04	0.01	0.01

Conc. adjusted protein (mg/mL)	Microsomes				LM			HM	
	0.02	0.01	0.005	1	0.1	0.04	1	0.1	0.04
Dog	11.74	13.63	15.77	3.45	4.87	5.62	1.31	1.70	1.95
	10.50	11.60	12.40	3.51	4.37	4.96	1.29	1.51	1.55
	11.49	10.30	10.80	3.49	4.48	5.07	1.27	1.50	1.57
Average	11.25	11.84	12.99	3.48	4.57	5.22	1.29	1.57	1.69
SEM	0.38	0.97	1.47	0.02	0.15	0.21	0.01	0.06	0.13
		12.03			4.90			1.63	
HRN	13.20	13.38	14.81	4.13	6.06	6.68	3.92	6.58	6.95
Mouse	13.24	13.71	13.84	4.10	5.98	6.79	3.84	6.21	7.04
	12.95	13.55	12.72	3.90	5.79	6.31	4.23	6.15	6.81
Average	13.13	13.55	13.79	4.04	5.95	6.59	4.00	6.31	6.93
SEM	0.09	0.09	0.60	0.07	0.08	0.15	0.12	0.13	0.07
		13.49			6.27			6.62	
Mouse	9.97	10.06	9.20	4.13	7.38	8.07	3.58	4.48	4.92
	9.84	9.34	8.88	4.30	7.56	8.94	3.73	4.64	4.88
	9.31	9.34	11.44	4.13	7.37	8.87	3.66	4.72	5.52
Average	9.71	9.58	9.84	4.19	7.44	8.63	3.66	4.61	5.11
SEM	0.20	0.24	0.81	0.06	0.06	0.28	0.04	0.07	0.21
		9.71			8.03			4.86	

N.B. Absorbance values at the top end of the samples (so all values for neat LM and neat HM), were outside the range of the protein standards, and appear to have been well outside the predictive range of the assay.

Disregard neat LM and HM values

APPENDIX 3

SDH ASSAY

LIGHT MITOCHONDRIAL PELLETS				
Strain code	**HRN**			
dilution	1	1	1	1
dilution factor	0.005	0.005	0.005	0.005
rate (s^{-1})	0.0004	0.0004	0	0
Prot conc (mg/ml)	6.27	6.27	6.27	6.27
SDH (nmol/min/mg protein)	38.864806	38.864806	0	0
MEAN	38.86			
SD	0			

HEAVY MITOCHONDRIAL PELLETS				
Strain code	**HRN**			
dilution	1	1	1	1
dilution factor	0.005	0.005	0.005	0.005
rate (s^{-1})	0.0008	0.0006	0.0006	0.0006
Prot conc (mg/ml)	6.62	6.62	6.62	6.62
SDH (nmol/min/mg protein)	73.567699	55.175775	55.175775	55.175775
MEAN	59.773756			
SD	9.1959624			

Strain code	**Wild-type**			
dilution	1	1	1	1
dilution factor	0.005	0.005	0.005	0.005
rate (s^{-1})	0.0004	0.0004		
Prot conc (mg/ml)	8.03	8.03	8.03	8.03
SDH (nmol/min/mg protein)	30.338333	30.338333	0	0
MEAN	30.34			
SD	0			

Strain code	**Wild-type**			
dilution	1	1	1	1
dilution factor	0.005	0.005	0.005	0.005
rate (s^{-1})	0.0002	0.0003	0.0004	0.0003
Prot conc (mg/ml)	4.86	4.86	4.86	4.86
SDH (nmol/min/mg protein)	25.070215	37.605323	50.140431	37.605323
MEAN	37.61			
SD	10.234873			

Strain code	**Dog**			
dilution	1	1	1	1
dilution factor	0.005	0.005	0.005	0.005
rate (s^{-1})	0.0002	0.0002	0.0002	
Prot conc (mg/ml)	4.90	4.90	4.90	4.90
SDH (nmol/min/mg protein)	24.884096	24.884096	24.884096	0
MEAN	24.88			

Strain code	**Dog**			
dilution	1	1	1	1
dilution factor	0.005	0.005	0.005	0.005
rate (s^{-1})	0.0001	0.0001	0.0001	0.0001
Prot conc (mg/ml)	1.63	1.63	1.63	1.63
SDH (nmol/min/mg protein)	37.371781	37.371781	37.371781	37.371781
MEAN	37.37			

APPENDIX 4

Cytochrome c reductase assay

code	dilution	dilution factor	rate	Prot conc (mg/ml)	CYTC Reductase (nmol/min/mg protein)
dog 5b 270412	5	0.009091	0.0022	12.03	57.20296
dog 10a 270412	10	0.004545	0.001	12.03	52.00269
DLMA	10	0.004545	0.0009	12.03	46.80243
DLMB	10	0.004545	0.001	12.03	52.00269
dog 5a 260412	5	0.009091	0.0016	12.03	41.60216
dog 5b.260412	5	0.009091	0.0014	12.03	36.40189
dog 10a2 260412	10	0.004545	0.0006	12.03	31.20162
dog 10b2. 260412	10	0.004545	0.0005	12.03	26.00135

code	dilution	dilution factor	rate (s-1)	Prot conc (mg/ml)	CYTC Reductase (nmol/min/mg protein)
hrn mouse 10a	10	0.004545	0.00007	13.49	3.246217
hrn mouse 10b	10	0.004545	0.0001	13.49	4.637453
hrn mouse 20 a	20	0.002273	0.0001	13.49	9.274906
hrn mouse 20b	20	0.002273	0.00009	13.49	8.347415
hrn_a1	10	0.004545	0.0002	13.49	9.274906
hrn_a2	10	0.004545	0.0002	13.49	9.274906
hrn_a3	10	0.004545	0.0002	13.49	9.274906

wt_1a	10	0.004545	0.0013	9.71	83.75594
wt_1b	10	0.004545	0.0014	9.71	90.1987
wt_1c	10	0.004545	0.0014	9.71	90.1987
MLMA	10	0.004545	0.0015	9.71	96.64147
MLMB	10	0.004545	0.0016	9.71	103.0842
MLMC	10	0.004545	0.0012	9.71	77.31317
mouse 10a.26.04	10	0.004545	0.0014	9.71	90.1987
mouse 10b	10	0.004545	0.0015	9.71	96.64147

APPENDIX 5

GENERIC INCUBATION PLAN FOR CVB ASSAY

	Biological sample	HRN	WT	Dog	HRN	WT	Dog	WT	Dog	HRN	WT	Dog	HRN	WT	Dog	final conc
								Incubation Plate								
	Description	Test Cpd		+ NADPH	Test Cpd		- NADPH	test compound+NADPH+ABT		Test Compound+UDPGA			Cmpd to be back-added at 1hr (250µl volume)			
Pre-Incubate	Microsomes	37.1	51.5	41.6	37.1	51.5	41.6	51.5	41.6	37.1	51.5	41.6	18.6	25.8	20.8	1mg/ml
	NADPH (20mM stock)	50.0	50.0	50.0	x	x	x	50	50	x	x	x	25.0	25.0	25.0	2mM
	UDPGA (50mM stock)	x	x	x	x	x	x	x	x	40.0	40.0	40.0	x	x	x	4mM
	ABT (50 mM stock)	x	x	x	x	x	x	10	10	x	x	x	x	x	x	1mM
	Buffer	x	x	x	50.0	50.0	50.0	x	x	x	x	x	x	x	x	
Add to start preincubation	Buffer (pre-warmed)	410.1	395.7	405.6	410.1	395.7	405.6	385.7	395.6	420.1	405.7	415.6	206.4	199.3	204.2	
Add at time 0	1.8 mM stock solution (µL)	2.8	2.8	2.8	2.8	2.8	2.8	2.8	2.8	2.8	2.8	2.8	x	x	x	10µM
TOTAL VOLUME		**500**	**500**	**500**	**500**	**500**	**500**	**500**	**500**	**500**	**500**	**500**	**250**	**250**	**250**	
Add at T=60	1.8 mM stock solution (µL)	x	x	x	x	x	x	x	x	x	x	x	1.4	1.4	1.4	10µM

TOTAL VOLUME REQUIRED (µl)

HRN mcsm	WT mcsm	Dog mcsm	NADPH	Buffer	ABT	UDPGA	Test cmpd
259.7	463.5	374.4	650	10410.7	40	240.0	70
2 aliquots	2 aliquots	2 aliquots					

microsome	prt conc mg/ml
Dog	12.03
HRN	13.49
WT	9.71

for 50mM ABT stock, weigh 1mg of ABT and dissolve in 150µl of buffer

for 50mM UDPGA stock, weigh 6.46mg of UDPGA and dissolve in 200µl of buffer

for 20mM NADPH stock, prepare a 16.67mg/ml solution

KEYS

HRN=HRN mouse microsome

WT= wild-type mouse microsome

buffer= 0.1 M Potassium phosphate buffer+ 10mM MgCl2

APPENDIX 6

GENERIC PLATE LAYOUT FOR CVB

Figure 1

Plate layout for microsomal incubation

Plate 1

Cpd Inc | Back-add

	1	2	3	4	5	6	7	8	9	10	11	12	vol of isotope (µl)	
A	HRN+N (a)	HRN+N (b)	WT+N+ABT(a)	WT+N+ABT(b)	HRN +N (a)	HRN +N (b)							11.2	0
B	WT +N (a)	WT +N (b)	Dog +N + ABT + (a)	Dog +N + ABT + (b)	WT + N (a)	WT + N (b)							11.2	0
C	Dog+N (a)	Dog+N (b)	HRN + UDPGA (a)	HRN + UDPGA (b)	Dog + N (a)	Dog + N (b)							11.2	0
D	HRN -N (a)	HRN -N (b)	WT + UDPGA (a)	WT + UDPGA (b)									11.2	
E	WT -N (a)	WT -N (a)	Dog + UDPGA (a)	Dog + UDPGA (b)									11.2	
F	Dog-N (a)	Dog-N (b)											5.6	
G														
H														

Plate layout for acetone quench

Plate 2

0 minutes | 60 minutes | Back-add (60 minutes)

	1	2	3	4	5	6	7	8	9	10	11	12	vol of isotope (µl)
A	HRN+N (a)	HRN+N (b)	WT+N+ABT(a)	WT+N+ABT(b)	HRN+N (a)	HRN+N (b)	WT+N+ABT(a)	WT+N+ABT(b)	HRN +N (a)	HRN +N (b)			1
B	WT +N (a)	WT +N (b)	Dog +N + ABT + (a)	Dog +N + ABT + (b)	WT +N (a)	WT +N (b)	Dog +N + ABT + (a)	Dog +N + ABT + (b)	WT + N (a)	WT + N (b)			1
C	Dog+N (a)	Dog+N (b)	HRN + UDPGA (a)	HRN + UDPGA (b)	Dog+N (a)	Dog+N (b)	HRN + UDPGA (a)	HRN + UDPGA (b)	Dog + N (a)	Dog + N (b)			1
D	HRN -N (a)	HRN -N (b)	WT + UDPGA (a)	WT + UDPGA (b)	HRN -N (a)	HRN -N (b)	WT + UDPGA (a)	WT + UDPGA (b)					
E	WT -N (a)	WT -N (a)	Dog + UDPGA (a)	Dog + UDPGA (b)	WT -N (a)	WT -N (a)	Dog + UDPGA (a)	Dog + UDPGA (b)					
F	Dog-N (a)	Dog-N (b)			Dog-N (a)	Dog-N (b)							
G													
H													

Plate layout for acetonitrile + formic acid (metabolite identification)

60 minutes

	1	2	3	4	5	6	7	8	9	10	11	12
A	HRN+N (a)	HRN+N (b)	WT+N+ABT(a)	WT+N+ABT(b)								
B	WT +N (a)	WT +N (b)	Dog +N + ABT + (a)	Dog +N + ABT + (b)								
	Dog+N (a)	Dog+N (b)	HRN + UDPGA (a)	HRN + UDPGA (b)								
C			WT + UDPGA (a)	WT + UDPGA (b)								
D			Dog + UDPGA (a)	Dog + UDPGA (b)								
E												
F												
G												
H												

APPENDIX 7

Generic Radioactivity count for ^{14}C-diclofenac

Cycle 1 Results

S#	Count Time	CPMA	DPM1	SIS	tSIE	MESSAGES
1	10.00	9	10	68.16	865.96	
2	10.00	34	36	66.28	786.81	
3	10.00	34	37	70.93	782.78	
4	10.00	111	118	68.58	779.13	
5	10.00	87	92	65.98	793.74	
6	10.00	18	19	74.00	783.58	
7	10.00	22	24	64.85	790.06	
8	10.00	29	31	62.45	789.76	
9	10.00	38	41	64.40	782.57	
10	10.00	648	689	70.31	794.63	
11	10.00	644	685	68.78	785.82	
12	10.00	245	261	69.68	783.30	
13	10.00	140	148	68.24	801.54	
14	10.00	19	20	75.01	783.90	
15	10.00	205	218	70.69	778.51	
16	10.00	40	43	65.16	778.94	
17	10.00	65	69	64.34	780.48	
18	10.00	93	99	69.21	777.49	
19	10.00	78	83	66.61	782.99	
20	10.00	85	90	64.41	779.42	
21	10.00	99	105	68.20	791.35	
22	10.00	242	258	68.26	773.74	
23	10.00	149	159	68.36	780.45	
24	10.00	50	53	73.84	791.42	
25	10.00	70	75	71.35	773.11	
26	10.00	105	112	64.79	778.93	
27	10.00	103	109	69.47	774.45	
28	10.00	80	85	67.59	783.05	
29	10.00	53	57	66.31	772.49	
30	10.00	56	60	67.93	775.61	
31	10.00	72	77	66.10	779.24	
32	10.00	255	272	71.45	780.77	
33	10.00	185	197	71.67	786.25	
34	10.00	10	10	85.44	778.87	
35	10.00	9	10	65.92	788.67	
36	10.00	12	12	71.51	793.53	
37	10.00	9	10	80.19	779.61	

APPENDIX 8

Generic Radioactivity count for 14C-Feclozic acid

S#	Count Time	CPMA	DPM1	SIS	tSIE	MESSAGES
1	10.00	7	7	41.02	880.88	
2	10.00	9	9	66.66	801.86	
3	10.00	10	10	61.46	785.76	
4	10.00	7	7	63.21	794.09	
5	10.00	9	9	72.63	775.86	
6	10.00	65	69	70.06	786.90	
7	10.00	66	71	68.61	778.74	
8	10.00	98	104	73.32	769.26	
9	10.00	84	90	66.59	779.66	
10	10.00	84	89	70.33	786.12	
11	10.00	88	94	72.10	786.21	
12	10.00	189	201	70.87	791.79	
13	10.00	309	329	71.98	783.59	
14	10.00	80	85	70.15	769.79	
15	10.00	77	82	71.23	773.74	
16	10.00	80	85	71.25	770.56	
17	10.00	150	160	74.75	772.11	
18	10.00	166	177	71.30	767.84	
19	10.00	185	197	70.96	779.18	
20	10.00	84	89	70.26	773.71	
21	10.00	75	79	74.06	777.35	
22	10.00	147	156	73.21	770.30	
23	10.00	100	106	67.77	774.75	
24	10.00	233	249	71.32	770.57	
25	10.00	170	180	61.84	838.82	
26	10.00	1807	1925	72.27	767.87	
27	10.00	2141	2281	72.64	772.17	
28	10.00	96	102	72.22	788.14	
29	10.00	110	117	70.43	766.05	
30	10.00	485	516	72.04	773.55	
31	10.00	417	444	73.00	774.01	
32	10.00	68	72	71.38	766.29	
33	10.00	58	61	72.69	768.94	
34	10.00	82	87	73.15	779.72	
35	10.00	93	99	74.48	777.35	
36	10.00	307	327	74.76	773.46	
37	10.00	393	419	74.14	765.07	
38	10.00	124	132	71.68	767.66	
39	10.00	136	145	71.44	765.04	
40	10.00	145	155	74.84	756.31	
41	10.00	126	135	74.78	768.01	
42	10.00	155	165	73.22	770.35	
43	10.00	131	140	73.35	764.47	
44	10.00	431	460	72.58	764.04	
45	10.00	510	543	72.98	758.02	
46	10.00	92	98	71.29	771.36	
47	10.00	104	110	70.46	761.22	
48	10.00	338	360	73.57	764.90	
49	10.00	321	342	74.99	770.78	
50	10.00	78	83	72.50	777.82	
51	10.00	89	95	70.37	762.92	
52	10.00	120	128	71.63	767.02	
53	10.00	206	219	73.30	759.74	
54	10.00	162	173	72.62	760.42	
55	10.00	149	157	54.32	885.36	

APPENDIX 9

GENERIC COVALENT BINDING CALCULATIONS

group	radioactivity(DPM)	corrected radioactivity	mole equivalent in sample	Pmole equivalent	protein concentration n)	mg protein in 0.95ml	CVB(pmole equiv/mg prote
BL1	7						
BL2 (background)	11						
BL3 (background)	13						
BL4 (background)	11						
BL5 (background)	8						
Average	**10.75**						
HRN +NADPH, T=0	52	41.25	3.38E-13	3.38E-01	81	0.08	4.41
	35	24.25	1.99E-13	1.99E-01	111	0.11	1.88
HRN +NADPH, T=60	98	87.25	7.15E-13	7.15E-01	105	0.10	7.17
	82	71.25	5.84E-13	5.84E-01	104	0.10	5.91
HRN -NADPH, T=0	60	49.25	4.03E-13	4.03E-01	143	0.14	2.96
	83	72.25	5.92E-13	5.92E-01	112	0.11	5.54
HRN -NADPH, T=60	110	99.25	8.13E-13	8.13E-01	130	0.12	6.57
	94	83.25	6.82E-13	6.82E-01	102	0.10	7.02
HRN + UDGPA, T=0	77	66.25	5.43E-13	5.43E-01	173	0.16	3.30
	80	69.25	5.67E-13	5.67E-01	189	0.18	3.15
HRN + UDGPA, T=60	122	111.25	9.11E-13	9.11E-01	132	0.13	7.28
	170	159.25	1.30E-12	1.30E+00	212	0.20	6.49
HRN BA	35	24.25	1.99E-13	1.99E-01	109	0.10	1.92
	51	40.25	3.30E-13	3.30E-01	158	0.15	2.20
WT + NADPH, T=0	48	37.25	3.05E-13	3.05E-01	119	0.11	2.70
	37	26.25	2.15E-13	2.15E-01	117	0.11	1.94
WT + NADPH, T=60	456	445.25	3.65E-12	3.65E+00	132	0.13	29.12
	434	423.25	3.47E-12	3.47E+00	125	0.12	29.18
WT + ABT, T=0	39	28.25	2.31E-13	2.31E-01	129	0.12	1.88
	27	16.25	1.33E-13	1.33E-01	152	0.14	0.92
WT + ABT, T=60	114	103.25	8.46E-13	0.8456175	122	0.12	7.32
	135	124.25	1.02E-12	1.0176075	141	0.13	7.60
WT -NADPH, T=0	88	77.25	6.33E-13	0.6326775	125	0.12	5.35
	74	63.25	5.18E-13	0.5180175	110	0.10	4.94
WT -NADPH, T=60	118	107.25	8.78E-13	0.8783775	89	0.08	10.38
	71	60.25	4.93E-13	0.4934475	38	0.04	13.81
WT + UDPGA, T=0	66	55.25	4.52E-13	0.4524975	136	0.13	3.49
	51	40.25	3.30E-13	0.3296475	146	0.14	2.38
WT + UDPGA, T=60	125	114.25	9.36E-13	0.9357075	85	0.08	11.60
	194	183.25	1.50E-12	1.5008175	133	0.13	11.89
WT BA	79	68.25	5.59E-13	0.5589675	113	0.11	5.20
	21	10.25	8.39E-14	0.0839475	73	0.07	1.21
DOG + NADPH, T=0	77	66.25	5.43E-13	0.5425875	84	0.08	6.83
	58	47.25	3.87E-13	0.3869775	96	0.09	4.26
DOG + NADPH, T=60	479	468.25	3.83E-12	3.8349675	66	0.06	61.25
	648	637.25	5.22E-12	5.2190775	96	0.09	57.41
DOG + ABT, T=0	31	20.25	1.66E-13	0.1658475	89	0.08	1.96
	34	23	1.88E-13	0.18837	112	0.11	1.76
DOG + ABT, T=60	191	180	1.47E-12	1.4742	76	0.07	20.31
	399	388	3.18E-12	3.17772	110	0.10	30.48
DOG -NADPH, T=0	95	84	6.88E-13	0.68796	72	0.07	9.99
	52	41	3.36E-13	0.33579	63	0.06	5.64
DOG -NADPH, T=60	87	76	6.22E-13	0.62244	64	0.06	10.22
	69	58	4.75E-13	0.47502	53	0.05	9.44
DOG + UDPGA, T=0	45	34	2.78E-13	0.27846	64	0.06	4.59
	40	29	2.38E-13	0.23751	60	0.06	4.16
DOG + UDPGA, T=60	233	222	1.82E-12	1.81818	52	0.05	36.79
	288	277	2.27E-12	2.26863	64	0.06	37.54
DOG BA	194	183	1.50E-12	1.49877	178	0.17	8.85
	71	60	4.91E-13	0.4914	139	0.13	3.71
BLANK	7						
Background	13						
	12						
	9						
	10	**11**					

molar radioactivity of (C-14) diclofenac mole /dpm	**8.19E-15**

APPENDIX 10

FENCLOZIC ACID COVALENT BINDING

Fenclozic acid Covalent binding assay data

	HRN -NADPH, T=0	HRN -NADPH, T=60	HRN +NADPH, T=0	HRN +NADPH, T=60	HRN + ABT, T=0	HRN + ABT, T=60	HRN + UDGPA, T=0	HRN + UDGPA, T=60	HRN BA
	4.94	7.02	7.21	23.79			10.92	7.03	21.75
Exp 1	5.88	8.70	4.52	23.62			5.52	5.64	15.74
	5.78	10.07	9.93	40.77			9.55	7.69	16.55
Exp 2	5.76	10.46	8.80	33.93			7.99	10.38	21.23
	7.34	11.72	12.73	36.40	10.430	26.222	7.23	11.56	12.02
Exp 3	6.03	10.91	10.91	34.36	10.456	32.089	7.67	12.63	10.59
mean	5.96	9.81	9.02	32.15	10.44	29.16	8.15	9.15	16.31
SD	0.78	1.70	2.89	6.97	0.02	4.15	1.88	2.77	4.59

	WT -NADPH, T=0	WT -NADPH, T=60	WT + NADPH, T=0	WT + NADPH, T=60	WT + ABT, T=0	WT + ABT, T=60	WT + UDPGA, T=0	WT + UDPGA, T=60	WT BA
	4.01	10.87	13.34	134.60	8.31	31.93	7.75	10.36	35.58
Exp 1	6.70	7.53	23.93	172.17	7.72	22.77	10.38	15.00	32.63
	8.22	12.36	25.59	204.03	12.56	46.97	6.16	9.18	35.39
Exp 2	7.97	7.70	16.64	208.91	10.57	44.47	4.64	9.74	46.40
	8.631	10.273	18.282	189.879	9.438	47.887	6.715	8.739	22.000
Exp 3	10.870	9.272	18.535	216.787	9.327	43.198	6.986	9.434	26.167
mean	7.73	9.67	19.39	187.73	9.65	39.54	7.10	10.41	33.03
SD	2.28	1.88	4.59	30.42	1.73	10.02	1.91	2.31	8.48

	DOG -NADPH, T=0	DOG -NADPH, T=60	DOG + NADPH, T=0	DOG + NADPH, T=60	DOG + ABT, T=0	DOG + ABT, T=60	DOG + UDPGA, T=0	DOG + UDPGA, T=60	DOG BA
	7.89	9.23	14.95	83.17	9.37	36.54	7.79	12.24	19.22
Exp 1	0.00	0.00	15.34	56.12	4.51	33.00	6.75	8.49	16.36
	16.12	21.17	23.63	87.92	13.94	44.81	9.24	18.07	12.91
Exp 2	8.924	10.750	17.149	72.149	12.723	26.135	8.535	13.027	10.551
	8.726	11.391	18.402	38.467	19.031	21.593	7.878	10.325	12.881
Exp 3	14.86	16.51	21.38	64.71	9.40	44.87	10.79	28.07	14.50
mean	9.42	11.51	18.47	67.09	11.50	34.49	8.50	15.04	14.40
SD	5.77	7.16	3.43	18.24	4.94	9.56	1.39	7.16	3.05

APPENDIX 11

COVALENT BING ASSAY IN [^{14}C]-DICLOFENAC

		HRN -NADPH, T=0	HRN -NADPH, T=60	HRN +NADPH, T=0	HRN +NADPH, T=60	HRN + ABT T=0	HRN + ABT T=60	HRN + UDGPA, T=0	HRN + UDGPA, T=6	HRN BA
		3.29	9.87	2.40	9.14			6.10	21.28	0.78
EXP 1	1005	5.69	7.32	3.16	8.18			5.21	12.43	0.96
		2.96	6.57	4.41	7.17			3.30	7.28	1.92
EXP2	1405	5.54	7.02	1.88	5.91			3.15	6.49	2.20
		10.137	14.512	11.980	18.448	10.418	16.731	11.636	22.571	13.983
EXP4	608	11.738	16.057	9.983	17.132	9.569	16.290	12.486	25.750	13.268
	means	6.56	10.22	5.64	11.00	9.99	16.51	6.98	15.97	5.52
	SD	3.61	4.11	4.27	5.39	0.60	0.31	4.10	8.31	6.31
	geomean	5.76	9.59	4.43	10.00	9.98	16.51	6.03	13.91	2.89
	SEM	1.47	1.68	1.74	2.20	0.42	0.22	1.67	3.39	2.58

		WT -NADPH, T=0	WT -NADPH, T=60	WT + NADPH, T=0	WT + NADPH, T=60	WT + ABT, T=0	WT + ABT, T=6	WT + UDPGA, T=0	WT + UDPGA, T=60	WT BA
		2.81	7.91	1.35	48.37	5.41	20.49	3.86	17.71	0.80
EXP2	1005	5.56	6.30	2.46	50.61	3.17	8.29	4.75	11.71	17.01
		5.35	10.38	2.70	29.12	1.88	7.32	3.49	11.60	5.20
EXP3	1405	4.94	13.81	1.94	29.18	0.92	7.60	2.38	11.89	1.21
		10.993	17.998	7.891	32.058	7.934	13.051	11.874	25.151	12.201
EXP5	608	13.728	16.678	6.938	32.526	5.365	16.919	11.374	26.087	15.145
	means	7.23	12.18	3.88	36.98	4.11	12.28	6.29	17.36	8.59
	SD	4.18	4.75	2.79	9.82	2.60	5.51	4.20	6.81	7.12
	geomean	6.29	11.36	3.14	35.98	3.29	11.31	5.24	16.30	5.01
	SEM	1.71	1.94	1.14	4.01	1.06	2.25	1.72	2.78	2.91

		DOG -NADPH, T=0	DOG -NADPH, T=60	DOG + NADPH, T=	DOG + NADPH, T=6	DOG + ABT, T=0	DOG + ABT, T=	DOG + UDPGA, T=0	DOG + UDPGA, T=6	DOG BA
		9.99	10.22	6.83	61.25	1.96	20.31	4.59	36.79	8.85
EXP2	1405	5.64	9.44	4.26	57.41	1.76	30.48	4.16	37.54	3.71
		8.59	12.70	5.35	85.92	3.91	46.49	10.09	46.52	11.27
EXP3	2505	5.59	10.70	7.38	73.10			10.21	56.26	7.18
		11.876	19.250	11.679	60.929	10.354	19.062	8.491	53.632	6.773
EXP4	608	7.653	19.890	14.689	48.026	8.334	13.370	6.557	61.505	6.793
	means	8.22	13.70	8.36	64.44	5.26	25.94	7.35	48.71	7.43
	SD	2.47	4.68	4.01	13.25	3.88	13.04	2.66	10.16	2.51
	geomean	7.91	13.09	7.63	63.35	4.11	23.61	6.92	47.80	7.05
	SEM	1.01	1.91	1.64	5.41	1.74	5.83	1.09	4.15	1.02

APPENDIX 12

BODY WEIGHT OF WILD-TYPE AND HRN MICE

C57BL6J Control

	day -2	day-1	day 1	day 2	day 3	day 4	day 5	day 6	day 7	day 8
1	23.6	23.8	23.1	22.9	23.7	23.1	23.4	23.6	23.7	24.4
2	22.7	23.5	23.1	22.1	22.2	21.8	22.4	22.9	22.5	22.8
3	22	22.4	22.4	22.2	22.7	22	21.9	21.8	22.1	22.2
4	25.1	25.5	24.6	23.8	24.4	24.5	24.3	24.5	25.1	25.9
5	21.9	21.7	20.9	21.2	21.6	21.7	21.6	22.2	22.6	23.1
mean	23.06	23.38	22.82	22.44	22.92	22.62	22.72	23	23.2	23.68
SD	1.3278	1.4550	1.3405	0.9711	1.1300	1.1904	1.1167	1.0840	1.2166	1.4789

C57BL6J Diclofenac 10 mg/kg

	day-2	day-1	day 1	day 2	day 3	day 4	day 5	day 6	day 7	day 8
6	20.8	21.3	21.3	21.3	21.5	21.5	21.7	21.5	21.9	22.5
7	23.8	24	23.8	24	23.9	24.1	24.8	25.1	24.9	25.4
8	24.7	24.7	24.3	24	24.3	24.8	24.6	24.8	24.9	25.4
9	24.1	24.7	25	24.6	25	25.1	24.8	25.2	25	25.6
10	21.6	21.8	21.9	21.4	21.6	22	21.7	21.8	21.8	22
mean	23	23.3	23.26	23.06	23.26	23.5	23.52	23.68	23.7	24.18
SD	1.6985	1.6325	1.5884	1.5805	1.6103	1.6477	1.6634	1.8620	1.6897	1.7726

C57BL6J Diclofenac 20mg/kg

	day-2	day-1	day 1	day 2	day 3	day 4	day 5	day 6	day 7	day 8
11	23.7	24.2	23.6	23.4	23.8	23.7	23.8	23.7	24.1	24.2
12	21.6	21.8	21.2	20.8	21.4	21.5	21.5	21.2	21.4	22
13	24.1	24.6	24.1	23.9	24.3	24.2	24.6	24.7	24.8	24.4
14	23.7	24	23.7	24	24.3	24.4	24.4	24.3	23.7	24.6
15	23.2	23.5	23.2	22.8	23.1	23.1	23	22.8	23.1	23.2
mean	23.26	23.62	23.16	22.98	23.38	23.38	23.46	23.34	23.42	23.68
SD	0.9813	1.0918	1.1415	1.3084	1.2112	1.1649	1.2602	1.3939	1.2872	1.0826

C57BL6J diclofenac 30mg/kg

	Day-2	Day-1	day 1	day 2	day 3	day 4	day 5	day 6	day 7	day 8
16	22.7	23.2	22.7	22.9	23	22.8	23.1	23.4	23.5	23.6
17	21.5	21.6	21.7	21.4	21.3	21.2	21.7	21.8	20.6	20
18	23.5	23.7	23.2	23.1	23.1	23.1	23.5	24	23.4	23.1
19	24.6	24.7	25	24.7	25.4	25.1	25.5	25.7	25.5	26.2
20	25.1	25	24.6	24.5	24.2	24.1	24.2	24.8	24.1	24.5
mean	23.48	23.64	23.44	23.32	23.4	23.26	23.6	23.94	23.42	23.48
SD	1.4498	1.3539	1.3612	1.3424	1.5248	1.4639	1.4000	1.4758	1.7852	2.2753

C57BL6J Fenclozic acid 50mg/kg

	day-2	day-1	day 1	day 2	day 3	day 4	day 5	day 6	day 7	day 8
21	21.8	22	22.2	21.9	22.2	21.7	22.2	22.3	22.7	23.3
22	23.5	24	23.7	23.2	23.4	23.3	23.5	23.6	23.5	24
23	23.9	24.3	24.9	24	24.6	23.8	24.1	24.5	24.4	24.8
24	23.8	23.7	23.9	22.9	23.8	23	23.4	23.8	23.7	23.9
25	23.5	23.6	23.3	22.9	23.2	23.4	23.7	23.4	23.3	23.8
mean	23.3	23.52	23.6	22.98	23.44	23.04	23.38	23.52	23.52	23.96
SD	0.8573	0.8927	0.9798	0.7530	0.8764	0.8019	0.7120	0.7981	0.6181	0.5413

C57BL6J Fenclozic acid 100mg/kg

	day-2	day-1	day 1	day 2	day 3	day 4	day 5	day 6	day 7	day 8
26	23.1	23.3	23	22.4	22.5	22.3	23.1	23.5	23.2	23.4
27	22.6	22.9	22.6	21.7	22	22	21.9	22.6	21.9	22.3
28	20.8	21.2	21.5	20.9	21.2	21	21.6	22.4	22.5	21.7
29	24.9	24.9	25.4	24	24.5	24.7	24.6	24.4	24.2	24.7
30	22.8	23.2	23.4	22.7	23.1	22.8	23.1	23.7	23.4	23.7
mean	22.84	23.1	23.18	22.34	22.66	22.56	22.86	23.32	23.04	23.16
SD	1.4605	1.3172	1.4290	1.1589	1.2422	1.3649	1.1887	0.8228	0.8792	1.1824

HRN control

	Day-2	Day-1	day 1	day 2	day 3	day 4	day 5	day 6	day 7	day 8
31	23.7	23.8	23.6	23.4	23.8	23.5	23.2	24.1	24.3	23.9
32	20.6	20.7	20.6	20.2	20.5	20.3	20	20.2	20.9	21.1
33	19.7	19.7	20	19.3	19.9	20.1	20.1	20.5	20.3	19.9
34	26.7	26.8	26.2	25.5	25.8	25.2	25.7	25.6	26	25.9
mean	22.675	22.75	22.6	22.1	22.5	22.275	22.25	22.6	22.875	22.7
SD	3.1837	3.2151	2.8705	2.8694	2.7893	2.4958	2.7380	2.6721	2.7281	2.7129

HRN diclofenac 30mg/kg

	day-2	day-1	day 1	day 2	day 3	day 4	day 5	day 6	day 7	day 8
35	19.2	19.3	18.6	18.5	18.8	18.8	18.9	18.9	19	19.1
36	25.6	25.7	25.8	24.9	26.4	25	25.7	25.1	25.2	25.2
37	21.1	21.1	21.1	20.9	21.1	20	20	19.4	19	19
38	25.9	25.6	25.4	25.6	25.7	25.2	24.5	24.2	24.8	25
39	24.4	24.6	24.7	23.8	24.9	23.8	25.6	26.4	25.9	25.2
mean	23.24	23.26	23.12	22.74	23.38	22.56	22.94	22.8	22.78	22.7
SD	2.9535	2.8971	3.1380	2.9720	3.2783	2.9645	3.2439	3.4271	3.4730	3.3332

HRN fenclozic acid 100mg/kg

	Day-2	Day-1	day 1	day 2	day 3	day 4	day 5	day 6	day 7	day 8
40	22.5	22.8	23.2	22.5	22.2	22.2	22.4	22.7	23	22.8
41	22.7	22.3	21.8	20.8	20.7	20.4	20.9	20.9	20.8	20.6
42	19.7	19.7	19.4	18.7	19.1	18.8	18.9	19.3	19.3	19.4
43	23.8	23.8	24.4	22.9	22.5	22.3	22.9	22.9	23.9	23.8
44	26.9	26.9	27.1	26.1	26.2	25.9	26.3	26.9	26.4	26.8
mean	23.12	23.1	23.18	22.2	22.14	21.92	22.28	22.54	22.68	22.68
SD	2.5985	2.6086	2.8744	2.7386	2.6425	2.6508	2.7335	2.8439	2.7563	2.8865

APPENDIX 13
FOOD INTAKE IN WILD-TYPE AND HRN MICE

food consumption

C57BL6J Control

	day -2	day -1	day 1	day 2	day 3	day 4	day 5	day 6	day 7	day 8
M 1	5	3.8	3.7	6.2	1.9	3.6	3.6	4.3	4.3	4.4
M 2	5	4.6	4.3	3.8	3.3	3.3	3.5	4.8	3.8	3.8
M 3	4	4.5	4.8	4	4.6	3.7	4.3	3.2	4.2	3.7
M 4	4	5.3	3.7	2.9	3.3	4.3	4.3	3.5	4.8	4.7
M 5	4	4.2	3.1	3.8	3.3	4.3	5.3	1.9	4.1	4.4
Mean	**4.4**	**4.48**	**3.92**	**4.14**	**3.28**	**3.84**	**4.2**	**3.54**	**4.24**	**4.2**
SD	0.5477	0.5541	0.6496	1.2280	0.9550	0.4450	0.7211	1.1149	0.3647	0.4301

C57BL6J Diclofenac 10mg/kg

	day -1	day 1	day 2	day 3	day 4	day 5	day 6	day 7	day 8	
M 6	4	4.5	4	4.1	3.5	3.9	3.8	3.4	3.7	5.9
M 7	5	4.8	4.5	4	3.7	4.3	4.5	4.4	4.1	4.3
M 8	4	5	4.1	3.7	4	4.1	4.2	3.9	4.3	4.2
M 9	4	4.7	5.1	4	4.3	4.4	4.7	3.7	4.5	4.8
M 10	3	4.7	4.5	3.5	3.8	4	4.8	2.6	4	3.8
Mean	**4**	**4.74**	**4.44**	**3.86**	**3.86**	**4.14**	**4.4**	**3.6**	**4.12**	**4.6**
SD	0.7071	0.1817	0.4336	0.2510	0.3050	0.2074	0.4062	0.6671	0.3033	0.8093

food consumption

C57BL6J Diclofenac 20mg/kg

	day -2	day -1	day 1	day 2	day 3	day 4	day 5	day 6	day 7	day 8
M 11	5.3	4.5	4.3	4.3	4.4	4.7	4.1	4.8	4.5	4.7
M 12	4.7	4.3	3.5	3.4	4.3	4.5	3.7	3.5	3.7	4.9
M 13	4.5	4.4	4	4	4.5	4.9	4.1	4.1	4.2	4.1
M 14	5	4.8	4.4	4.7	5.5	5.6	4.4	5.2	4.2	5.8
M 15	3.9	4.7	3.8	4	5	5.8	3.8	3.1	4	3.9
Mean	**4.68**	**4.54**	**4**	**4.08**	**4.74**	**5.1**	**4.02**	**4.14**	**4.12**	**4.68**
SD	0.5310	0.2074	0.3674	0.4764	0.5030	0.5701	0.2775	0.8735	0.2950	0.7497

C57BL6J Diclofenac 30 mg/kg

	day -2	day -1	day 1	day 2	day 3	day 4	day 5	day 6	day 7	day 8
M 16	4.9	4.3	4.6	4.4	4.5	4.3	4.4	4.2	4.9	4.7
M 17	4.4	4.6	4.3	4.6	4	3.7	3.1	3.1	3.1	3.4
M 18	4.6	4.7	4.2	4.6	4.1	3.8	3.9	4.5	4	4.2
M 19	5.9	7	6.1	4.9	5.8	4.9	5.4	5.2	4.7	5
M 20	5.6	4.2	4.8	4.4	4.1	4.4	3.9	4.1	4.4	4.5
Mean	**5.08**	**4.96**	**4.8**	**4.58**	**4.5**	**4.22**	**4.14**	**4.22**	**4.22**	**4.36**
SD	0.6458	1.1589	0.7649	0.2049	0.7517	0.4868	0.8444	0.7596	0.7120	0.6107

food consumption

C57BL6J Fenclozic acid 50mg/kg

	day -2	day -1	day 1	day 2	day 3	day 4	day 5	day 6	day 7	day 8
M 21	4	4.3	4.1	3.8	3.6	3.5	3.7	4.1	4	4.5
M 22	4	3.6	4.6	3.6	3.2	3.5	3.5	4.2	3.8	4.1
M 23	4	5	5.5	4.4	4	3.8	3.6	4.7	4.8	4.4
M 24	4	4.8	4.6	3.4	3.9	3.8	3.3	4.8	4.1	3.8
M 25	4	4.5	4.3	4.2	3.2	3.8	3.6	4.5	4.2	3.9
Mean	**4**	**4.44**	**4.62**	**3.88**	**3.58**	**3.68**	**3.54**	**4.46**	**4.18**	**4.14**
SD	0.0000	0.5413	0.5357	0.4147	0.3768	0.1643	0.1517	0.3050	0.3768	0.3050

C57BL6J Fenclozic acid 100mg/kg

	day -2	day -1	day 1	day 2	day 3	day 4	day 5	day 6	day 7	day 8
M 26	5.1	5.3	4.2	4	4	4	4.8	4.4	5	4.6
M 27	5.4	4.8	4.1	3.9	4.8	4.6	4	4.5	3.9	4.4
M 28	5.2	4.7	4.2	4.7	4.3	4.6	4.5	4.3	4.8	4.6
M 29	5.9	5.1	5.3	4	4.6	5.4	5.3	4.5	5.2	5.7
M 30	5.1	5	4.1	4.5	4.6	5.4	4.8	5.2	4.2	5.3
Mean	**5.34**	**4.98**	**4.38**	**4.22**	**4.46**	**4.8**	**4.68**	**4.58**	**4.62**	**4.92**
SD	0.3362	0.2387	0.5167	0.3564	0.3130	0.6000	0.4764	0.3564	0.5495	0.5541

HRN Control

	day -2	day -1	day 1	day 2	day 3	day 4	day 5	day 6	day 7	day 8
M 31	5.3	5	4.7	4.8	4.4	4.6	4.2	5.7	4.9	5.3
M 32	4.5	4.7	4.7	3.7	4.4	4.1	3.8	4.7	4.8	5.2
M 33	4.5	4.5	4.1	4.3	4.9	4.6	4.7	4.9	4.2	5.1
M 34	5.6	5.3	4.8	5	4.6	4.6	4.6	5.2	5.2	4.9
Mean	**4.975**	**4.875**	**4.575**	**4.45**	**4.575**	**4.475**	**4.325**	**5.125**	**4.775**	**5.125**
SD	0.5620	0.3500	0.3202	0.5802	0.2363	0.2500	0.4113	0.4349	0.4193	0.1708

HRN Diclofenac 30 mg/kg

	day -2	day -1	day 1	day 2	day 3	day 4	day 5	day 6	day 7	day 8
M 35	4.8	4.8	4	4.2	4.4	4.4	4	5.1	4.6	4.4
M 36	5.9	6.1	5.8	5.4	5.7	4.9	4.7	5	5.6	4.8
M 37	4.1	4.1	4.1	4.3	3.8	2.2	3.4	8.1	4.7	5.7
M 38	5.5	5.1	5.1	5.3	4.6	3.9	1.5	4.1	5	6.4
M 39	5.4	5.3	5.4	5.1	5.3	3.4	4.3	3.6	5.2	6.5
Mean	**5.14**	**5.08**	**4.88**	**4.86**	**4.76**	**3.76**	**3.58**	**5.18**	**5.02**	**5.56**
SD	0.7021	0.7294	0.7981	0.5683	0.7503	1.0359	1.2558	1.7484	0.4025	0.9397

HRN Fenclozic acid 100 mg/kg

	day -2	day -1	day 1	day 2	day 3	day 4	day 5	day 6	day 7	day 8
M 40	5.1	5.5	5.5	4.9	4.5	5	5.3	5.3	4.9	4.7
M 41	7.2	5.6	5.3	4.4	4.8	4.8	4.8	5.2	4.4	4.7
M 42	4.8	5	4.8	4	4.5	4.4	3.8	5.1	4.5	4.2
M 43	5.6	5	5.2	3.8	3.6	4.1	4.7	5.3	5.6	5.4
M 44	4.8	4.5	5.4	4.7	4.6	5.3	4.8	6	5.2	6
Mean	**5.5**	**5.12**	**5.24**	**4.36**	**4.4**	**4.72**	**4.68**	**5.38**	**4.92**	**5**
SD	1.0050	0.4438	0.2702	0.4615	0.4637	0.4764	0.5450	0.3564	0.4970	0.7036

APPENDIX 14
LIVER-TO-BODY WEIGHT RATIO IN
WILD-TYPE AND HRN MICE

C57BL6J control

SN	BW	LW	LW:BW
1	24	1.05	0.044
2	22.1	1.26	0.057
3	21.8	1.21	0.056
4	25	1.63	0.065
5	22.8	1.33	0.058
mean	23.14	1.29	0.06
SD	1.341	0.213	0.008

C57BL6J diclofenac 10mg/kg

SN	BW	LW	LW:BW
6	22	1.55	0.070
7	24.7	1.34	0.054
8	24.6	1.47	0.060
9	25.4	1.55	0.061
10	21.8	1.31	0.060
mean	23.70	1.44	0.06
SD	1.673	0.112	0.006

C57BL6J diclofenac 20mg/kg

SN	BW	LW	LW:BW
11	23.5	1.58	0.067
12	21.5	1.45	0.067
13	23.9	1.61	0.067
14	23.8	1.58	0.066
15	22.2	1.36	0.061
mean	22.98	1.52	0.07
SD	1.071	0.109	0.003

C57BL6J diclofenac 30mg/kg

SN	BW	LW	LW:BW
16	23	1.52	0.066
17	19.7	1.03	0.052
18	22.9	1.47	0.064
19	25.7	1.74	0.068
20	24.1	1.51	0.063
mean	23.08	1.45	0.06
SD	2.200	0.256	0.006

C57BL6J Fenclozic acid 50mg/kg

SN	BW	LW	LW:BW
21	22.8	1.37	0.060
22	23.5	1.34	0.057
23	24.5	1.47	0.060
24	23.2	1.31	0.056
25	23.4	1.41	0.060
mean	23.48	1.38	0.06
SD	0.630	0.062	0.002

C57BL6J Fenclozic acid 100mg/kg

SN	BW	LW	LW:BW
26	23.1	1.48	0.064
27	21.7	1.39	0.064
28	21.5	1.50	0.070
29	24	1.77	0.074
30	23	0.00	0.000
mean	22.66	1.23	0.05
SD	1.045	0.701	0.031

HRN control

SN	BW	LW	BW:LW
31	23.9	2.55	0.107
32	20.7	2.32	0.112
33	20.3	2.06	0.101
34	25.3	2.84	0.112
mean	22.55	2.44	0.11
SD	2.441	0.333	0.005

HRN diclofenac 30mg/kg

SN	BW	LW	LW:BW
35	19.2	2.25	0.117
36	25.2	2.49	0.099
37	20	2.25	0.113
38	25.6	2.46	0.096
39	24.6	2.79	0.113
mean	22.92	2.45	0.11
SD	3.065	0.222	0.010

HRN Fenclozic acid 100mg/kg

SN	BW	LW	LW:BW
40	22.6	2.59	0.115
41	20.6	2.34	0.114
42	19.1	2.03	0.106
43	23.9	2.90	0.121
44	26.3	2.84	0.108
mean	22.50	2.54	0.11
SD	2.810	0.362	0.006

APPENDIX 15

TIME-DEPENDENT CHANGE IN ALT IN WILD-TYPE AND HRN MICE

WILD-TYPE

Day of treatment	**CONTROL**				
	Day -1	Day 2	Day 4	Day 8	Day 8 auto
M 1	20.6	13.3	24.1	31.6	59
M 2	25.1	24.5	22.2	21.6	32
M 3	22.2	21.4	25.5	18.4	39
M 4	19.7	20.4	26.7	18.2	29
M 5	19.3	8.1	16.2	15.0	29
mean	21.4	17.5	22.9	20.9	37.6
SD	2.4	6.7	4.1	6.4	12.6
Geomean	21.27	16.31	22.58	14.64	36.17

	DICLOFENAC (10MG/KG)				
	Day -1	Day 2	Day 4	Day 8	Day 8 auto
M 6	21.92	16.45	14.07	15.73	32
M 7	38.03	27.03	15.38	11.70	22
M 8	23.36	21.16	24.20	25.71	27
M 9	18.38	9.60	12.54	15.47	25
M 10	28.89	36.39	28.99	30.48	40
mean	26.12	22.13	19.03	19.82	29.20
SD	7.66	10.22	7.18	7.90	7.05
geomean	25.29	20.11	18.03	18.61	28.56

Day of treatment	**DICLOFENAC 20 mg/kg**				
	Day -1	Day 2	Day 4	Day 8	**Day 8 Auto**
M 11	23.87	15.03	22.10	20.33	28
M 12	21.92	21.92	21.92	34.20	42
M 13	22.10	18.56	18.56	13.26	24
M 14	27.18	16.66	13.15	18.41	20
M 15	27.18	21.92	27.18	34.20	36
mean	24.5	18.8	20.6	24.1	30.0
SD	2.6	3.1	5.2	9.6	8.9
Geomean	24.34	18.61	20.02	22.53	28.95

	DICLOFENAC 30 mg/kg				
	Day -1	Day 2	Day 4	Day 8	**Auto**
M 16	38.51	31.95	31.95	30.32	34
M 17	33.59	30.32	33.59	27.04	28
M 18	33.59	27.04	27.04	41.79	46
M 19	22.12	20.48	20.48	25.40	26
M 20	33.59	20.48	22.12	38.51	33
mean	32.3	26.1	27.0	32.6	33.4
SD	6.1	5.4	5.8	7.2	7.8
Geomean	31.76	25.60	26.53	31.99	32.73

	FENCLOZIC ACID (50MG/KG)				
	Day -1	Day 2	Day 4	Day 8	Day 8 auto
M 21	14.12	18.68	16.37	20.72	41
M 22	15.71	21.05	20.33	20.26	40
M 23	14.05	15.64	19.28	20.72	42
M 24	19.40	21.20	21.63	15.58	31
M 25	20.49	21.92	22.64	33.61	51
mean	16.75	19.70	20.05	22.18	41.00
SD	3.01	2.58	2.42	6.75	7.11
geomean	16.54	19.55	19.93	21.46	40.50

	FENCLOZIC ACID 100 mg/kg				
	Day -1	Day 2	Day 4	Day 8	**Day 8 Auto**
M 26	23.68	27.18	32.44	51.74	54
M 27	25.43	28.94	32.44	39.46	46
M 28	16.66	21.92	30.69	92.07	100
M 29	20.33	27.40	39.78	69.84	88
M 30	34.20	39.46	35.95	35.95	44
mean	24.06	28.98	34.26	57.81	66.40
SD	6.59	6.43	3.63	23.29	25.82
geomean	23.37	28.45	34.11	54.30	62.61

HRN MICE

	CONTROL				
	Day -1	Day 2	Day 4	Day 8	**Day 8 Auto**
M 31	1817.1	2461.8	2754.9	1172.3	868
M 32	646.0	1409.5	1761.8	3934.8	2900
M 33	830.2	889.5	1186.0	2312.7	1862
M 34	237.2	1067.4	1363.9	1719.7	1426
mean	882.6	1457.1	1766.7	2284.9	1764.0
SD	670.5	703.8	701.4	1194.5	859.7
Geomean	693.39	1347.25	1673.92	2069.58	1607.88

	DICLOFENAC 30 mg/kg				
	Day -1	Day 2	Day 4	Day 8	**Day 8 Auto**
M 35	357.7822	715.5644	1355.005	639.4406	910
M 36	395.8442	190.3097	1119.021	943.9361	242
M 37	662.2777	266.4336	677.5025	1324.555	284
M 38	553.4925	204.7164	856.7761	1046.328	178
M 39	606.5672	894.6866	614.1493	166.806	188
mean	515.2	454.3	924.5	824.2	360.4
SD	132.7	327.7	310.3	441.7	310.2
Geomean	500.77	366.86	884.23	769.6	353.2

	fenclozic acid 100mg/Kg				
Day of treatment	Day -1	Day 2	Day 4	Day 8	**Day 8 Auto**
M 40	487.19281	1004.835	1370.23	274.046	276
M 41	213.14685	578.5415	578.5415	1004.835	1562
M 42	578.54146	548.0919	304.4955	1644.276	2790
M 43	424.59701	606.5672	727.8806	272.9552	382
M 44	1243.4627	1182.806	1213.134	515.5821	648
mean	589.4	784.2	838.9	742.3	1131.6
SD	389.6	290.3	443.9	586.0	1056.3
Geomean	501.49	744.41	734.07	576.58	784.81

APPENDIX 16

BIOCHEMICAL PARAMETERS IN WILD-TYPE AND HRN MICE

C57BL6J Control

GROUP 1	Animal	UREA (mmol/L)	CRE (µmol/L)	GLU (mmol/L)	CHOL (mmol/L)	TG (mmol/L)	Ca (mmol/L)	TP (g/L)	ALB (g/L)	GLOB (g/L)	TBIL	ALP (IU/L)	AST (IU/L)	ALT (IU/L)	GLDH (IU/L)	BA(µmol/L)	HAEM
	M 1	0	0	0	0	0	0	0	0	0	4	164	94	59	26	151	1
	M 2	0	0	0	0	0	0	0	0	0	1	169	45	32	19	0	1
	M 3	0	0	0	0	0	0	0	0	0	2	124	52	39	0	0	1
	M 4	0	0	0	0	0	0	0	0	0	1	139	43	29	19	0	-
	M 5	0	0	0	0	0	0	0	32	0	1	216	46	29	12	3	-
	Mean	0	0	0	0	0	0	0	6.4	0	1.8	162.4	56	37.6	15.2	30.8	
	SD	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	14.3108	0.0000	1.3038	35.1468	21.5058	12.6412	9.8336	67.2064	
	geomean	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	1.19	158.38	46.38	32.01	#NUM!	#NUM!	
	SEM	0.00										15.72	9.62	5.65	4.40	30.06	

C57BL6J Diclofenac 10 mg/kg

GROUP 2	Animal	UREA (mmol/L)	CRE (µmol/L)	GLU (mmol/L)	CHOL (mmol/L)	TG (mmol/L)	Ca (mmol/L)	TP (g/L)	ALB (g/L)	GLOB (g/L)	TBIL	ALP (IU/L)	AST (IU/L)	ALT (IU/L)	GLDH (IU/L)	BA(µmol/L)	HAEM
	M 6	7.7	5	17.8	2.8	2.25	2.73	48	31	17	1	167	42	32	13	2	-
	M 7	0	0	16.8			2.5		22		1	80	47	22	9	66	-
	M 8	0	0	0								115	40	27			-
	M 9	0	0	0								144	40	25			-
	M 10	0	0	0									54	40			-
	Mean	1.54	1	6.92	2.8	2.25	2.615	48	26.5	17	1	126.5	44.6	29.2	11	34	
	SD	3.4435	2.2361	9.4822	#DIV/0!	#DIV/0!	0.1626	#DIV/0!	6.3640	#DIV/0!	0.0000	37.5988	5.9833	7.0498	2.8284	45.2548	
	geomean	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	2.50	#NUM!	22.00	#NUM!	1.00	109.83	44.89	27.76	9.00	66.00	
	SEM											18.80	2.68	3.15	2.00	32.00	

C57BL6J Diclofenac 20 mg/kg

GROUP 3	animal number	UREA (mmol/L)	CRE (µmol/L)	GLU (mmol/L)	CHOL (mmol/L)	TG (mmol/L)	Ca (mmol/L)	TP (g/L)	ALB (g/L)	GLOB (g/L)	ALP (IU/L)	AST (IU/L)	ALT (IU/L)	GLDH (IU/L)	BA(µmol/L)
	M 11	6.4	6	23.4	2.68	1.92	2.52	48.2	32	16.2	132	42	28	14	4
	M 12	8.4	6	19	2.52	1.72	2.56	48.2	32	16.2	132	52	42	30	2
	M 13	7	6	21.6	2.96	1.46	2.54	45.6	30	15.6	80	30	24	8	
	M 14	7.6	8	22.2	2.74	1.94	2.66	47.4	30	17.4	86	32	20	12	
	M 15	7	8	25.4	2.7	1.58	2.52	49	34	15	112	44	36	16	4
	Mean	7.28	6.8	22.32	2.72	1.724	2.56	47.68	31.6	16.08	108.4	40	30	16	3.33333333
	SD	0.7563	1.0954	2.3563	0.1581	0.2095	0.0583	1.2931	1.6733	0.8899	24.6739	9.0554	8.9443	8.3666	1.1547
	geomean	7.25	6.73	22.22	2.72	1.71	2.56	47.67	31.56	16.06	106.07	39.17	28.95	14.52	3.17
	SEM	0.34	0.49	1.05	0.07	0.09	0.03	0.58	0.75	0.40	11.03	4.05	4.00	3.74	0.67

C57BL6J Diclofenac 30mg/kg

GROUP 4	Animal	UREA (mmol/L)	CRE (µmol/L)	GLU (mmol/L)	CHOL (mmol/L)	TG (mmol/L)	Ca (mmol/L)	TP (g/L)	ALB (g/L)	GLOB (g/L)	TBIL	ALP (IU/L)	AST (IU/L)	ALT (IU/L)	GLDH (IU/L)	BA(µmol/L)
	M 16	6.8	6	22.4	2.9	2.06	2.62	47.6	30	17.6	0	78	44	34	12	0
	M 17	17	6	13	2.82	1.2	2.46	44.2	28	16.2	2	48	68	28	12	2
	M 18	9.2	6	21	2.86	3.08	2.62	46	28	18	0	84	56	46	14	2
	M 19	7.8	6	16.8	2.44	2.06	2.54	43.6	28	15.6	0	80	32	26	8	0
	M 20	6.9	9	15.9	2.64	1.44	2.13	43.8	27	16.8	0	78	51	33	15	9
	Mean	9.54	6.6	17.82	2.732	1.968	2.474	45.04	28.2	16.84	0.4	73.6	50.2	33.4	12.2	2.6
	SD	4.2800	1.3416	3.8421	0.1911	0.7284	0.2034	1.7170	1.0954	0.9839	0.8944	14.5190	13.4239	7.7974	2.6833	3.7148
	geomean	9.58	6.64	16.43	2.68	1.82	2.43	44.39	27.75	16.63	#NUM!	70.82	49.93	32.42	11.93	#NUM!
	SEM	1.91	0.60	1.72	0.09	0.33	0.09	0.77	0.49	0.44	0.40	6.49	6.00	3.49	1.20	1.66

C57BL6J Fenclozic acid 50 mg/kg

GROUP 5	Animal	UREA (mmol/L)	CRE (µmol/L)	GLU (mmol/L)	CHOL (mmol/L)	TG (mmol/L)	Ca (mmol/L)	TP (g/L)	ALB (g/L)	GLOB (g/L)	TBIL	ALP (IU/L)	AST (IU/L)	ALT (IU/L)	GLDH (IU/L)	BA(µmol/L)	
	M 21	8.9	0	20.9	2.37	1.88	2.96	55.8	36	19.8	1	176	55	41	9	2	1
	M 22	11.7	10	27.3	2.18	1.69	2.92	52	34	18	1	127	44	40	12	4	-
	M 23	0	0	0	0	0	0	0	0	0	1	133	48	42	0	0	-
	M 24	0	0	0	0	0	0	0	0	0	1	112	37	31	0	0	-
	M 25	0	0	0	0	0	0	0	0	0	1	133	61	51	0	0	-
	Mean	4.12	2	9.64	0.91	0.714	1.176	21.56	14	7.56	1	136.2	49	41	4.2	1.2	
	SD	5.7277	4.4721	13.3926	1.2479	0.9800	1.6104	29.5528	19.1833	10.3715	0.0000	23.8474	9.3541	7.1063	5.8481	1.7889	
	geomean	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	1.00	125.95	46.73	40.37	#NUM!	#NUM!	

APPENDIX 16

BIOCHEMICAL PARAMETERS IN WILD-TYPE AND HRN MICE

	SEM	2.56	2.00	5.99	0.56	0.44	0.72	13.22	8.58	4.64	0.00	10.66	4.18	3.18	2.62	0.80

Fenclozic acid 100 mg/kg

GROUP 6	**animal number**	**UREA (mmol/L)**	**CRE (µmol/L)**	**GLU (mmol/L)**	**CHOL (mmol/L)**	**TG (mmol/L)**	**Ca (mmol/L)**	**TP (g/L)**	**ALB (g/L)**	**GLOB (g/L)**	**ALP (IU/L)**	**AST (IU/L)**	**ALT (IU/L)**	**GLDH (IU/L)**	**BA(µmol/L)**
	M 26	9	10	18.8	1.74	1.88	2.6	47	34	13	122	80	54	14	6
	M 27	8.4	10	18.4	1.98	1.62	2.6	48.8	34	14.8	98	54	46	14	10
	M 28	7.8	12	20.6	2.44	1.74	2.58	47.4	32	15.4	162	104	100	26	6
	M 29	9	10	20.6	2	1.8	2.5	49.8	34	15.8	116	70	88	14	4
	M 30	7.4	10	22.2	1.94	1.56	2.58	47.6	34	13.6	142	48	44	16	4
	Mean	8.32	10.4	20.12	2.02	1.72	2.572	48.12	33.6	14.52	128	71.2	66.4	16.8	6
	SD	0.7155	0.8944	1.5401	0.2565	0.1304	0.0415	1.1541	0.8944	1.1883	24.6577	22.2980	25.8225	5.2154	2.4495
	geomean	8.13	10.47	20.40	2.08	1.68	2.56	48.39	33.49	14.88	127.17	65.91	64.96	16.90	5.57
	SEM	0.32	0.40	0.69	0.11	0.06	0.02	0.52	0.40	0.53	11.03	9.97	11.55	2.33	1.10

HRN Control

GROUP 7	**Animal**	**UREA (mmol/L)**	**CRE (µmol/L)**	**GLU (mmol/L)**	**CHOL (mmol/L)**	**TG (mmol/L)**	**Ca (mmol/L)**	**TP (g/L)**	**ALB (g/L)**	**GLOB (g/L)**	**TBIL**	**ALP (IU/L)**	**AST (IU/L)**	**ALT (IU/L)**	**GLDH (IU/L)**	**BA(µmol/L)**
	M 31	9.8	8	18.6	1.76	1.16	3.06	60	36	24	6	374	452	868	218	8
	M 32	9.4	10	15.8	2.38	0.94	3.1	59.6	36	23.6	14	546	2004	2900	990	18
	M 33	10.8	10	16.6	1.4	0.6	2.9	51.2	32	19.2	20	432	1258	1862	492	12
	M 34	7.2	8	19.4	1.58	0.64	2.8	52.8	32	20.8	24	424	898	1426	386	12
	Mean	9.3	9	17.6	1.78	0.835	2.965	55.9	34	21.9	16	444	1153	1764	521.5	12.5
	SD	1.52	1.15	1.68	0.43	0.26	0.14	4.55	2.31	2.29	7.83	72.68	656.16	859.68	332.08	4.12
	geomean	9.20	8.94	17.54	1.74	0.80	2.96	55.76	33.94	21.81	14.17	439.77	1005.77	1607.88	449.95	12.00
	SEM	0.76	0.58	0.84	0.21	0.13	0.07	2.28	1.15	1.15	3.92	36.34	328.08	429.84	166.04	2.06

HRN Diclofenac 30 mg/kg

GROUP 8	**Animal**	**UREA (mmol/L)**	**CRE (µmol/L)**	**GLU (mmol/L)**	**CHOL (mmol/L)**	**TG (mmol/L)**	**Ca (mmol/L)**	**TP (g/L)**	**ALB (g/L)**	**GLOB (g/L)**	**TBIL**	**ALP (IU/L)**	**AST (IU/L)**	**ALT (IU/L)**	**GLDH (IU/L)**	**BA(µmol/L)**
	M 35	9.8	14	16.8	1.75	1.05	2.52	53.9	35	18.9	7	406	525	910	392	14
	M 36	7.8	6	26.6	0.72	0.52	2.78	47.8	28	19.8	2	144	144	242	136	2
	M 37	11.2	6	16	0.14	0.46	3.04	48.4	26	22.4	2	116	316	284	172	2
	M 38	9.6	6	15.6	0.38	0.66	2.72	47.4	26	21.4	2	90	166	178	92	2
	M 39	8.4	2	20.4	0.84	0.74	2.82	47.8	28	19.8	2	126	134	188	66	2
	Mean	9.36	6.8	19.08	0.766	0.686	2.776	49.06	28.6	20.46	3	176.4	257	360.4	171.6	4.4
	SD	1.3221	4.3818	4.6122	0.6158	0.2317	0.1873	2.7291	3.7148	1.4100	2.2361	129.8260	166.9461	310.2044	129.7413	5.3666
	geomean	9.16	4.56	19.18	0.42	0.58	2.84	47.85	26.98	20.82	2.00	117.32	178.37	218.99	109.17	2.00
	SEM	0.59	1.96	2.06	0.28	0.10	0.08	1.22	1.66	0.63	1.00	58.06	74.66	138.73	58.02	2.40

HRN Fenclozic acid 100mg/kg

GROUP 9	**Animal**	**UREA (mmol/L)**	**CRE (µmol/L)**	**GLU (mmol/L)**	**CHOL (mmol/L)**	**TG (mmol/L)**	**Ca (mmol/L)**	**TP (g/L)**	**ALB (g/L)**	**GLOB (g/L)**	**TBIL**	**ALP (IU/L)**	**AST (IU/L)**	**ALT (IU/L)**	**GLDH (IU/L)**	**BA(µmol/L)**
	M 40	13	10	20	1.04	2.2	2.88	54.6	34	20.6	2	342	190	276	88	6
	M 41	11.2	12	24.4	1.4	0.74	3.1	55.6	34	21.6	8	370	1396	1562	444	10
	M 42	7.2	12	23.2	0.86	0.88	2.74	43.8	28	15.8	2	308	2576	2790	150	8
	M 43	11.4	8	14.4	1.44	1.1	2.7	49.8	32	17.8	4	318	296	382	100	4
	M 44	10	10	28.8	0.98	1.38	2.76	55	34	21	4	290	370	648	90	6
	Mean	10.56	10.4	22.16	1.144	1.26	2.836	51.76	32.4	19.36	4	325.6	965.6	1131.6	174.4	6.8
	SD	2.1606	1.6733	5.3636	0.2605	0.5784	0.1621	5.0128	2.6077	2.4674	2.4495	31.1256	1022.6548	1056.3327	152.8097	2.2804
	geomean	9.79	10.36	22.01	1.14	1.00	2.82	50.82	31.90	18.90	4.00	320.18	792.19	1019.13	156.47	6.62
	SEM	0.97	0.75	2.40	0.12	0.26	0.07	2.24	1.17	1.10	1.10	13.92	457.35	472.41	68.34	1.02

APPENDIX 17

STAT ANALYSIS
BIOCHEMICAL PARAMETERS IN WILD-TYPE

ALKALINE PHOSPHATASE ASSAY

t-Test: Two-Sample Assuming Unequal Variance

	CONTROL	*Diclofenac 10mg/kg*
Mean	162.4	126.5
Variance	1235.3	1413.667
Observatio	5	4
Hypothesiz	0	
df	6	
t Stat	1.46503	
P(T<=t) one	0.09663	
t Critical on	1.94318	
P(T<=t) two	0.19326	
t Critical tw	2.446912	

t-Test: Two-Sample Assuming Unequal Variances

	CONTROL	*diclofenac 20mg/kg*
Mean	162.4	108.4
Variance	1235.3	608.8
Observatio	5	5
Hypothesiz	0	
df	7	
t Stat	2.811814	
P(T<=t) one	0.013039	
t Critical on	1.894579	
P(T<=t) two	0.026078	
t Critical tw	2.364624	

t-Test: Two-Sample Assuming Unequal Variances

	CONTROL	*diclofenac 30mg/kg*
Mean	162.4	73.6
Variance	1235.3	210.8
Observatio	5	5
Hypothesiz	0	
df	5	
t Stat	5.221542	
P(T<=t) one	0.001703	
t Critical on	2.015048	
P(T<=t) two	0.003406	
t Critical tw	2.570582	

t-Test: Two-Sample Assuming Unequal Variances

	CONTROL	*fenclozic acid 50mg/kg*
Mean	162.4	136.2
Variance	1235.3	568.7
Observatio	5	5
Hypothesiz	0	
df	7	
t Stat	1.37933	
P(T<=t) one	0.105124	
t Critical on	1.894579	
P(T<=t) two	0.210247	
t Critical tw	2.364624	

t-Test: Two-Sample Assuming Unequal Variances

	CONTROL	*fenclozic acid 100mg/kg*
Mean	162.4	128
Variance	1235.3	608
Observatio	5	5
Hypothesiz	0	
df	7	
t Stat	1.791618	
P(T<=t) one	0.058151	
t Critical on	1.894579	
P(T<=t) two	0.116302	
t Critical tw	2.364624	

ASPARTATE AMINOTRANSFERASE

t-Test: Two-Sample Assuming Unequal Variances

	CONTROL	*Diclofenac 10mg/kg*
Mean	56	44.6
Variance	462.5	35.8
Observatio	5	5
Hypothesiz	0	
df	5	
t Stat	1.141943	
P(T<=t) one	0.152599	
t Critical on	2.015048	
P(T<=t) two	0.305198	
t Critical tw	2.570582	

t-Test: Two-Sample Assuming Unequal Variances

	CONTROL	*diclofenac 20mg/kg*
Mean	56	40
Variance	462.5	82
Observatio	5	5
Hypothesiz	0	
df	5	
t Stat	1.533226	
P(T<=t) one	0.092899	
t Critical on	2.015048	
P(T<=t) two	0.185798	
t Critical tw	2.570582	

t-Test: Two-Sample Assuming Unequal Variances

	CONTROL	*fenac 30mg/kg*
Mean	56	50.2
Variance	462.5	180.2
Observatio	5	5
Hypothesiz	0	
df	7	
t Stat	0.511574	
P(T<=t) one	0.312343	
t Critical on	1.894579	
P(T<=t) two	0.624687	
t Critical tw	2.364624	

t-Test: Two-Sample Assuming Unequal Variance

	CONTROL	*zic acid 50mg/kg*
Mean	56	49
Variance	462.5	87.5
Observatio	5	5
Hypothesiz	0	
df	5	
t Stat	0.667424	
P(T<=t) one	0.267032	
t Critical on	2.015048	
P(T<=t) two	0.534064	
t Critical tw	2.570582	

t-Test: Two-Sample Assuming Unequal Variances

	CONTROL	*zic acid 100mg/kg*
Mean	56	71.2
Variance	462.5	497.2
Observatio	5	5
Hypothesiz	0	
df	8	
t Stat	-1.09714	
P(T<=t) one	0.152252	
t Critical on	1.859548	
P(T<=t) two	0.304504	
t Critical tw	2.306004	

ALANINE AMINOTRANSFERASE

t-Test: Two-Sample Assuming Unequal Variances

	CONTROL	*Diclofenac 10mg/kg*
Mean	37.6	29.2
Variance	159.8	49.7
Observatio	5	5
Hypothesiz	0	
df	6	
t Stat	1.297694	
P(T<=t) one	0.121023	
t Critical on	1.94318	
P(T<=t) two	0.242047	
t Critical tw	2.446912	

t-Test: Two-Sample Assuming Unequal Variances

	CONTROL	*diclofenac 20mg/kg*
Mean	37.6	30
Variance	159.8	80
Observatio	5	5
Hypothesiz	0	
df	7	
t Stat	1.097423	
P(T<=t) one	0.154383	
t Critical on	1.894579	
P(T<=t) two	0.308766	
t Critical tw	2.364624	

t-Test: Two-Sample Assuming Unequal Variances

	CONTROL	*diclofenac 30mg/kg*
Mean	37.6	33.4
Variance	159.8	60.8
Observatio	5	5
Hypothesiz	0	
df	7	
t Stat	0.632312	
P(T<=t) one	0.273636	
t Critical on	1.894579	
P(T<=t) two	0.547272	
t Critical tw	2.364624	

t-Test: Two-Sample Assuming Unequal Variance

	CONTROL	*fenclozic acid 50mg/kg*
Mean	37.6	41
Variance	159.8	50.5
Observatio	5	5
Hypothesiz	0	
df	6	
t Stat	-0.52426	
P(T<=t) one	0.309447	
t Critical on	1.94318	
P(T<=t) two	0.618895	
t Critical tw	2.446912	

t-Test: Two-Sample Assuming Unequal Variances

	CONTROL	*fenclozic acid 100mg/kg*
Mean	37.6	66.4
Variance	159.8	666.8
Observatio	5	5
Hypothesiz	0	
df	6	
t Stat	-2.23991	
P(T<=t) one	0.033178	
t Critical on	1.94318	
P(T<=t) two	0.066356	
t Critical tw	2.446912	

APPENDIX 18 DICLOFENAC

COVALENT BINDING ASSAY

t-Test: Two-Sample Assuming Equal Variances

	HRN -NADPH, T	*+NADPH, T=60*
Mean	10.22435	10.99579
Variance	20.01105	35.75078
Observatio	3	3
Pooled Var	27.88091	
Hypothesiz	0	
df	4	
t Stat	-0.17893	
P(T<=t) one	0.433344	
t Critical or	2.131847	
P(T<=t) two	0.866687	
t Critical tw	2.776445	

t-Test: Two-Sample Assuming Unequal Variances

	HRN + UDGPA,	*+ UDGPA, T=60*
Mean	6.982181	15.96503
Variance	20.8217	75.2342
Observatio	3	3
Hypothesiz	0	
df	3	
t Stat	-1.5875	
P(T<=t) one	0.1053	
t Critical or	2.353363	
P(T<=t) two	0.2106	
t Critical tw	3.182446	

t-Test: Two-Sample Assuming Equal Variances

	WT -NADPH, T=	*+ NADPH, T=60*
Mean	9.181171	39.50165
Variance	6.753729	103.4711
Observatio	3	3
Pooled Var	55.11244	
Hypothesiz	0	
df	4	
t Stat	-5.00215	
P(T<=t) one	0.00374	
t Critical or	2.131847	
P(T<=t) two	0.007479	
t Critical tw	2.776445	

t-Test: Two-Sample Assuming Equal Variances

	WT + UDPGA, =	*+ UDPGA, T=60*
Mean	3.789013	12.7035
Variance	0.5512	3.022867
Observatio	3	3
Pooled Var	1.787034	
Hypothesiz	0	
df	4	
t Stat	-8.16725	
P(T<=t) one	0.000612	
t Critical or	2.131847	
P(T<=t) two	0.001224	
t Critical tw	2.776445	

t-Test: Two-Sample Assuming Unequal Variances

	WT + NADPH, TT	*+ ABT, T=60*
Mean	39.50165	9.887867
Variance	103.4711	15.23517
Observatio	3	3
Hypothesiz	0	
df	3	
t Stat	4.707795	
P(T<=t) one	0.00907	
t Critical or	2.353363	
P(T<=t) two	0.018139	
t Critical tw	3.182446	

t-Test: Two-Sample Assuming unqual Varianc

	DOG -NADPH, T	*+ NADPH, T=60*
Mean	13.7	64.43896
Variance	26.71121	176.2395
Observatio	3	3
Pooled Var	101.4754	
Hypothesiz	0	
df	4	
t Stat	-6.16889	
P(T<=t) one	0.001753	
t Critical or	2.131847	
P(T<=t) two	0.003506	
t Critical tw	2.776445	

t-Test: Two-Sample Assuming Equal Variances

	DOG + UDPGA,	*+ UDPGA, T=60*
Mean	7.351351	48.70855
Variance	8.362312	109.4573
Observatio	3	3
Pooled Var	58.90979	
Hypothesiz	0	
df	4	
t Stat	-6.59938	
P(T<=t) one	0.001366	
t Critical or	2.131847	
P(T<=t) two	0.002732	
t Critical tw	2.776445	

t-Test: Two-Sample Assuming Unequal Variances

	DOG + ABT, T=	*+ NADPH, T=60*
Mean	29.36579	64.43896
Variance	240.8824	176.2395
Observatio	3	3
Hypothesiz	0	
df	4	
t Stat	-2.97443	
P(T<=t) one	0.020482	
t Critical or	2.131847	
P(T<=t) two	0.040964	
t Critical tw	2.776445	

APPENDIX 19

STAT ANALYSIS FOR FENCLOZIC ACID COVALENT BINDING

t-Test: Two-Sample Assuming Equal Variances

	HRN -NADPH,	*HRN +NADPH, T=60*
Mean	9.811	32.1451
Variance	3.14231	54.4088
Observat	3	3
Pooled \	28.7756	
Hypothes	0	
df	4	
t Stat	-5.0992	
P(T<=t)	0.00349	
t Criti	2.13185	
P(T<=t)	0.00699	
t Criti	2.77645	

t-Test: Two-Sample Assuming Unequal Variances

	HRN + UDGPA,	*HRN + UDGPA, T=60*
Mean	8.14701	9.15243
Variance	0.43895	8.30497
Observat	3	3
Hypothes	0	
df	2	
t Stat	-0.5889	
P(T<=t)	0.30779	
t Criti	2.91999	
P(T<=t)	0.61557	
t Criti	4.30265	

t-Test: Two-Sample Assuming Unequal Variances

	WT -NADPH, ! WT + NADPH, T=60	
Mean	9.66728	187.729
Variance	0.18253	887.133
Observat	3	3
Hypothes	0	
df	2	
t Stat	-10.354	
P(T<=t)	0.0046	
t Criti	2.91999	
P(T<=t)	0.0092	
t Criti	4.30265	

t-Test: Two-Sample Assuming Unequal Va

	WT + NADPH,	*WT + ABT, T=60*
Mean	187.729	39.5365
Variance	887.133	111.414
Observat	3	3
Hypothes	0	
df	2	
t Stat	8.12272	
P(T<=t)	0.00741	
t Criti	2.91999	
P(T<=t)	0.01482	
t Criti	4.30265	

t-Test: Two-Sample Assuming Unequal Variances

	WT + UDPGA,	*WT + UDPGA, T=60*
Mean	7.10392	10.4086
Variance	3.4093	3.89826
Observat	3	3
Hypothes	0	
df	4	
t Stat	-2.1174	
P(T<=t)	0.05082	
t Criti	2.13185	
P(T<=t)	0.10164	
t Criti	2.77645	

t-Test: Two-Sample Assuming Une

	DOG -NADPH,	*DOG + NADPH, T=60*
Mean	11.5082	67.0877
Variance	36.6418	207.242
Observat	3	3
Hypothes	0	
df	3	
t Stat	-6.1643	
P(T<=t)	0.0043	
t Criti	2.35336	
P(T<=t)	0.00859	
t Criti	3.18245	

t-Test: Two-Sample Assuming Une

	DOG + NADPH,	*DOG + ABT, T=60*
Mean	67.0877	34.492
Variance	207.242	1.3154
Observat	3	3
Hypothes	0	
df	2	
t Stat	3.90938	
P(T<=t)	0.02982	
t Criti	2.91999	
P(T<=t)	0.05964	
t Criti	4.30265	

t-Test: Two-Sample Assuming Unequal Variances

	DOG + UDPGA,	*DOG + UDPGA, T=60*
Mean	8.49722	15.0356
Variance	1.17504	19.7186
Observat	3	3
Hypothes	0	
df	2	
t Stat	-2.4776	
P(T<=t)	0.06576	
t Criti	2.91999	
P(T<=t)	0.13152	
t Criti	4.30265	

APPENDIX 20

LIVER-TO-BODY-WEIGHT RATIO
IN WILD-TYPE AND HRN MICE

WILD-TYPE

t-Test: Two-Sample Assuming Unequal Varian

	CONTROL	*DFC 10mg/kg*
Mean	0.05589	0.06113
Variance	6.1E-05	3.3E-05
Observat	5	5
Hypothes	0	
df	7	
t Stat	-1.2098	
P(T<=t)	0.13281	
t Critic	1.89458	
P(T<=t)	0.26562	
t Critic	2.36462	

t-Test: Two-Sample Assuming Unequal Varian

	CONTROL	*FC 20mg/kg*
Mean	0.05589	0.06594
Variance	6.1E-05	7.4E-06
Observat	5	5
Hypothes	0	
df	5	
t Stat	-2.718	
P(T<=t)	0.02094	
t Critic	2.01505	
P(T<=t)	0.04188	
t Critic	2.57058	

t-Test: Two-Sample Assuming Unequal Varian

	CONTROL	*FC 30mg/kg*
Mean	0.05589	0.06255
Variance	6.1E-05	3.5E-05
Observat	5	5
Hypothes	0	
df	7	
t Stat	-1.5221	
P(T<=t)	0.0859	
t Critic	1.89458	
P(T<=t)	0.1718	
t Critic	2.36462	

t-Test: Two-Sample Assuming Unequal Varian

	CONTROL	*A 50 mg/kg*
Mean	0.05589	0.05873
Variance	6.1E-05	3.3E-06
Observat	5	5
Hypothes	0	
df	4	
t Stat	-0.794	
P(T<=t)	0.23581	
t Critic	2.13185	
P(T<=t)	0.47162	
t Critic	2.77645	

t-Test: Two-Sample Assuming Unequal Varian

	CONTROL	*100 mg/kg*
Mean	0.05589	0.06797
Variance	6.1E-05	2.2E-05
Observat	5	4
Hypothes	0	
df	7	
t Stat	-2.88	
P(T<=t)	0.01183	
t Critic	1.89458	
P(T<=t)	0.02365	
t Critic	2.36462	

HRN

t-Test: Two-Sample Assuming Equal Variance

	CONTROL	*FC 30mg/kg*
Mean	0.10809	0.10765
Variance	2.7E-05	9.1E-05
Observat	4	5
Pooled V	6.4E-05	
Hypothes	0	
df	7	
t Stat	0.08141	
P(T<=t)	0.4687	
t Critic	1.89458	
P(T<=t)	0.93739	
t Critic	2.36462	

t-Test: Two-Sample Assuming Equal Variances

	CONTROL	*100 mg/kg*
Mean	0.10809	0.11273
Variance	2.7E-05	3.5E-05
Observat	4	5
Pooled V	3.2E-05	
Hypothes	0	
df	7	
t Stat	-1.2288	
P(T<=t)	0.12942	
t Critic	1.89458	
P(T<=t)	0.25884	
t Critic	2.36462	

APPENDIX 21

STAT FOR CLINICAL CHEMISTRY DATA HRN MICE

UREA

t-Test: Two-Sample Assuming Unequa

	CONTROL	DFC 30 mg/kg
Mean	9.3	9.36
Variance	2.30667	1.748
Observat	4	5
Hypothes	0	
df	6	
t Stat	-0.0623	
P(T<=t)	0.47616	
t Critic	1.94318	
P(T<=t)	0.95232	
t Critic	2.44691	

t-Test: Two-Sample Assuming Unequal Varian

	CONTROL	FA 100 mg/kg
Mean	9.3	10.56
Variance	2.30667	4.668
Observat	4	5
Hypothes	0	
df	7	
t Stat	-1.0253	
P(T<=t)	0.16967	
t Critic	1.89458	
P(T<=t)	0.33935	
t Critic	2.36462	

CREATINE

t-Test: Two-Sample Assuming Unequa

	CONTROL	DFC 30 mg/kg
Mean	9	6.8
Variance	1.33333	19.2
Observat	4	5
Hypothes	0	
df	5	
t Stat	1.07691	
P(T<=t)	0.16536	
t Critic	2.01505	
P(T<=t)	0.33071	
t Critic	2.57058	

t-Test: Two-Sample Assuming Unequal Variances

	CONTROL	FA 100 mg/kg
Mean	9	10.4
Variance	1.33333	2.8
Observat	4	5
Hypothes	0	
df	7	
t Stat	-1.4812	
P(T<=t)	0.09105	
t Critic	1.89458	
P(T<=t)	0.1821	
t Critic	2.36462	

TRIGLYCERIDES

t-Test: Two-Sample Assuming Unequa

	CONTROL	DFC 30 mg/kg
Mean	0.835	0.686
Variance	0.06997	0.05368
Observat	4	5
Hypothes	0	
df	6	
t Stat	0.88685	
P(T<=t)	0.20465	
t Critic	1.94318	
P(T<=t)	0.4093	
t Critic	2.44691	

t-Test: Two-Sample Assuming Unequal Varian

	CONTROL	FA 100 mg/kg
Mean	0.835	1.26
Variance	0.06997	0.3346
Observat	4	5
Hypothes	0	
df	6	
t Stat	-1.4628	
P(T<=t)	0.09692	
t Critic	1.94318	
P(T<=t)	0.19384	
t Critic	2.44691	

GLUCOSE

t-Test: Two-Sample Assuming Unequa

	CONTROL	DFC 30 mg/kg
Mean	17.6	19.08
Variance	2.82667	21.272
Observat	4	5
Hypothes	0	
df	5	
t Stat	-0.6645	
P(T<=t)	0.2679	
t Critic	2.01505	
P(T<=t)	0.5358	
t Critic	2.57058	

t-Test: Two-Sample Assuming Unequal Variances

	CONTROL	FA 100 mg/kg
Mean	17.6	22.16
Variance	2.82667	28.768
Observat	4	5
Hypothes	0	
df	5	
t Stat	-1.7941	
P(T<=t)	0.06638	
t Critic	2.01505	
P(T<=t)	0.13277	
t Critic	2.57058	

CHOLESTEROL

t-Test: Two-Sample Assuming Unequa

	CONTROL	DFC 30 mg/kg
Mean	1.78	0.766
Variance	0.1816	0.37918
Observat	4	5
Hypothes	0	
df	7	
t Stat	2.91221	
P(T<=t)	0.01129	
t Critic	1.89458	
P(T<=t)	0.02259	
t Critic	2.36462	

t-Test: Two-Sample Assuming Unequal Varian

	CONTROL	FA 100 mg/kg
Mean	1.78	1.144
Variance	0.1816	0.06788
Observat	4	5
Hypothes	0	
df	5	
t Stat	2.6189	
P(T<=t)	0.02358	
t Critic	2.01505	
P(T<=t)	0.04716	
t Critic	2.57058	

TOTAL PROTEIN

t-Test: Two-Sample Assuming Unequa

	CONTROL	DFC 30 mg/kg
Mean	55.9	49.06
Variance	20.7333	7.448
Observat	4	5
Hypothes	0	
df	5	
t Stat	2.64788	
P(T<=t)	0.02277	
t Critic	2.01505	
P(T<=t)	0.04554	
t Critic	2.57058	

t-Test: Two-Sample Assuming Unequal Varian

	CONTROL	FA 100 mg/kg
Mean	55.9	51.76
Variance	20.7333	25.128
Observat	4	5
Hypothes	0	
df	7	
t Stat	1.29572	
P(T<=t)	0.11808	
t Critic	1.89458	
P(T<=t)	0.23616	
t Critic	2.36462	

GLOBULIN

t-Test: Two-Sample Assuming Unequa

	CONTROL	DFC 30 mg/kg
Mean	21.9	20.46
Variance	5.26667	1.988
Observat	4	5
Hypothes	0	
df	5	
t Stat	1.09982	
P(T<=t)	0.16076	
t Critic	2.01505	
P(T<=t)	0.32152	
t Critic	2.57058	

t-Test: Two-Sample Assuming Unequal Variances

	CONTROL	FA 100 mg/kg
Mean	21.9	19.36
Variance	5.26667	6.088
Observat	4	5
Hypothes	0	
df	7	
t Stat	1.59554	
P(T<=t)	0.07731	
t Critic	1.89458	
P(T<=t)	0.15462	
t Critic	2.36462	

TOTAL BILIRUBIN

t-Test: Two-Sample Assuming Unequa

	CONTROL	DFC 30 mg/kg
Mean	16	3
Variance	61.3333	5
Observat	4	5
Hypothes	0	
df	3	
t Stat	3.21667	
P(T<=t)	0.02435	
t Critic	2.35336	
P(T<=t)	0.04871	
t Critic	3.18245	

t-Test: Two-Sample Assuming Unequal Variances

	CONTROL	FA 100 mg/kg
Mean	16	4
Variance	61.3333	6
Observat	4	5
Hypothes	0	
df	3	
t Stat	2.95122	
P(T<=t)	0.02998	
t Critic	2.35336	
P(T<=t)	0.05997	
t Critic	3.18245	

ALANINE AMINOTRANSFERASE

t-Test: Two-Sample Assuming Unequa

	CONTROL	DFC 30 mg/kg
Mean	1764	360.4
Variance	739053	96226.8
Observat	4	5
Hypothes	0	
df	4	
t Stat	3.10756	
P(T<=t)	0.01798	
t Critic	2.13185	
P(T<=t)	0.03596	
t Critic	2.77645	

t-Test: Two-Sample Assuming Unequa

	CONTROL	FA 100 mg/kg
Mean	1764	1131.6
Variance	739053	1115839
Observat	4	5
Hypothes	0	
df	7	
t Stat	0.99014	
P(T<=t)	0.17754	
t Critic	1.89458	
P(T<=t)	0.35509	
t Critic	2.36462	

ALKALINE PHOSPHATASE

t-Test: Two-Sample Assuming Unequa

	CONTROL	DFC 30 mg/kg
Mean	444	176.4
Variance	5282.67	16854.8
Observat	4	5
Hypothes	0	
df	6	
t Stat	3.90683	
P(T<=t)	0.00396	
t Critic	1.94318	
P(T<=t)	0.00792	
t Critic	2.44691	

t-Test: Two-Sample Assuming Unequal Variances

	CONTROL	FA 100 mg/kg
Mean	444	325.6
Variance	5282.67	968.8
Observat	4	5
Hypothes	0	
df	4	
t Stat	3.04248	
P(T<=t)	0.01915	
t Critic	2.13185	
P(T<=t)	0.03831	
t Critic	2.77645	

ASPARTATE AMINOTRANSFERASE

t-Test: Two-Sample Assuming Unequa

	CONTROL	DFC 30 mg/kg
Mean	1153	257
Variance	430551	27871
Observat	4	5
Hypothes	0	
df	3	
t Stat	2.66294	
P(T<=t)	0.03807	
t Critic	2.35336	
P(T<=t)	0.07615	
t Critic	3.18245	

t-Test: Two-Sample Assuming Unequal Varian

	CONTROL	FA 100 mg/kg
Mean	1153	965.6
Variance	430551	1045823
Observat	4	5
Hypothes	0	
df	7	
t Stat	0.33295	
P(T<=t)	0.37446	
t Critic	1.89458	
P(T<=t)	0.74892	
t Critic	2.36462	

BILE ACID

t-Test: Two-Sample Assuming Unequa

	CONTROL	DFC 30 mg/kg
Mean	12.5	4.4
Variance	17	28.8
Observat	4	5
Hypothes	0	
df	7	
t Stat	2.56017	
P(T<=t)	0.01877	
t Critic	1.89458	
P(T<=t)	0.03754	
t Critic	2.36462	

t-Test: Two-Sample Assuming Unequal Varian

	CONTROL	FA 100 mg/kg
Mean	12.5	6.8
Variance	17	5.2
Observat	4	5
Hypothes	0	
df	4	
t Stat	2.47826	
P(T<=t)	0.03417	
t Critic	2.13185	
P(T<=t)	0.06834	
t Critic	2.77645	

APPENDIX 21

STAT FOR CLINICAL CHEMISTRY DATA HRN MICE

CALCIUM

ces t-Test: Two-Sample Assuming Unec t-Test: Two-Sample Assuming Unequal Variances

	CONTROL 'C	*30 mg/kg*
Mean	2.965	2.776
Variance	0.01957	0.03508
Observat	4	5
Hypothes	0	
df	7	
t Stat	1.732	
P(T<=t)	0.06344	
t Critic	1.89458	
P(T<=t)	0.12688	
t Critic	2.36462	

	CONTROL	*100 mg/kg*
Mean	2.965	2.836
Variance	0.01957	0.02628
Observat	4	5
Hypothes	0	
df	7	
t Stat	1.28058	
P(T<=t)	0.12057	
t Critic	1.89458	
P(T<=t)	0.24114	
t Critic	2.36462	

ALBUMIN

ces t-Test: Two-Sample Assuming Unec t-Test: Two-Sample Assuming Unequal Variances

	CONTROL 'C	*30 mg/kg*
Mean	34	28.6
Variance	5.33333	13.8
Observat	4	5
Hypothes	0	
df	7	
t Stat	2.66904	
P(T<=t)	0.01602	
t Critic	1.89458	
P(T<=t)	0.03204	
t Critic	2.36462	

	CONTROL	*100 mg/kg*
Mean	34	32.4
Variance	5.33333	6.8
Observat	4	5
Hypothes	0	
df	7	
t Stat	0.97493	
P(T<=t)	0.18104	
t Critic	1.89458	
P(T<=t)	0.36207	
t Critic	2.36462	

GLUTAMATE DEHYDROGENASE

ıl Variances t-Test: Two-Sample Assuming Unec t-Test: Two-Sample Assuming Unequal Variances

	CONTROL '	*30 mg/kg*
Mean	521.5	171.6
Variance	110278	16833
Observat	4	5
Hypothes	0	
df	4	
t Stat	1.98935	
P(T<=t)	0.05877	
t Critic	2.13185	
P(T<=t)	0.11754	
t Critic	2.77645	

	CONTROL	*100 mg/kg*
Mean	521.5	174.4
Variance	110278	23350.8
Observat	4	5
Hypothes	0	
df	4	
t Stat	1.93312	
P(T<=t)	0.06268	
t Critic	2.13185	
P(T<=t)	0.12536	
t Critic	2.77645	

ces