Investigation of novel urinary markers of hepatotoxicity

A thesis submitted in partial fulfilment of the requirements for the degree of Doctor of Philosophy

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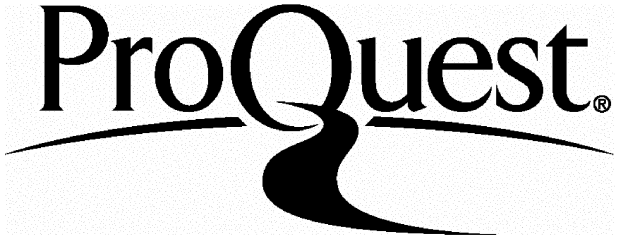

Abstract

The aim of this study was to identify novel, sensitive, and specific protein markers of hepatotoxicity in rat urine. Collection of urine is non-invasive compared to biopsy or serum analysis and therefore preferable when screening for toxicity. Carbon tetrachloride (CCl_4) was used both acutely to produce hepatotoxicity and chronically to produce a rat model of liver fibrosis.

An optimal dose of CCl_4, and the time post-dosing for maximum acute liver injury, were established by histopathological examination and by assaying serum enzyme markers of liver injury. Urine was analysed using Surface Enhanced Laser Desorption/Ionisation (SELDI) ProteinChip® technology. SELDI revealed the appearance of a protein peak at 15.7 KDa in response to CCl_4-treatment, while one-dimensional sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) identified an 18.4 KDa protein in urine from CCl_4-treated rats. This protein was identified by in-gel digestion and tandem mass spectrometry as Cu/Zn superoxide dismutase (SOD-1). SOD-1 catalyses the destruction of the superoxide anion and acts as a defence against oxidative damage. SOD-1 exists as a 32.5 KDa homodimer and it was concluded that the 15.7 KDa SELDI and 18.4 KDa SDS PAGE proteins are the SOD-1 subunit that runs at an anomalous MW on SDS PAGE.

SOD-1 in the rat urine was confirmed by Western blotting with a commercial antibody and by measuring SOD activity. Further studies revealed that SOD-1 was increased in urine from CCl_4-treated rats between 12 hours and 60 hours post-dosing, at dose levels as low as 0.4 ml/kg CCl_4. Western blots of homogenates showed that SOD-1 was being lost from rat liver presumably by necrosis. Although the enzyme SOD-1 is not specifically located in the liver, its appearance in the rat urine following hepatotoxicity is a novel finding. Since changes in SOD-1 levels were detected following low dose levels of CCl_4 and the response was measured using non-invasive methods suggests that SOD-1 is thus a potential marker of hepatotoxicity

Liver fibrosis was induced by repeat dosing with CCl_4 and confirmed by histopathological examination. Analysis of the urine samples by SDS-PAGE revealed an increase in SOD-1 in fibrotic rats but no other differences were evident. Examination of urine samples from rats with fibrosis, or acute hepatotoxicity, by two-dimensional gel electrophoresis revealed a number of proteins that were increased in these models. Two-dimensional gel electrophoresis of liver homogenates from the acute model showed a number of proteins that decreased. These results suggest that although the urinary proteins have not yet been identified it is highly probable that one or more will be a specific marker for the non-invasive identification of hepatotoxicity.

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ADH	anti-diuretic hormone
A/D	analogue to digital converter
ALP	alkaline phosphatase
ALT	alanine aminotransferase
ANIT	α-naphthylisothiocyanate
Arg	arginine
AST	asparate aminotransferase
BDL	bile duct ligation
BSA	bovine serum albumin
Ca	calcium
CAPS	3-(cyclohexylamino)-1-propanesulfonic acid
CCl_4	carbon tetrachloride
CCl3•	trichloromethyl radical
C/D	collision-induced dissociation
CHAPS	3-(3-cholamidopropyl)dimethylammonio)-1-propanesulfonate
CK	creatine kinase
CTGF	connective tissue growth factor
Cu	copper
CYP	cytochrome
Cys	cysteine
1D	one dimensional
2DGE	two dimensional gel electrophoresis
DIGE	difference gel electrophoresis
DMN	dimethylnitrosamine
DTT	dithiothreitol
EAM	energy absorbing molecule
ECL	enhanced chemiluminescence
ECM	extracellular matrix components
EDTA	ethylene-diamine tetra acetic acid

EGF	epidermal growth factor
ESI	electrospray ionisation
Fe	iron
FPLC	fast performance liquid chromatography
FT-MS	fourier transform ion cyclotron
GFAP	glial fibrillary acid protein
GLDH	glutamte dehydrogenase
HAP-E	haptoglobin
H&E	haematoxylin and eosin
HGF	hepatocyte growth factor
His	histidine
HPLC	high performance liquid chromatography
HRP	horse radish peroxidase
HSC	hepatic stellate cell
HST	high salt tween
H_2O_2	hydrogen peroxidase
ICAT	isotope-coded affinity tag
IEF	isoelectric focussing
IL-1	interleukin-1
IL-6	interleukin-6
IMAC	immobilised metal affinity capture
I.N.T	2-(4-iodophenyl)-3-(4-nitrophenyl)-5-phenyltetrazolium chloride
IPG	immobilised pH gradient
LC-MS	liquid chromatography mass spectrometry
LDH	lactate dehydrogenase
MALDI	matrix assisted laser desorption ionisation
MDA	malonaldehyde
MMP	metalloproteinase
Mn	manganese
m/z	mass to charge ratio
MS/MS	tandem mass spectrometry
NaCl	sodium chloride
NADPH	nicotinamide adenine dinucleotide phosphate
Nano-ES-MS/MS	nano-electrospray tandem mass spectrometry

Ni	nickel
NP-1	normal phase
O_2	oxygen
$O_2^{\bullet}$	superoxide free radical
$\bullet OOCCl_3$	trichloromethyl peroxy radical
OTA	ochratoxin
PA-1	plasminogen activator inhibitor
PAGE	polyacrylamide gel electrophoresis
PGDG	platelet-derived growth factor
pI	isoelectric point
PVDF	polyvinylidene difluoride
PMSF	phenylmethanesulfonyl fluoride
PSD	post source decay
SELDI	surface enhanced laser desorption ionisation
SiO_2	silicon dioxide
SD	standard deviation
α-SMA	alpha smooth muscle actin
SOD	superoxide dismutase
SDS	sodium dodecyl sulphate
SDH	sorbital dehydrogenase
SBTI	soya bean trypsin inhibitor
TAA	thioacetamide
TAP	tandem affinity purification
TBP	tributylphosphine
TCA	trichloroacetic acid
TCDD	2, 3, 7, 8-tetrachlorodibenzene-p-dioxin
TEMED	N, N, N', N'-tetramethyethylenediamine
TFA	trifluoroacetic acid
TGF	transforming growth factor
TIMP	tissue inhibitors of metalloproteinase
TOF	time of flight
TNF	tumour necrosis factor
Tris	Tris (hydroxymethyl) methylamine

VLDL	very low density lipoprotein
WBC	white blood cell
XOD	xanthine oxidase
Zn	zinc

Chapter one

Introduction

1.1 Liver: Structure and Function

1.1.1 Anatomy of the liver

The liver is one of the largest organs in the body (Evans and Lake, 1998) and as a result of its portal blood supply it is also the first major organ to be exposed to ingested toxicants (Popp and Cattley, 1991). The normal liver is dark red/brown in colour due to the large quantity of blood it contains. The liver is situated in the abdominal cavity and is in contact with the diaphragm, stomach, right kidney and other parts of the gastrointestinal tract. It is connected to the diaphragm and abdominal walls by five ligaments: the falciform, coronary, right and left triangular ligaments and the round ligament. Figure 1.1 shows a posterior view of a human liver with each of the lobes indicated: left, right, quadrate and caudate. On the anterior side of the liver, the falciform ligament separates the right and left lobes and the inferior vena cava marks the division between the right and caudate lobe on the posterior side. The quadrate lobe lies beneath the caudate lobe. The rat liver has four specific lobes; it has a bile duct but no gall bladder.

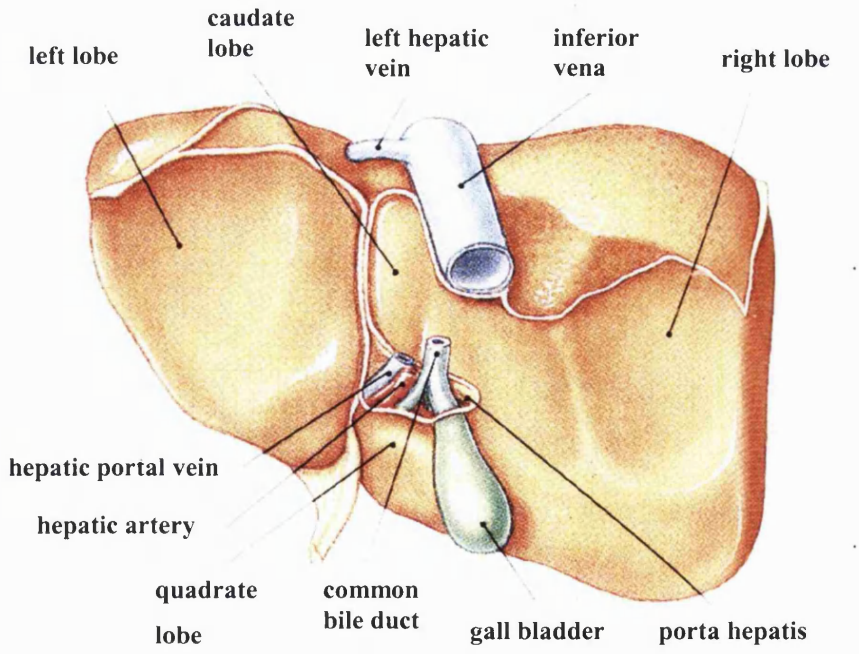


Figure 1.1 A posterior view of a human liver showing the position of each of the four different lobes: left, right, caudate and quadrate. (Martini, 2001)

1.1.2 Blood supply to the liver

The liver has a dual blood supply: the hepatic artery supplies arterial blood, and venous blood flows from the gastrointestinal tract into the hepatic portal vein and then to the liver. Blood then leaves the liver via the hepatic vein (Evans and Lake, 1998). Therefore the hepatic portal system consists of blood that contains substances absorbed by the stomach and intestines. These absorbed substances are transported to the liver for storage, metabolic conversion or excretion. Veins supplying the hepatic portal system include the inferior mesenteric vein, left colic vein and superior rectal vein, splenic vein and the superior mesenteric vein. The hepatic portal vein is formed as a fusion of the superior mesenteric and splenic veins. The hepatic artery and hepatic portal vein enter the liver at the hilus and then branch to supply the different lobes of the liver. The blood leaves the liver via the hepatic vein, which joins the inferior vena cava.

1.1.3 Histological organisation of the liver

Hepatocytes (the parenchymal cells of the liver) make up most of the liver volume. Hepatocytes are polyhedral cells with large nuclei, which are spherical or ovoid in shape. The size of the nuclei varies from cell to cell and occasionally binucleate cells are present (Leeson et al., 1985). The hepatocytes are organised into cords of single rows of cells one cell thick, which are exposed on each side to blood flowing in the sinusoidal spaces (Hinton and Grasso, 1993). Sinusoids between adjacent cords empty into the central vein. Two main concepts have been proposed for the functional organisation of hepatocytes in relation to the vascular supply of the liver: the lobular and the acinar organisation (Plaa and Charbonneau, 2001). Figure 1.2 illustrates both of these functional organisations.

In the lobular organisation, each lobe of the liver is divided into liver lobules, which are roughly hexagonal in shape. Each liver lobule has a central vein (terminal hepatic venule) and located at the corners of the lobule are portal tracts containing a branch of the portal vein, hepatic arteriole and a bile duct. Each lobule is separated by a thin layer of collagenous tissue and is composed of three regions: centrilobular, midzonal and periportal (Molsen, 1986).

Rappaport (1979) described a second concept for the functional organisation of hepatocytes; he proposed that the fundamental unit of the liver was the hepatic acinus. The acinus is described as having a polyhedral shape with a portal tract forming the central axis (Glaister, 1986). Structurally the acinus has three zones; zone one has the greatest vascular supply and zone three is closest to the terminal hepatic vein (central vein) and therefore receives the least oxygenated blood (Telford and Bridgman, 1995).

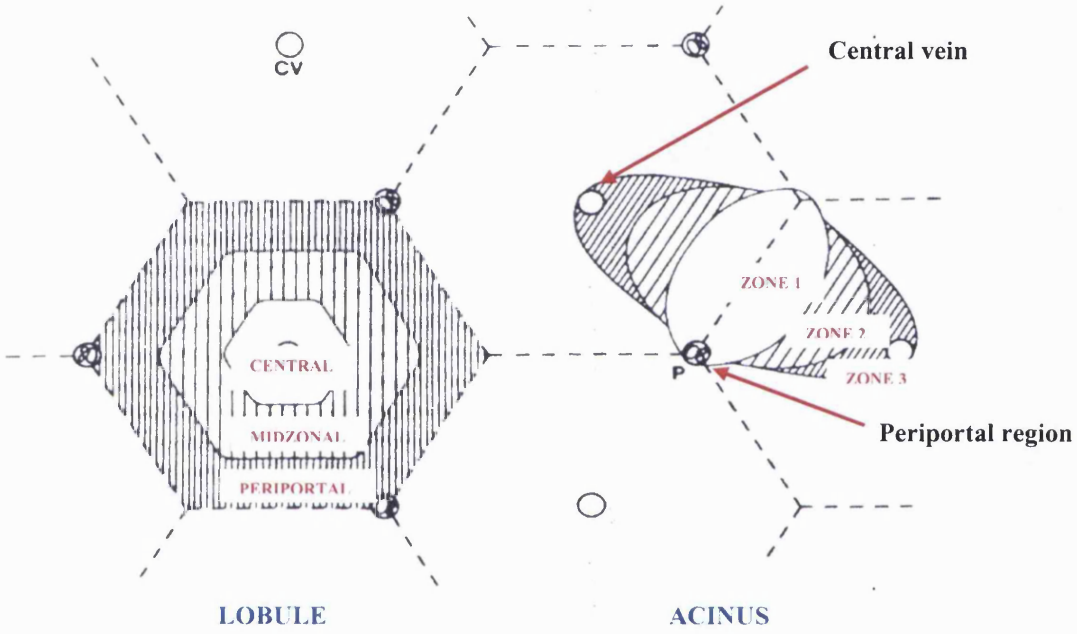


Figure 1.2 Functional organisation of the hepatocytes according to the concepts of lobular versus acinar structure (Popp and Cattley, 1991). In the lobular structure the liver consists of three lobular regions: central, midzonal and periportal. The central lobular region encloses the central vein (CV) whereas the periportal region is furthest from the central vein. The acinar structure has three zones; zone three is closest to the central vein and therefore is the least oxygenated zone.

The hepatic sinusoids are lined by a discontinuous layer of cells, which are separated from the hepatocytes by a narrow space (space of Disse) (Figure 1.3). The space of Disse drains into the lymphatics of the portal tracts. Three main types of cells can be identified within the sinusoidal space: endothelial cells, Kupffer and Ito cells. The endothelial cells form a discontinuous lining to the sinusoids with many pores between them. These pores and the absence of a true basement lining allow the exchange of

molecules between the blood flowing along the sinusoids and the hepatocytes (Hinton and Grasso, 1993). Kupffer cells are the macrophages of the liver. These cells are actively phagocytic and engulf pathogens, cell debris, and damaged blood cells (Martini, 2001). Ito cells are fat-storing cells that synthesise collagen and are situated in the space of Disse between the endothelial lining of the sinusoids and the hepatocytes (Molsen, 1986).

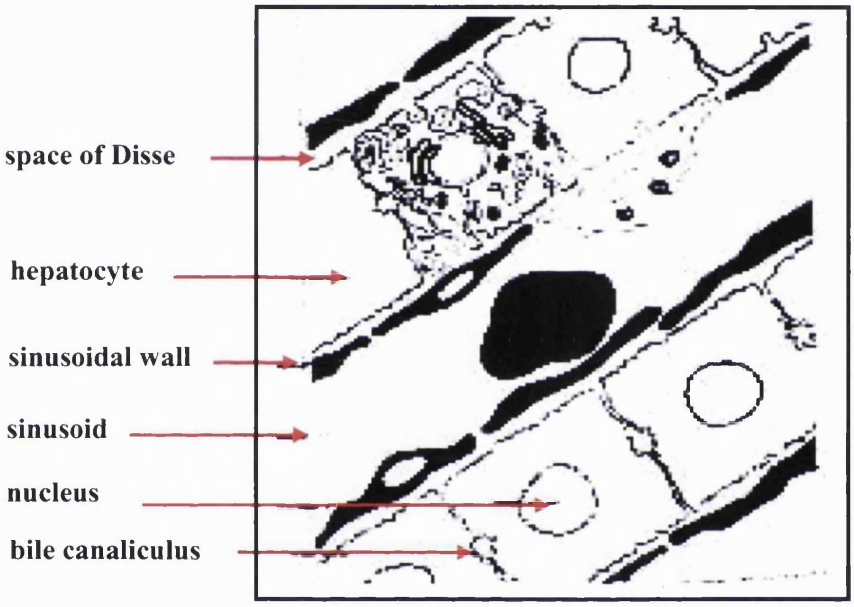


Figure 1.3 A cross-sectional view showing the distribution of a sinusoid between rows of hepatocytes (Adapted from Vandenberge, 1995). The sinusoids are lined with a sinusoidal wall composed of endothelial cells and Kupffer cells. The sinusoidal wall and the hepatocytes are separated by the space of Disse. The bile canaliculi situated between the plasma membranes of adjacent hepatocytes are small tubular canals into which bile flows.

Blood enters the sinusoids via small branches of the hepatic portal vein and hepatic artery (Figure 1.4). As the blood flows through the sinusoids towards the central vein of the lobule, hepatocytes absorb solutes from the plasma and secrete materials such as plasma proteins (Leeson et al., 1985). The central veins of the liver lobule merge to form the hepatic veins, which empty into the inferior vena cava.

Bile ducts form part of the portal tract and drain bile from the hepatocytes (Evans and Lake, 1998). A network of bile canaliculi is situated between the plasma membranes of adjacent hepatocytes into which bile drains (Figure 1.4). These canaliculi have no

structure (Molsen, 1986) but extend outwards away from the central vein. From the canaliculi the bile drains into a system of bile collecting ducts (canals of Hering) situated in the portal tracts. The collecting ducts drain into the common bile duct and then to the duodenum. Bile therefore flows in the opposite direction to the flow of blood along the sinusoids.

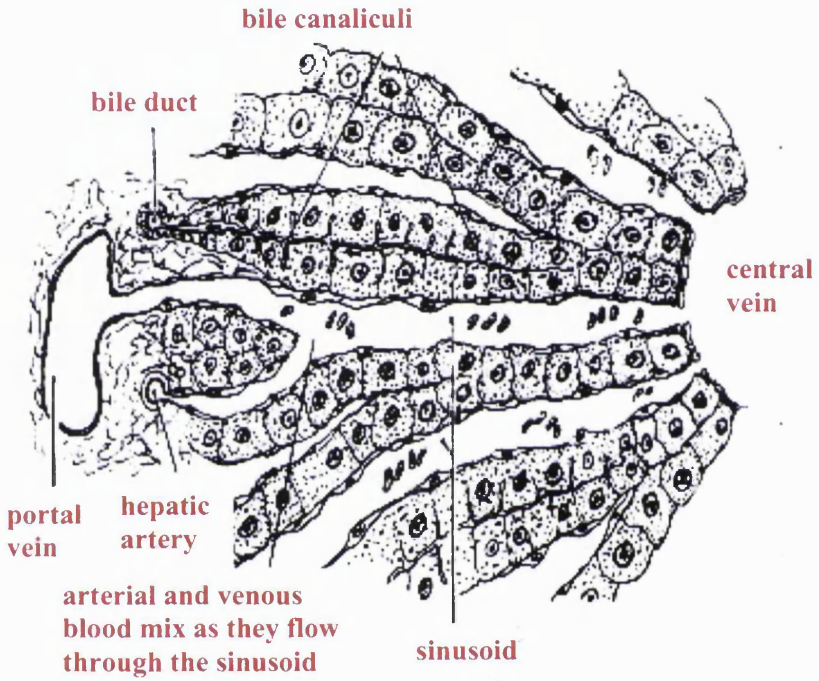


Figure 1.4 Representation of a hepatic lobule showing the sinuoids and bile canaliculi (Adapted from Ham, 1974). Blood enters sinusoids from the hepatic artery and portal vein and flows towards the central vein. Bile forms in the bile canaliculi and flows towards the bile ducts. Therefore blood and bile flow in opposite directions.

1.1.4 Physiology of the liver

The liver performs many vital functions, including metabolic regulation, synthesis of proteins and bile production (Hinton and Grasso, 1993). The liver regulates the metabolism of carbohydrates. When the glucose level in the blood is high, glycogen is deposited in the liver. Glycogen can then be broken down to release glucose into the blood when the blood glucose levels are low (glycogenolysis). The liver can also synthesise glucose from e.g. amino acids, this process is known as gluconeogenesis.

The liver also regulates circulating levels of triglycerides, fatty acids and cholesterol, and removes excess amino acids from blood. Hepatocytes synthesize and secrete very low-density lipoproteins (VLDL), which are metabolised to low-density lipoproteins (LDL). These lipoproteins are major sources of triglycerides and cholesterol, respectively. Therefore the liver is a major source of synthesis of cholesterol in the body. The liver stores fat-soluble vitamins and vitamin B_{12} and also has reserves of iron.

The liver is the major site for the synthesis of most plasma proteins, including albumin, blood clotting factors, acute phase proteins and proteins involved in lipid transport (Telford and Bridgman, 1995). Bile is synthesised and secreted by the liver. Bile consists mainly of water, bilirubin, cholesterol and bile salts; these constituents are all synthesised and secreted by hepatocytes into the bile canaliculi. Bile acids and salts emulsify lipids, thereby increasing the surface area for lipolytic enzymes prior to absorption and aiding absorption by the intestinal epithelium.

The liver transforms and excretes many hormones, drugs and toxins (Leeson et al., 1985). When proteins and amino acids are broken down the liver strips off the amino groups, a process known as deamination. This process produces ammonia, which is toxic and can not be further metabolised by most tissues. The liver converts ammonia to urea, a less harmful compound, which can be excreted by the kidneys. Hepatocytes contain systems of enzymes and cofactors, which are able to convert many substances to inactive forms. Other enzymes present in the endoplasmic reticulum of the hepatocytes can catalyse the conjugation of many compounds with glucuronic acid, glycine or glutathione, rendering them more water-soluble for excretion by the kidney. The conversion of lipophilic, water-insoluble materials to water-soluble conjugates usually involves the generation of an electrophilic species, this is described as phase I metabolism. The major phase I reactions that occur in the liver are oxidation, reduction and hydrolysis. Most of the phase I oxidation reactions are catalysed by the cytochrome P-450 monooxygenase system. Phase II metabolism involves the conjugation of phase I metabolites with a functional group such as a glucuronic acid or glutathione (Hinton and Grasso, 1993). The most important phase II reactions are glucuronidation, sulphation, acetylation, methylation and amino acid and glutathione conjugation (Evans and Lake, 1998).

1.2 Toxic responses of the liver

The liver is a common target organ for the toxic effects of many drugs and chemicals. As a result of the presence of the portal blood supply, the liver is exposed to higher levels of ingested toxicant than any other organ outside the gastrointestinal tract (Lu, 1996). The liver also has a high concentration of xenobiotic metabolising enzymes (e.g. cytochrome P450) and these enzymes may generate a high level of toxic intermediates during the metabolism of toxic compounds (Hinton and Grasso, 1993).

The centrilobular region is the most common site of hepatocellular injury. This is because the area has a relatively low oxygen concentration as a result of its proximity to the central vein, and there is also a high concentration of metabolising cytochrome P-450 enzymes present in the centrilobular hepatocyte (Evans and Lake, 1998). The mid-zonal region is the least susceptible to hepatic injury. Hepatic injury may be confined to a single hepatocyte, or extend to involve adjacent hepatocytes or even involve the whole of the liver lobule from the centrilobular to the periportal region.

1.2.1 Hydropic changes

Hydropic degeneration is a common reversible pathological response of the hepatocyte to injury. Hepatocytes depend on an ATPase-dependent sodium-potassium membrane pump to maintain an extracellular sodium concentration that is higher than within the cells, and similarly to also maintain an intracellular level of potassium, which is higher in the cells than in the extracellular fluid. The pump can be inhibited specifically or non-specifically, for example decreased ATP production causes a decrease in the pumping of sodium out of the cell and potassium into the cell. This results in an overall increase in the number of solutes inside the cell and therefore causes excess water to enter the cell, leading to cellular swelling, or hydropic degeneration (Brown, 1998). Hydropic degeneration can be identified histologically as swollen cells with a pale cytoplasm. Severe swelling of the cell leads to the formation of single large vacuoles in the cytoplasm, which can burst resulting in death of the cell (Glaister, 1986).

1.2.2 Fatty liver (steatosis)

By definition a fatty liver is one that contains more than 5% lipid by weight (Lu, 1996), and occurs commonly after an acute exposure but is often reversible. Fatty liver can result from an excess delivery of free fatty acids to the liver, an increased production of lipids by the liver or a decrease in the production of very low-density lipoproteins (VLDL) (Plaa and Charbonneau, 2001). Excess delivery of free fatty acids is due to an increased level in the blood as a result of an increased mobilization of adipose tissue, which can be caused by starvation or diabetes. Excessive alcohol consumption can result in an increase in the production of triglycerides. A decrease in the formation and secretion of VLDL can be a result of reduced levels of apoproteins, which are necessary for the production of VLDL. The decrease in apoprotein may be a result of protein deficiency or may be caused by hepatotoxic agents such as CCl_4 (Brown, 1998).

Histologically, fatty change appears intracellularly in the hepatocytes as empty vacuoles of varying sizes. This is because the intracellular fat in the vacuoles dissolves in the solvents used to process the paraffin sections. The excess fat can be determined histologically by the use of special techniques and by using fat-soluble dyes such as oil red O or Sudan black (Glaister, 1986).

In some cases the cytoplasm of the hepatocyte may contain small fat droplets inside lysosomes (microvesicular steatosis). However, the accumulation of lipid may be described as macrovesicular when a few large droplets cause the nucleus to become abnormally displaced to the side of the cell. (Vandenberghe, 1995) Macrovesicular steatosis may cause the hepatocyte to swell, which may consequently disrupt the blood flow through the sinusoids. Steatosis can be induced by a number of hepatotoxic chemicals such as phosphorus, tetracycline and ethanol. Microvesicular steatosis tends to occur as a result of an acute toxic response, whereas a more chronic injury will induce macrovesicular steatosis (Brown, 1998).

1.2.3 Cholestasis

The term cholestasis refers to an impairment of bile flow, either as a decrease in the volume produced or as impaired secretion (Vandenberghe, 1995; Ramm et al., 2000).

Cholestasis may result from hepatocellular dysfunction or from biliary obstruction. Two types exist: canalicular and cholangiolitic cholestasis. In canalicular cholestasis, bile is deposited within bile ductules forming bile plugs. Whereas in cholangiolitic cholestasis bile plugs are formed in the bile ducts but there is also evidence of other histopathological changes in the form of inflammatory cell infiltrates of the portal tracts and swollen hepatocytes (Glaister, 1986). Cholestasis tends to occur initially in the centrilobular regions but may extend to other areas. The bile pigment accumulates within the hepatic parenchyma causing dilation of the bile canaliculi, leading to degeneration of hepatocytes.

Prolonged biliary obstruction can lead to a number of alterations in liver function and morphology, including hepatocellular dysfunction, proliferation of biliary epithelial cells, portal tract fibrosis and cirrhosis (Ramm et al., 2000). Biochemical changes indicating cholestasis are elevated levels of bile acids, bilirubin and alkaline phosphatase in the serum (Popp and Cattley, 1991). Cholestasis is caused by a variety of compounds e.g. α-naphthylisothiocyanate (ANIT), chlorpromazine and androgens (Lu, 1996).

1.2.4 Hypertrophy and hyperplasia

An organ can adapt to injury in many different ways, these adaptations can include changing the size of cells, number of cells or the type of cells (Brown, 1998). An increase in the size of cells, and therefore the size of the organ is termed hypertrophy whereas a decrease in cell and organ size is known as atrophy. Cells may become more numerous (hyperplasia) or less numerous (hypoplasia). Hypertrophy and hyperplasia are often induced in the liver following a toxic response as part of the reparative process.

Hypertrophy is an increase in the size of individual cells without an increase in cell numbers (Evans and Lake, 1998). In hypertrophy, therefore there is a reduction in the number of nuclei per unit volume of liver tissue. The increase in cell size is due to an increase in number and size of cell organelles coupled with an increase in protein synthesis (Brown, 1998). Liver hypertrophy is often a focal response and frequently affects the centrilobular area. This area contains a high concentration of cytochrome

P450 enzymes, which are responsible for the metabolism of many toxic compounds (Glaister, 1986).

Hyperplasia is defined as an increase in cell numbers and may result in the enlargement of liver size. Hyperplasia may be identified by an increase in mitosis and cytoplasmic changes indicating enzyme induction. Total DNA is elevated in hyperplasic responses but this is not the case in hypertrophy (Evans and Lake, 1998). Hyperplasia may be focal or diffuse.

Both hypertrophy and hyperplasia may cause an increase in liver weight (mass). These adaptive processes of an increase in hepatocyte size or proliferation are usually reversible and liver weights often return to normal when exposure to the toxic compound is withdrawn.

1.2.5 Cell death

Severe or continuing cell injury may eventually result in cell death; this will occur when the cellular changes that have been induced become irreversible. Cell death is due to one of two different mechanisms: apoptosis or necrosis. Apoptosis is referred to as an active form of cell death since it requires RNA and protein synthesis and is controlled by both pro-apoptotic and anti-apoptotic genes (Plaa and Charbonneau, 2001). Apoptosis involves scattered single cells and cell shrinkage is observed. Apoptotic cells lack inflammatory cell infiltrates and can be characterised as densely eosinophilic structures, containing condensed chromatin (Popp and Cattley, 1991).

Necrosis may involve either a single cell or a large number of cells in one area; it involves the denaturation of proteins and an enzymatic digestion of organelles and other cytosolic components (Brown, 1998).

Necrosis can be determined histologically by the changes that occur in both the cytoplasm and nucleus. The cytoplasm becomes eosinophilic and pink in colour, and this change in colour is due to the dissociation of ribosomes from the endoplasmic reticulum and the denaturation of proteins. The nuclear changes that occur usually follow a pattern of either pyknosis or karyolysis (Evans and Lake, 1998). The process

of pyknosis involves shrinkage of the nucleus, which then becomes densely stained. The nucleus may then break into smaller fragments, a process known as karyorrhexis. In karyolysis the nucleus gradually fades and dissolves as the nuclear material is digested.

Hepatocyte necrosis occurs quite frequently after liver injury. Cytoplasmic oedema, dilatation of the endoplasmic reticulum and a disaggregation of polysomes precede necrosis (Lu, 1996). Protein synthesis is inhibited and eventually the plasma membrane ruptures causing a leakage of intracellular enzymes into the plasma e.g. alanine aminotransferase (ALT) and asparate aminotransferase (AST). The release of the cell contents induces an inflammatory response, which causes greater injury mediated by inflammatory cells, cytokines and oxygen species (Rosser and Gores, 1995). Cell death can be focal, zonal or panlobular (Molsen, 1986). Focal necrosis involves the death of single hepatocytes or small numbers of cells. Panlobular necrosis refers to a massive death of hepatocytes involving many lobules. Most hepatotoxicants cause zonal death and the centrilobular region is the most commonly affected (Glaister, 1986).

Centrilobular cells are more susceptible to toxicants since they are relatively hypoxic and have higher concentrations of cytochrome P-450 enzymes resulting in greater levels of the metabolised chemicals in the area. In centrilobular necrosis the necrotic cells completely encircle the central vein, with the extent of the lesion depending on the toxic agent administered. Centrilobular necrosis can be repaired rapidly since the adjacent hepatocytes begin to regenerate within 24 hours, and therefore the new hepatocytes move into the necrotic area (Popp and Cattley, 1991).

Midzonal necrosis is the least common type of hepatic necrosis and is associated with a band of necrotic tissue half way between the central vein and the portal triad. Midzonal necrosis generally affects only two to three cells in the lobule (Popp and Cattley, 1991). Periportal necrosis is more common than midzonal but less frequent than centrilobular. In periportal necrosis necrotic cells surround the portal area, and can involve a single row of hepatocytes or up to one-third of the lobule.

Massive necrosis occurs when animals are exposed to extremely large doses of toxicants and involves the entire liver lobule extending from the central vein to the portal tracts, although not every lobule will be affected equally.

In most cases a process of repair will take place following hepatocyte necrosis involving the division of surrounding undamaged cells. However in the case of massive necrosis, complete death of the hepatocytes in a lobule renders that lobule incapable of undergoing restoration (Evans and Lake, 1998). In such cases lobules in less affected areas will carry out most of the reparative process but this usually involves the deposition of fibrous tissue.

Centrilobular necrosis is observed following treatment with CCl_4, thioacetamide and bromobenzene. Compounds such as allyl alcohol and aflatoxin induce periportal necrosis. Midzonal necrosis is rare but can occur following exposure to phosphorus. Paracetamol and isoniazid are examples of drugs that can induce centrilobular necrosis (Lu, 1996).

Table 1.1 Summary of the main types of hepatic injury and examples of drugs and chemicals that induce these toxic responses.

Hepatic injury	Drug/Chemical
Hydropic degeneration	CCl_4, carbon disulfide
Steatosis	CCl_4, phosphorus, tetracycline, ethanol, methotrexate
Cholestasis	ANIT, chlorpromazine, anabolic steroids, tolbutamide, phalloidin
Necrosis	CCl_4, thioacetamide, bromobenzene, allyl alcohol, aflatoxin, paracetamol, isoniazid,
Fibrosis and cirrhosis	CCl_4, thioacetamide, diethylnitrosamine, D-galactosamine, alcohol
Carcinoma	Androgens, alfatoxins, nitrosamines

1.2.6 Fibrosis

Fibrosis is defined as an increased amount of newly formed reticulin and collagen fibres and is characterised by a net increase in the hepatic content of collagens and other extracellular matrix proteins (Benyon et al., 1996). Liver fibrosis can be described as a wound healing or scarring process in response to injury (Li and Friedman, 1999). It occurs as a result of an imbalance between the synthesis and degradation of matrix proteins (Kauser et al., 1998). The early stages of the process of fibrosis consist of inflammation and necrosis, which may initiate hepatocyte regeneration and repair. But with repeated injury this will lead to an accumulation of matrix proteins, e.g. collagen I, III and IV (Friedman, 1993) and alterations in the architecture of hepatic lobules. A single injury to the liver will result in regeneration of the hepatocytes. However, if the injury is repetitive the response is likely to lead to the formation of fibrotic scar tissue. The scar tissue consists of collagens I and III, proteoglycans, fibronectin and hyaluronic acid (Friedman, 1993). Fibrosis is characterised by excess deposition of collagen fibres resulting in disruption of the normal liver structure, which may progress to cirrhosis. The liver becomes distorted by dense bands of collagen, which link vascular structures. Fibrosis and cirrhosis can accompany any chronic liver disease, such as viral hepatitis and biliary tract diseases (Freidman, 2003; Kershenobich Stalnikowitz and Weissbrod, 2003).

Hepatic injury stimulates hepatic stellate cells (HSC's) (also known as lipocytes, perisinusoidal or Ito cells) situated in the space of Disse. In normal liver these cells contain intracellular droplets containing vitamin A. Stimulation causes these HSC's to change from a lipid-filled perisinusoidal cell to a myofibroblast-like cell; a process called activation (Iredale et al., 1998; Kauser et al., 1998). The HSC's are thought to change firstly into transitional cells and then following repeated injury they become myofibroblast-like cells (Cassiman et al., 2002). In the activated state the stellate cells contain a decreased amount of vitamin A (Nanni et al., 2000). Activation is thought to occur in at least two steps (Figure 1.5); initiation: in which the cells enlarge; α-smooth muscle actin (α-SMA) is secreted; cytokine receptors (e.g. platelet-derived growth factor (PDGF) and transforming growth factor-β1 (TGF-β1) receptors) are induced making the cells more responsive to cytokines. The second step known as perpetuation reflects the effects of cytokines on the cells and the stimulation in response to the

perisinusoidal fibrosis and correlates with the degree of fibrosis. Other growth factors may be secreted, for example: connective tissue growth factor (CTGF), and these growth factors are thought to play an additional role in the fibrogenesis process (Paradis et al., 1999). Although collagen types I, III and IV are all increased, it is type I that is increased most (Benyon and Iredale, 2000).

α-SMA is a marker of myofibroblasts and smooth muscle cells, and studies have shown that the liver cells acquire this marker during activation following liver injury (Rockey et al., 1992). Nestin, desmin, vimentin and GFAP are members of the intermediate filament family but their role in the fibrosis process is unknown (Niki et al., 1996). These ECM components mainly interact with cells via integrin receptors, a family of transmembrane proteins (Wang et al., 1996; Kim et al., 1997).

In normal liver the interstitial ECM is confined to the areas surrounding the large vessels and portal areas, and the space of Disse contains a basement membrane-like matrix consisting of non-fibril-forming collagens (e.g. types IV and VI). However during fibrosis this basement membrane changes to an interstitial ECM composed of fibril-forming collagens (e.g. types I and III) (Li and Friedman, 1999; Kershenobich stalnilowitz and Weissbrod, 2003). The process of fibrosis therefore disturbs the normal function of the hepatocyte and can lead to sinusoidal capillarization resulting in severe functional impairment of the sinusoids (Hahn et al., 1980). The endothelial cells lose their fenestrations and become supported by a continuous basement membrane composed of collagens and fibronectin (Burt, 1993; Friedman, 1993). This basement membrane increases the diffusion barriers. Fibrosis therefore impedes the exchange of materials between the sinusoidal space and the hepatocytes (Hahn et al., 1980; McGuire et al., 1992; Hung et al., 2002). It is thought that the activated HSC's may contribute to this basement membrane formation by the deposition of collagen I in the space of Disse, which may act as an inducer of the sinusoidal endothelial defenestration process (Herbst et al., 1997).

Fibrosis leads to portal hypertension by distorting the normal liver architecture. Vascular modifications also occur during fibrosis with new blood vessels developing between nodules, and such vessels then connect afferent and efferent blood vessels. In this way a new vascular network is created which encircles the nodules (Gaudio et al., 1997).

It has been suggested that CCl_4-induced injury causes increased amounts of circulatory endotoxin and either this endotoxin or the free radicals produced by CCl_4 metabolism leads to the activation of Kupffer cells. Oxidative stress is also thought to play a role in Kupffer cell activation and fibrogenesis (Rivera et al., 2001). Activated Kupffer cells release cytokines including tumour necrosis factor-α (TNF-α) and transforming growth factor-β1 (TGF-β1) and a transforming growth factor-α (TGF-α)-like peptide (Burt, 1993). The release of these cytokines induces changes in the acute phase response causing plasma levels of acute phase proteins such as haptoglobin and fibrinogen to rise (Fulop et al., 2001).

The most profibrogenic cytokine released from the Kupffer cells is TGF-β1, which stimulates proliferation and the production of collagen types I and III by HSC's, and is highly expressed in fibrotic livers (Inagaki et al., 1995; Rivera et al., 2001; Tang et al., 2002 and Ezquerro et al., 2003). TGF-β1 is a 50-amino acid peptide, which can be released from Kupffer cells, activated HSC's, endothelial and inflammatory cells. TGF-β1 may bind to the same cell-surface receptor as epidermal growth factor (EGF) (Kokudo et al., 1992; Oguey et al., 1994). It has been suggested that TGF-β1 induces the production of hydrogen peroxide (H_2O_2) and that this acts as a signalling mediator for tumour necrosis factor (TNF), interleukin-1 (IL-1) and TGF-β1. TGF-β1 is also up-regulated by interleukin-6 (IL-6), which is released by Kupffer cells. IL-6 induces hepatic inflammation and increases collagen synthesis (Choi et al., 1994; Salazar-Montes, 1999). Kupffer cells are also known to generate reactive oxygen species, which may enhance stellate cell activation and collagen synthesis (Friedman, 2003).

Normally quiescent HSC's produce small amounts of collagen but upon activation this amount greatly increases. However the hepatocytes themselves are thought to produce only small amounts of collagen during the process of fibrosis (Ogata et al., 1991).

Activated HSC'S secrete leptin, a hormone secreted by adipocytes that regulates obesity by reducing food intake and increasing energy use (Tang et al., 2002). It has been suggested that the secretion of leptin may play an active role in fibrosis (Friedman, 2003). Studies have indicated that leptin increases the expression of TGF-β1 in cultured sinusoidal endothelial cells and Kupffer cells and also increases the production of collagen type-I (Tang et al., 2002). It has been suggested that mast cells, which are

present in liver may play a role in the fibrotic process, perhaps by the release of histamine. Histamine can increase the synthesis of collagen by fibroblasts (Akiyoshi and Terada, 1998). In CCl_4-induced fibrosis the cells in the fibrotic bands and regenerating hepatocyte nodules express both desmin (an intermediate filament protein specific for muscle cells) and α-SMA. As the severity of fibrosis increases there is an increase in both desmin and α-SMA positive cells.

Altered degradation of the extracellular proteins may play a role in fibrogenesis. Matrix metalloproteinases (MMP) are a family of zinc dependent endoproteinases, which consist of interstitial collagenase (MMP-I), type IV collagenase, gelatinases and stromelysin and are expressed by HSC's and Kupffer cells (Arthur, 1997; Benyon and Iredale, 2000). The MMP's are the key enzymes responsible for the degradation of interstitial collagens I, II and III (Gaudio et al., 1997). Tissue inhibitors of metalloproteinases (TIMP's, e.g. TIMP-I and TIMP-II), and proteinase scavengers (e.g. α-2 macroglobulin) regulate these interstitial collagenases (Li and Friedman, 1999). TIMP's bind to collagenase and block its activity (Murawaki et al., 1993; Knittel et al., 1999). There is evidence that expression of both α-2 macroglobulin and TIMP's by HSC's is increased after CCl_4-induced fibrosis (Gaudio et al., 1997; Kauser et al., 1998; Li and Friedman, 1999), thereby allowing fibrosis to progress. As fibrosis increases and becomes irreversible collagenase activity decreases. α-2 macroglobulin is a plasma globulin and acts as an inhibitor of the coagulation and fibrinolytic systems. α-2 macroglobulin binds to and inactivates metalloproteases; it is secreted by hepatocytes as part of the acute phase response and is increased in response to IL-6. TGF-β1 inhibits the synthesis of collagenases and enhances the production of TIMP's (Kauser et al., 1998; Tahashi et al., 2002). In this way the TGF-β1 and α-2 macroglobulin complex may act synergistically.

Other inhibitory mechanisms of MMP's may enhance fibrosis; MMP's are released as pro-enzymes requiring the cleavage of the inhibitory N-terminal peptide for activity (Benyon and Iredale, 2000). The protease plasmin is required for the activation of pro-MMP-1. However activated HSC's synthesize plasminogen activator inhibitor (PA-1), which inhibits plasmin synthesis (Matrisian, 1992; Kim et al., 1997; Benyon and Iredale, 2000). The activation of HSC's has been shown to produce a reduction in the MMP expression and an increase in TIMP expression (Knittel et al., 1999). Therefore,

the amount of collagen deposited, and the severity of fibrosis, depends on the balance of the synthesis of collagen and the amount of collagen degraded by collagenases (MMP's).

Hepatocyte growth factor (HGF) is known to accelerate hepatocyte regeneration following hepatocellular injury (Noji et al., 1990; Masson et al., 1999b). Studies have shown that the administration of HGF can prevent the formation of liver fibrosis, which has been induced by some hepatotoxins (Masuda et al., 1994, Sato et al., 2000).

By removing the fibrogenic stimulus the process of fibrosis may be reversed with the recovery brought about by a decrease in HSC proliferation and apoptosis of the HSC's (Li and Friedman, 1999). During the fibrotic process the turnover of HSC's is increased, as both mitosis and apoptosis are elevated, but proliferation predominates resulting in a net increase in HSC's. However, during the recovery stage apoptosis outweighs proliferation due to the withdrawal of the fibrotic stimulus and therefore there is a loss of HSC's from the liver (Iredale et al., 1998; Benyon and Iredale, 2000). Complete recovery from fibrosis would require the remodelling and breakdown of the ECM and degradation of collagen fibres. The excess collagens need to be broken down by interstitial collagenase, which in humans is known as MMP-1 but the degradation of collagen I and III in the rat appears to be carried out by MMP-13 (Iredale et al., 1998). Whereas during fibrosis the actions of MMP's are inhibited by TIMP's, during recovery this is not the case. The level of MMP's remains the same throughout fibrosis and recovery but the level of TIMP's is greatly enhanced as fibrosis progresses but during recovery this expression is diminished. Therefore providing fibrosis has not reached an advanced stage it may be reversed.

1.2.7 Cirrhosis

Liver cirrhosis is the end result of fibrosis, hepatocellular regeneration, and necrosis; and is characterised by the loss of normal liver architecture. Early alterations include fat accumulation and the presence of thin fibrous trabeculae (Zaki, 1966). Cirrhosis of the liver is accompanied by liver cell hyperplasia, chronic inflammation and nodule formation. Splenomegaly and ascites are usually present in the human (Perez Tamayo, 1983). The formation of ascites is preceded by sodium retention, which occurs when

the liver function is diminished (Wensing et al., 1990). Sodium retention may result from a dysregulation of water channels and ion transporters (Fernandez-Llama et al., 2000). The hepatocytes are aggregated to form large nodules that are separated from each other by bands of fibrotic septa containing either fine or dense collagen fibres (More, 1973; MacSween, 1985). Eventually the nodules increase in size and number and in doing so compress blood vessels and bile ducts in the area adding further distortion to the liver architecture (Zaki, 1966). The portal tracts become interconnected by fibrotic tissue. This disruption of liver organisation leads to interference of blood flow through the liver and a reduced liver function (Gaudio et al., 1997; Evans and Lake, 1998). The liver may be enlarged during the early phase but as the disease progresses there tends to be a loss of cells and therefore the liver shrinks in size (MacSween, 1985). The cirrhotic liver is generally heavily infiltrated by lymphocytes.

Cirrhosis may be classified as micronodular in which the nodules are of approximately the same size and separated by thin septa, or macronodular. Macronodular cirrhosis consists of larger nodules of variable size encircled by bands of collagen (Vandenberghe, 1995). However both types often co-exist in the same liver. The colour of the cirrhotic liver varies depending on the degree of fatty change, on cholestasis and on the presence or absence of congestion. Normally the cirrhotic liver is a pale tan colour when there is a high fat infiltration and may be reddish in colour if there is excess iron deposition and necrosis. If there is a lot of scarring and jaundice then the liver may be grey or green (Conn, 1982).

In humans cirrhosis is also often classified according to the aetiology of the disease including alcoholic, post-viral and biliary cirrhosis (MacSween, 1985). Alcoholic cirrhosis presents with a mixed or predominantly macronodular appearance with narrow septa separating the nodules. The liver tends to be enlarged with a pebbled surface. Thin bands of connective tissue connect portal areas to each other and typical nodules are less than one lobule in size (Conn, 1982). The connective tissue appears to start at the portal areas and migrate towards the centre of the lobule. Central veins may therefore be difficult to locate. The alcoholic cirrhotic liver often contains hyaline bodies in the centrilobular region of the liver, and fat deposits tend to be localized to this area also (Conn, 1982).

Post-viral cirrhosis (also known as post-hepatitic) presents as a smaller liver with little or no fat deposits and is predominately macronodular in appearance (MacSween, 1985). The liver is divided into nodules of varying sizes by broad bands of connective tissue. The architecture of the post-viral cirrhotic liver is not completely lost; the portal and central veins become distorted but remain recognizable (Conn, 1982). Infiltration of lymphocytes is normal as is necrosis.

Biliary cirrhosis is a condition resulting from long standing cholestasis, whereby there is a retention of bile. The liver is usually dark green in colour, firm and nodular (Conn, 1982). There are two types of biliary cirrhosis: primary and secondary. In primary biliary cirrhosis there is an infiltration of lymphocytes and plasma cells around the epithelium of the smaller intrahepatic bile ducts (MacSween, 1985). As the disease progresses the inflammatory cell infiltrate expands and involves the periportal area, this is usually accompanied by portal fibrosis and cholestasis. Secondary biliary cirrhosis occurs as a result of the obstruction of the flow of bile out of the liver, and therefore bile accumulates in the hepatocytes, bile canaliculi and Kupffer cells. Eventually, this leads to inflammatory cell infiltrate and bile duct and fibroblast proliferation with the development of fibrous septa (MacSween, 1985). Secondary biliary cirrhosis presents as an enlarged liver with micronodular cirrhosis and an increase in the number of interlobular bile ducts.

Fibrosis and cirrhosis can be induced in animals by compounds such as dimethylnitrosamine (DMN), CCl_4 and thioacetamide and D-galactosamine (Proctor and Chatamra, 1983; Jeong et al., 2001; Bruck et al., 2001; Sato et al., 2000; George et al., 2001; Al-Bader et al., 2000; Masson et al., 1999b). In humans the most common cause of cirrhosis is alcohol abuse (Popp and Cattley 1991).

Cirrhosis has been classified as irreversible due to the increased deposition of connective tissue causing impaired hepatic function and reduced blood flow, which decreases the regeneration capability of the cirrhotic liver. Fischer-Nielsen et al. (1991) demonstrated that cirrhosis resulting from treatment with both phenobarbital and carbon tetrachloride is histologically irreversible and liver function measurements remain decreased. However, there is now evidence that cirrhosis could potentially be reversed (Hernández-Muñoz et al., 2001; Friedman, 2003).

1.2.8 Carcinogenesis

In man, primary carcinoma of the liver is relatively uncommon and is mostly associated with pre-existing cirrhosis. The most common neoplasms of the liver are hepatocellular carcinoma and cholangiocarcinoma (bile duct carcinoma) (Lu, 1996). In rodents hepatocellular carcinomas are said to develop from altered hepatocyte foci e.g. hepatocytes showing increased eosinophilia or basophilia (Evans and Lake, 1998). These altered foci can develop into hepatic nodules, which are able to grow and acquire structural features typical of neoplasms. Cholangiocarcinoma arises from the bile duct epithelia and can spread along the portal tracts or form nodular growths in the liver (Evans and Lake, 1998). Hepatocellular carcinoma can arise as a result of exposure to androgens, alfatoxins and nitrosamines.

1.3 Serum biomarkers of toxic injury

1.3.1 Serum enzymes

Enzyme tests are carried out mainly using serum samples because plasma has clotting abilities and most of the commonly used anticoagulants (e.g. ethylene-diamine-tetraacetic acid and heparin) are enzyme inhibitors (H¢rder and Wilkinson, 1979). Plasma is the fluid in which the blood cells are suspended. Serum is the fluid that separates from clotted blood; it is similar to plasma except it lacks fibrinogen and other blood clotting factors.

Serum enzymes are important as markers of organ toxicity. When tissues are damaged cytoplasmic enzymes may leak from the cells into the plasma (Plaa and Charbonneau, 2001). Cell necrosis is followed by a breakdown in the cell membrane therefore the intracellular enzymes are released into the extracellular fluid. Inflammation of a cell may result in the cell membrane becoming more permeable allowing enzymes to leak into the extracellular fluid. Enzyme activity is generally expressed as units per litre (U/L), where the unit describes the amount of catalytic activity, which converts one micromole of substrate per minute (H¢rder and Wilkinson, 1979).

Since intracellular enzymes are often distributed in a number of tissues the results of serum enzyme analysis can be relatively non-specific (Marshall, 1992). Therefore it is often necessary to measure an appropriate combination of serum enzyme activities in order to determine the nature and origin of tissue damage.

This project is concerned with CCl_4-induced hepatotoxicity and therefore we wish to measure the serum enzymes that increase as a result of hepatic injury. The activity of each of these enzymes can reflect a particular type of injury for example; alkaline phosphatase (ALP) activity increases in response to cholestatic injury (Zimmerman, 1978). Some serum enzymes are more specific for the liver than others; sorbitol dehydrogenase (SDH) and alanine aminotransferase (ALT) are found mainly in the liver whereas asparate aminotransferase (AST) and lactate dehydrogenase (LDH) are relatively non-specific and may reflect injury to other organs.

ALT and AST are commonly used in diagnostic enzymology (Marshall, 1992). The activity of each enzyme varies for different tissues. ALT is found in many tissues but its concentration is greatest in the liver and therefore is useful for identifying inflammation and necrosis in the liver. ALT is sensitive to necrotic lesions and it has been shown that increases in ALT activity are closely correlated with the severity of the injury (Balaza et al., 1961). ALT is more specific for the liver than AST (Ellis et al., 1978).

AST is widely distributed throughout different tissues, for example in the liver, skeletal muscle and heart. High AST activity is found in skeletal muscle, cardiac muscle and liver (Wroblewski, 1959). Therefore increased serum AST levels could indicate damage to a number of organs.

AST in found in both the liver microsomes and mitochondria, while ALT is located mainly in the microsomal fraction of the liver. The baseline activity of both enzymes in the liver is generally similar, however in most other tissues AST activity is greater than ALT. In acute liver injury the serum ALT activity is greater than AST, whereas chronic hepatic disease produces a much greater AST activity than ALT in the serum (Whitby et al., 1988). Significantly elevated values for serum AST and ALT nearly always imply injury to the liver and acute necrosis of hepatocytes (Jones and Berk, 1979). In viral

hepatitis and necrosis the ALT and AST levels can reach up to 100 times greater than normal.

An increase in serum activity of SDH is a relatively sensitive indicator of hepatic injury since SDH is mainly found in the liver (Awad et al., 1963). Korsrud and Trick (1973) studied the effect of several compounds on the liver and the subsequent serum enzyme response and concluded that SDH was the most sensitive enzyme when minimal damage had been induced.

Glutamate dehydrogenase (GLDH) is an enzyme found in the mitochondria (Schmidt and Schimdt, 1983), and is relatively liver specific, although it can also be found at lower levels in the kidney and muscle tissue. The activity of GLDH is much greater in the centrilobular region of hepatocytes than any other region and therefore it is a good marker of centrilobular necrosis.

Measurements of serum enzymes are useful for assessing injury to other organs such as the heart. The main enzymes measured in the determination of cardiotoxicity are creatine kinase (CK), lactate dehydrogenase (LDH) and AST. However, none of these enzymes are specific for the heart.

CK levels are useful for the detection of skeletal muscle and cardiac damage. Small amounts of the enzyme are found in the gastrointestinal tract, kidney and liver. CK is a dimeric enzyme with two subunits; B (brain) and M (muscle) and three isoenzymes exist; BB found in the brain and thyroid, MM found in skeletal muscle, and MB, which is present in high concentration in cardiac muscle (Neumeier, 1981). In humans CK levels are elevated by approximately 7-12 times following myocardial infarction. Skeletal muscle has a very high total concentration of CK; therefore total serum CK activity is raised as a result of damage to skeletal muscle (Lang, 1982).

Lactate dehydrogenase (LDH) is a tetrameric enzyme with five isoenyzmes, and these can be found in different organs, e.g. heart, liver and skeletal muscle. Isoenzyme patterns can be useful in the determination of specific tissue damage (Wilkinson, 1970). LDH has two monomers, H and M, which can combine in different ways to form each of the five isoenzymes (Markert et al., 1975). The isoenzymes differ in their biological, chemical and physical properties (Whitby et al., 1988). Increases in total LDH activity

reflect damage to a number of organs including the liver, skeletal muscle, heart, spleen, pancreas and kidney. The serum isoenzyme pattern differs according to the organ damaged and this pattern can be used to locate the origin of the increased serum LDH activity (Cornish et al., 1970). In humans myocardial infarction may result in increases of LDH activity by up to 10 times, this degree of elevation may also occur as a result of liver cirrhosis.

The tissue distribution of the enzyme alkaline phosphatase (ALP) lacks specificity (Zimmerman, 1978) and is found in the intestines, kidney, liver and bone. In the liver ALP activity is localized to the membranes forming the canaliculus, the sinusoidal membranes and central vein endothelial cells. Serum ALP levels tend to be elevated due to cholestatic lesions as a result of an increased synthesis of ALP by biliary ductile cells (Kaplan, 1986). Other forms of hepatic injury tend not to cause an increase in serum ALP activity since the enzyme is membrane bound and therefore will not readily leak into the plasma like other enzymes (Whitby et al., 1988). However, ALP activity tends to be high under control conditions and may be affected by other factors such as diet (Gutman, 1959).

For the determination of hepatic injury the most commonly measured enzymes include AST, ALT, GLDH and SDH; and in most liver injury studies the activities of at least two of these enzymes are measured. Levels of the aminotransferases (ALT and AST) are usually 25-1000 times higher than normal.

1.3.2 Non-enzymatic markers

A change in the concentration of an individual protein in the plasma or serum may be due to changes in the rate of synthesis, the rate of catabolism, in plasma volume, in the distribution of the protein or rate of excretion (Jones and Berk, 1979). Many plasma proteins are synthesised on the rough endoplasmic reticulum of liver parenchymal cells, therefore hepatic injury can cause the concentration of these proteins in the plasma to fall.

The most abundant plasma protein is albumin, which is synthesized by the liver. Albumin is a low affinity transport protein for many substances for example; thyroid

hormones, calcium and fatty acids and also many drugs are bound to albumin in the blood stream (Marshall, 1992). In acute hepatic injury the plasma level of albumin tends to be greatly elevated (Evans and Lake, 1998). Whereas in chronic liver injury plasma albumin concentration may be decreased, resulting from a decreased synthesis and an increase in the volume of distribution due to the formation of ascites (Marshall, 1992).

Other plasma proteins that are synthesized by the liver include acute phase proteins, such as fibrinogen and haptoglobin. The concentration of these proteins in the plasma or serum increases in response to inflammation (Jones and Berk, 1979).

Increased levels of serum bilirubin may reflect chemically induced hepatic injury but it is a relatively insensitive indicator of injury. However, compounds that produce bile duct obstruction often cause elevations in serum bilirubin (Plaa and Charbonneau, 2001). Serum bile acids levels are often measured as an indicator of cholestasis (Gopinath et al., 1980).

Serum levels of creatinine and urea are useful measurements of glomerular function and therefore reliable indicators of kidney injury and function. Both urea and creatinine are normally filtered from the blood and then usually reabsorbed by the kidney, however small amounts of both are excreted into the urine under normal conditions. Conditions which cause the glomerular filtration rate to decrease result in increased levels of urea and creatinine in the blood.

Therefore, serum analysis is extremely useful in the identification of tissue damage. However, none of the serum parameters are tissue specific resulting in the need for a combination of tests to identify the origin of tissue damage. The aim of this project is to identify liver specific markers of injury using non-invasive methods. The analysis of urine as a non-invasive method has had some success in the discovery of urinary markers of disease and tissue damage.

1.4 The kidney

1.4.1 Structure of the kidney

The kidneys are regulatory and excretory organs (Koeppen and Stanton, 1996). The kidneys have several major functions including the regulation of: body fluid osmolarity and volume, electrolyte balance, acid-base balance, the excretion of metabolic products and foreign substances, and the production and secretion of hormones. Each kidney is composed of two regions: an outer cortex and an inner medullary region. Both the medulla and cortex are composed of nephrons (functional units of the kidneys), blood vessels, lymphatics and nerves. Figure 1.6 shows the gross anatomy of the human kidney.

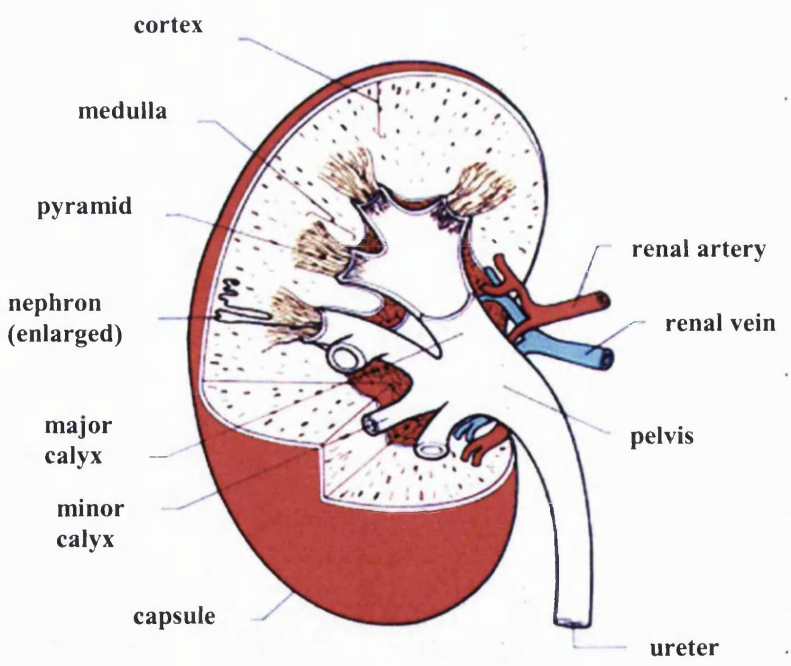


Figure 1.6 Cross-sectional view showing the structure of the human kidney (Modified from Marsh, 1983).

The nephron is the functional unit of the kidney and each consists of a renal corpuscle, a proximal tubule, a loop of Henle, a distal tubule and a collecting duct system. The structure of a nephron is shown in Figure 1.7.

The renal artery enters the kidneys alongside the ureter and branches into the interlobar arteries, arcuate arteries and interlobular arteries. The interlobular arteries branch to form afferent arterioles, which lead to the glomerular capillaries. Blood leaves the glomerulus via efferent arterioles leading into peritubular capillaries, which supply the proximal and distal convoluted tubules of the nephron (Koeppen and Stanton, 1996).

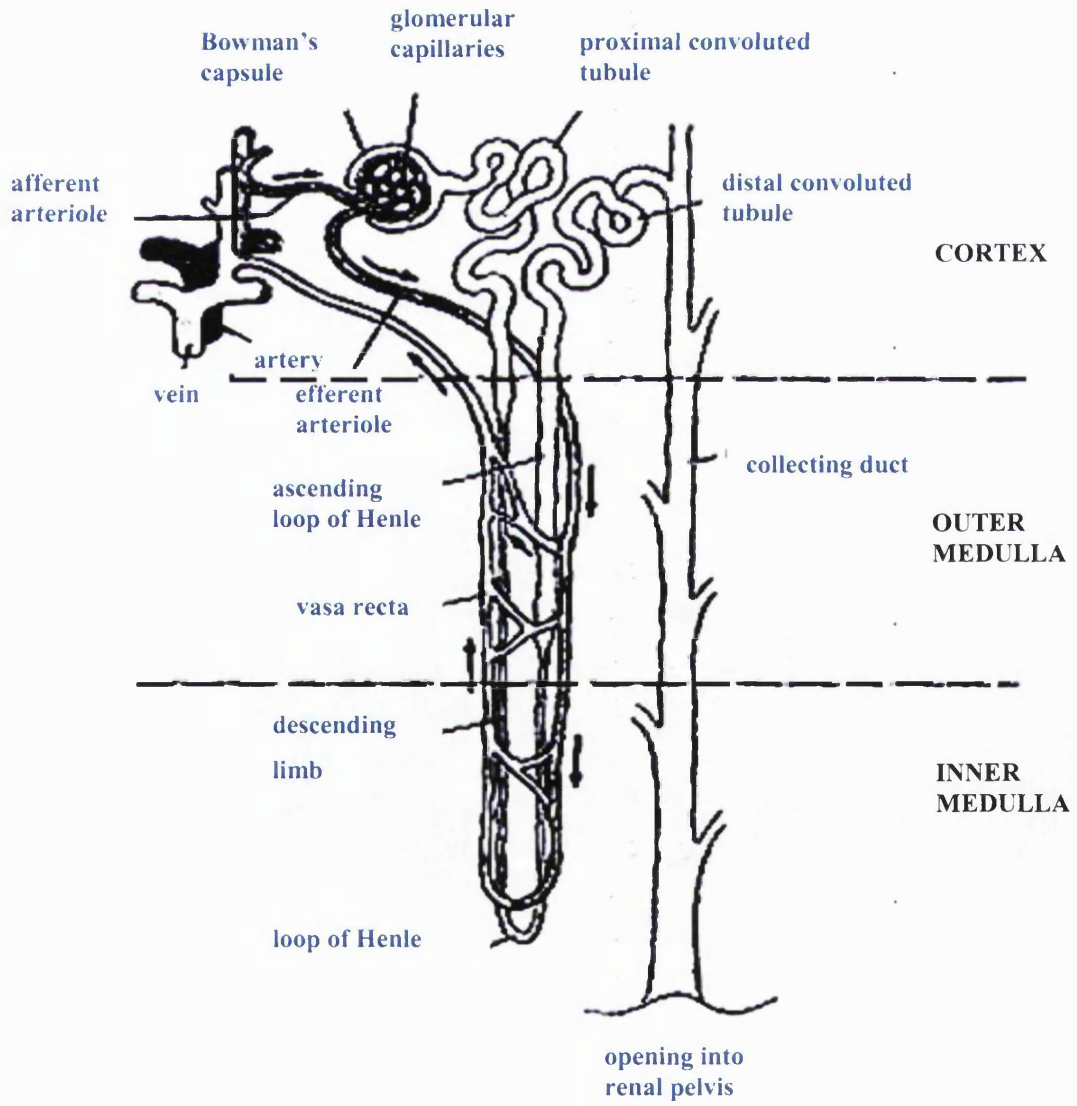


Figure 1.7 Structure of a nephron with its blood supply (Eckert and Randall, 1983)

1.4.2 Urine formation

All parts of the nephron and the collecting system participate in the process of urine formation. Urine formation involves three basic processes: ultrafiltration of plasma by the glomerulus, reabsorption of water and solutes, and secretion of solutes (Koeppen and Stanton, 1996). The first step in urine production begins with the ultrafiltration of plasma across the glomerulus (Koeppen and Stanton, 1996) and the accumulation of urine in the Bowman's capsule (Eckert and Randall, 1997). The glomerular capillaries have diameters, which are large enough to allow the diffusion of small plasma proteins but small enough to prevent the passage of blood cells. The glomerular epithelium is composed of podocytes separated by gaps, called filtration slits (Figure 1.8). This epithelium forms the visceral side of a capsular space known as Bowman's capsule. The endothelial cells of the glomerular capillaries are covered by a basement membrane, which is surrounded by podocytes; therefore the capillary endothelium, basement membrane and podocytes form a filtration barrier (Koeppen and Stanton, 1996).

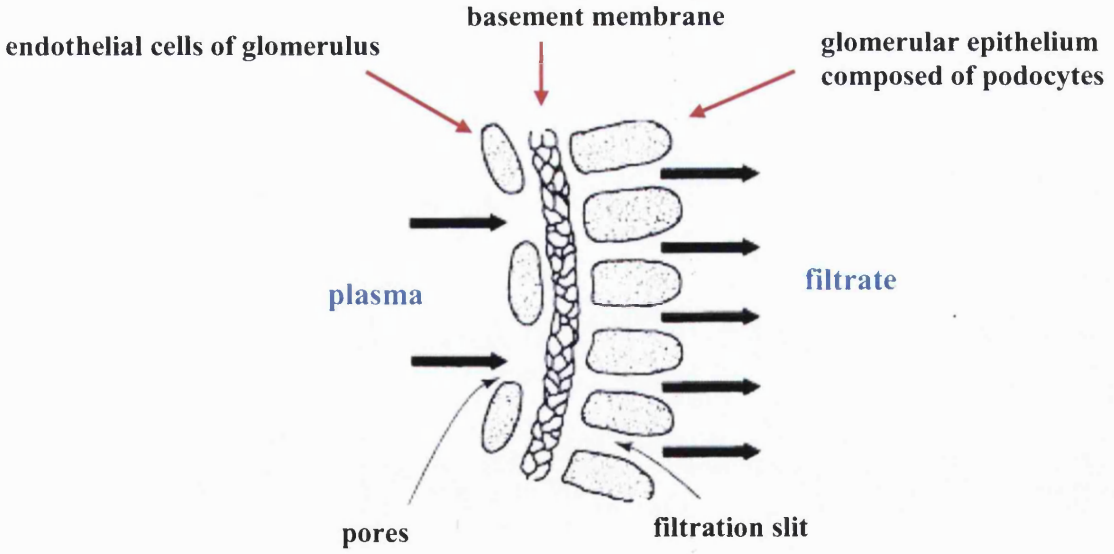


Figure 1.8 Representation of the glomerulus filtration barrier. (Adapted from Guyton, 1991). A basement membrane covers the endothelial cells of the glomerulus, preventing the passage of large plasma proteins. The glomerular epithelium is composed of podocytes separated by small filtration slits, thus allowing only small plasma proteins to end up in the filtrate.

The basement membrane and filtration slits contain negatively charged glycoproteins, therefore some molecules are held back on the basis of size and charge. The forces behind glomerular filtration are similar to those acting on all capillary beds, and include hydrostatic and osmotic pressure (Koeppen and Stanton, 1996). The hydrostatic pressure in the glomerular capillary promotes the movement of fluid out of the glomerulus into Bowman's space, and this is opposed by the hydrostatic pressure acting in Bowman's space. The blood osmotic pressure in the capillaries tends to draw water out of the filtrate and into the plasma. Therefore, the net filtration pressure at the glomerulus is the difference between the net hydrostatic pressure and the net osmotic pressure. The glomerular filtration rate is the amount (volume) of filtrate produced in the kidneys per minute (Martini, 2001).

Reabsorption from, and secretion into, the glomerular filtrate is achieved using a combination of diffusion, osmosis and carrier-mediated transport. The lining of the proximal convoluted tubule consists of simple cuboidal epithelial cells, which actively absorb nutrients, ions and plasma proteins from the tubular fluid (Martini, 2001). Proteins that have been filtered by the glomerulus are reabsorbed by the proximal convoluted tubule. Normally, virtually all the filtered proteins are reabsorbed but since the mechanism is easily saturated, proteins appear in the urine if the amount filtered increases.

In the proximal convoluted tubule and descending limb of the loop of Henle, water leaves the tubular fluid as a result of the osmotic gradient established by sodium ion reabsorption, leaving a reduced volume of concentrated tubular fluid. The ascending limb of the loop of Henle contains sodium-potassium-ATPase pumps in the membrane, which actively pump sodium and chloride ions out of the tubular fluid and into the interstitial fluid (Martini, 2001). The distal tubule and collecting ducts reabsorb sodium, chloride and calcium ions, secrete potassium and hydrogen ions and reabsorb variable amounts of water. The reabsorption of water in the distal tubule and collecting ducts depends on the concentration of anti-diuretic hormone (ADH) since the ascending limb of the loop of Henle is impermeable to water (Koeppen and Stanton, 1996). Urea diffuses out of the tubular fluid (urine) via the papillary ducts. The collecting ducts receive urine from many nephrons and carry it toward the renal sinus, and into the renal pelvis, and from here urine travels to the bladder via the ureters. The removal of water from the urine in the distal tubule and collecting ducts concentrates the urine. The loop

of Henle is present in the nephrons of all animals capable of producing a hypertonic urine (i.e. mammals and birds) and the longer the loop of Henle the greater the concentrating power of the kidneys (Eckert and Randall, 1997).

The kidneys function as a means of eliminating waste products, regulating plasma ion concentrations, regulating blood volume and pressure, and conserving nutrients (Martini, 2001).

1.5 Urinary biomarkers of toxic injury

Urine analysis provides a means of testing for disease states and toxicity using non-invasive methods to obtain a sample for analysis, therefore causing a minimal amount of distress to the animal. Urinary biomarkers may be detected before other clinical signs of distress or toxicity are evident in the animal. In animal studies biomarkers are routinely quantitatively expressed in relation to urine volume, urine creatinine levels, or 24-hour total urinary measurements, (Poon and Chu, 1998). This is because the excretion of protein in the urine is variable throughout the day, so a 24-hour total value eliminates this variation (Kashif et al., 2003). Expressing the urinary marker as a ratio to the amount of creatinine is useful since animals generally excrete similar amounts of creatinine daily unless there is renal dysfunction or a change in muscle mass (Poon and Chu, 1998).

Urine samples can be analysed using microscopic, physical or chemical testing. Microscopic analysis of urine involves examining a sample of urine under a light microscope for evidence of crystals, casts or squamous cells. Visualising the urine under a high power light microscope can identify cells and bacteria.

The first analysis of urine is usually a direct visual observation; normal urine is pale to dark yellow in colour and is clear. Cloudy or turbid urine is often due to the presence of excess cellular material, protein, bacteria or lipids in the urine. The presence of red blood cells produces a urine sample, which is both cloudy and red in colour. Urine that has a blue-green colour indicates excess bilirubin (Martini, 2001).

A simple and straightforward method of chemical urinalysis involves the use of urine dipsticks. These strips provide a qualitative and semi-quantitative analysis of the urine sample by testing for pH, specific gravity, protein, glucose, ketones, nitrite, blood, urinobilinogen and leucocytes. To obtain the results a colour change of each test on the dipstick is compared to a colour chart after a specified time.

Specific gravity measures urine density and is directly proportional to urine osmolality, which is directly proportional to the osmotic work being carried out by the kidney (Koeppen and Stanton, 1996). Blood in the urine samples indicates injury to the kidneys or urinary tract system. Ketones appear in the urine in humans as a result of poorly controlled diabetes mellitus and during both starvation and dehydration (Martini, 2001). A high specific gravity indicates dehydration, liver disease, diabetes mellitus and heart failure, whereas a low specific gravity occurs when there is renal failure and diabetes insipidus. A positive result for glucose in the urine is a sign of diabetes. A positive nitrite test indicates the presence of bacteria in the urine.

Normally urine contains only small amounts of low molecular weight proteins therefore the presence of large amounts of protein in the urine is a good indicator of the presence of a disease state. Proteinuria in humans is an indicator of a number of conditions for example, liver disease, cancer, diabetes, pregnancy, urinary tract obstruction, and toxaemia. An increase in the amount of protein in the urine can be due to an increase in glomerular filtration, inadequate tubular reabsorption, overflow and increased tubular secretion. Increased glomerular filtration is due to altered glomerular permeability and a defect in the tubular epithelial cells can result in proteinuria (Kashif et al., 2003). An overflow of protein occurs when an excess amount of protein is filtered and the tubular cells are unable to reabsorb all the protein. This may occur when there is increased expression of protein by an organ in response to injury; or when proteins leak from a cell during necrosis into the blood.

Currently, urine analysis is a quick and effective means of determining toxicity or the progression of a disease state. Many biomarkers are already measured in urine such as the measurement of urinary aldehydes, catecholamines and nitric oxide. Urinary levels of hydrogen peroxide may serve as a marker for oxidative stress and have been associated with malignancy (Banerjee et al., 2003). Aldehyde levels in urine, and in particular malondialdehdye (MDA) are currently measured as indicators of oxidative

stress (Hermanns et al., 1998; Draper et al., 2000). Levels of nitrates and nitrites (markers of nitric oxide production) are elevated in the urine from patients with multiple sclerosis (Smith and Lassman, 2002). Isoprostane is elevated in the urine samples of smokers (Obata et al., 2000).

However, these biomarkers are relatively non-specific and there is a need for urinary markers, that reflect a change in a particular organ or a disease state. Studies have been carried out to identify urinary markers of bladder cancer (Kageyama et al., 2004). These workers detected an elevated amount of calreticulin (CRT) in both the cancer tissue and in the urine samples when compared to non-cancerous samples. Dare and colleagues (2002) identified parvalbumin as a marker of skeletal muscle toxicity in rats that had been dosed with 2, 3, 5, 6-tetramethyl-p-phenylenediamine (TMPD).

The aim of this project is to identify urinary markers of specific liver injury in the rat. In order to induce hepatotoxicity carbon tetrachloride will be administered to the rats and urine samples analysed for marker proteins.

1.6 Carbon tetrachloride (CCl_4)

Carbon tetrachloride (CCl_4) is one of the most studied hepatotoxicants (de Zwart et al., 1998). It is a colourless liquid, which is immiscible with water. It is very lipid soluble, allowing it to cross cell membranes resulting in a good distribution throughout the body (Halliwell and McGutteridge, 1998). CCl_4 causes damage to a number of organs (e.g. kidney and lungs) but the liver is the main target. Other tissues can accumulate higher concentrations of CCl_4 (Slater, 1966) but the major damage induced occurs in the liver since CCl_4 is metabolised here to form toxic metabolites (Slater, 1978). The main toxicological event in the case of CCl_4 appears to be the initiation of lipid peroxidation, involving free-radical reactions and not the covalent binding of the metabolites of CCl_4 (Recknagel et al., 1977)

CCl_4 is metabolised in the liver endoplasmic reticulum (Slater, 1966; Recknagel, 1967, Castro et al., 1973; Recknagel and Glende, 1973) by the cytochrome P-450 system (Wolf et al., 1977; Masuda and Murano, 1978), in particular by the ethanol inducible cytochrome P-4502E1 (de Zwart et al., 1998; Edwards et al., 1993). The cytochrome

P450 system is an electron transport system located in the endoplasmic reticulum of the liver and consists of a NADPH-specific flavoprotein, which transfers electrons from NADPH to various cytochromes (Recknagel et al., 1977). Studies have shown that CCl_4 is not toxic to newborn rats since they do not have a well-developed hepatic cytochrome P450 system, but by seven days after birth the rats acquire sensitivity (Recknagel et al., 1977).

The cytochrome P-450 system acts as an electron donor causing homolytic cleavage of one carbon chlorine bond in CCl_4 to form a trichloromethyl free radical ($\bullet CCl_3$), which is a carbon free radical, and a chloride ion (Recknagel et al., 1977; Poyer et al., 1978; Lai et al., 1979; Mico and Pohl, 1982; Mico et al., 1983). The trichloromethyl free radical may then undergo several reactions both aerobically and anaerobically (Figure 1.9).

In the absence of oxygen the trichloromethyl free radical may directly bind to microsomal lipids and proteins (Rao and Recknagel, 1969). The $\bullet CCl_3$ radical may react with methylene groups on polyunsaturated fatty acids in the smooth endoplasmic reticulum and abstract a hydrogen atom in the formation of chloroform (Figure 1.9) (Glende et al., 1976). The $\bullet CCl_3$ radical can undergo further reduction reactions to form carbon monoxide (Wolf et al., 1977). Cell membranes contain large amounts of polyunsaturated fatty acid side chains; reactive free radicals are able to abstract a hydrogen atom from a methylene group (CH_2), leaving behind an unpaired electron on the carbon. This carbon free radical can either combine with another carbon radical or react with oxygen to form a peroxy radical. The hydrogen atoms on methylene carbons are particularly susceptible to attack by a free radical (Pryor and Stanley, 1975).

Various studies suggest that the $\bullet CCl_3$ radical is not reactive enough to produce the damage caused by CCl_4 (Slater, 1978). The $\bullet CCl_3$ radical in the presence of oxygen undergoes a cascade of events that play an important role in the damage produced by CCl_4 (Slater, 1978). The $\bullet CCl_3$ radical can be oxygenated by the mixed-function oxidase system to produce trichloromethanol, a precursor to phosgene (Shah et al., 1979), which can then homolytically cleave to produce carbon dioxide.

Figure 1.9 shows that the trichloromethyl radical can react with oxygen to form the unstable and highly reactive trichloromethyl peroxy radical ($\bullet OOCCl_3$) (Reynolds and

Molsen, 1979; Halliwell and McGutteridge, 1998). Studies have indicated that the trichloromethyl peroxy radical can be formed by a reaction of the trichloromethyl radical with the superoxide anion ($O_2^{-\bullet}$) (Packer et al., 1978). This unstable $\bullet OOCCl_3$ radical can cleave homolytically to form new free radicals, which attack methylene groups of membrane lipids and abstract hydrogen forming an organic free radical (Recknagel, 1967; Rao and Recknagel, 1969). The newly formed free radical is then able to abstract a methylene hydrogen from a neighbouring unsaturated fatty acid yielding another free radical and a hydroperoxide (Recknagel et al., 1977; Halliwell and Mc Gutteridge, 1998). In this way a linear chain of free radicals is propagated and lipid peroxidation is initiated (Recknagel et al., 1977; Recknagel, 1983; Mehendale, 1989; Recknagel et al. 1989). Lipid peroxidation will eventually lead to a break up of the fatty acid chain, with the production of malonaldehyde (MDA) and various aldehydes and ketones (Dahle et al., 1962 and Pryor and Stanley, 1975, Koster and Slee, 1980). The process can lead to the breakdown of membrane structure and loss of organelle and cell functions.

The process of lipid peroxidation occurs quite rapidly and can be detected within minutes after dosing rats orally with CCl_4 (Recknagel and Glende, 1973; Poli et al., 1990). Within minutes CCl_4 metabolites covalently bind to lipids and proteins in the endoplasmic reticulum (Reynolds and Moslen, 1979). During the first 10 to 15 minutes, the hepatic secretion of triglycerides is blocked, whereas new triglycerides are continuously synthesized, leading to the characteristic fatty liver (Recknagel et al., 1977). Protein synthesis is reduced, the endoplasmic reticulum loses ribosomes, and hepatic protein synthesis fades (Reynolds and Molsen, 1979). The activity of enzymes located in the endoplasmic reticulum e.g. glucose-6-phosphate decreases (Koster and Slee, 1980), and there is a reduction in the ability of the endoplasmic reticulum to sequester Ca^{2+} ions by the Ca^{2+} ATPase, leading to a rise in intracellular Ca^{2+} (Halliwell and Mc Gutteridge, 1998). One to three hours after dosing, triglycerides accumulate as fat droplets and the rough endoplasmic reticulum continues to lose ribosomes, eventually the plasma membrane ruptures. Liver enzymes appear in the plasma and the hepatic electrolyte balance becomes disturbed resulting in an increase in the liver water content (Recknagel et al., 1977). A large dose of CCl_4 will cause widespread hepatocellular necrosis.

1. $CCl_4 \xrightarrow{\text{P-450(2E1)}} \bullet CCl_3 + Cl^-$

Trichloromethyl radical

2. $\bullet CCl_3 \xrightarrow{H^+} CHCl_3 + R^\bullet$

Chloroform

3. $\bullet CCl_3 \xrightarrow{O_2} \bullet CCl_3O_2$

Trichloromethyl peroxy radical

4. $\bullet CCl_3O_2 \xrightarrow{+e^-} \bullet CCl_3O_2^- \xrightarrow{2H^+} CCl_3OH + OH^-$

Trichloromethanol

5. $CCl_3OH \rightarrow \underset{\text{Phosgene}}{COCl_2} + HCl \rightarrow 2HCl + CO_2$

Figure 1.9 Metabolism of carbon tetrachloride by cytochrome P4502E1.
Carbon tetrachloride is metabolised by cytochrome P-4502E1 to form a trichloromethyl free radical and a chloride ion (1). The trichloromethyl free radical is then able to form chloroform by the addition of a hydrogen ion and an electron (2). Under aerobic conditions the trichloromethyl radical reacts with oxygen and methylene groups on membrane lipids to form a series of free radicals involved in lipid peroxidation (3,4). The trichloromethyl peroxy radical can react with hydrogen with the eventual production of phosgene gas (5).

The earliest change after dosing with CCl_4 can be seen in the endoplasmic reticulum of hepatocytes in the centrilobular zone of liver lobules (Slater, 1978). This localisation is a result of the high concentration of cytochrome P-450 enzymes in this area and the very short half-life of the trichloromethyl free radical. The half-life of the trichloromethyl free radical has been calculated to be about 100 μs (Slater, 1976) therefore the damage produced by the radical is limited to the vicinity of the P-450

enzymes. This high reactivity and short half-life would ensure that the trichloromethyl radical reacts rapidly at the site of origin. However, since the trichloromethyl free radical is chemically very reactive it readily reacts with neighbouring membrane lipids forming free radicals, which have longer half-lives and can diffuse within the endoplasmic membrane causing damage (Slater, 1978). The process of lipid peroxidation produces short chain peroxides, which have longer half-lives than the primary products of the homolytic clevage of the CCl_3-Cl bond, and can diffuse out of the endoplasmic reticulum membrane and cause damage further away from the centrilobular region (Recknagel et al., 1977).

Small doses of CCl_4 act by destroying hepatic cytochrome P-450 and such a response may therefore protect the liver from further larger doses (Glende, 1972). The administration of a small dose of CCl_4 provides maximum protection against a lethal dose 24 hours later when the cytochrome P450 content is reduced to 30-35 % of normal. This period of protection lasts for about 4 days (Glende, 1972; Recknagel et al., 1977). It is possible that the trichloromethyl free radical or other free radicals derived from it directly attack the cytochrome P450 (Halliwell and Mc Gutteridge, 1998). Studies have shown that the loss of liver cytochrome P450 and microsomal glucose-6-phosphatase are dependent on lipid peroxidation and not on covalent binding of the products of CCl_4 metabolism (Glende, 1972; Glende et al., 1976). It had been suggested that this pre-treatment with CCl_4 induces a higher liver regenerative activity following the high dose exposure, thereby ensuring minimal damage to the high dose exposure (Rao and Mehendale, 1991).

CCl_4 induces fatty liver and hydropic degeneration of hepatocytes, but the primary hepatic lesion induced by CCl_4 is centrilobular necrosis and the severity of this change is dependent on the dose of CCl_4. The main damage to the liver occurs in the centrilobular area since this region contains the highest concentration of cytochrome P450 metabolising enzymes. CCl_4 may also induce the activation of Kupffer cells by increasing intracellular calcium. CCl_4 has been shown to inhibit the release of calcium from the hepatocyte endoplasmic reticulum, therefore the cytosolic calcium concentration rises (Long and Moore, 1986). Waller et al., (1983) demonstrated that calcium sequestration is sensitive to both lipid peroxidation and covalent binding of CCl_4 carbon. They inhibited lipid peroxidation using promethazine, or induced lipid peroxidation in the absence of CCl_4 and the results show that calcium sequestration is

decreased in both circumstances. They proposed that CCl_4 may have a toxic effect even in the absence of lipid peroxidation and also that the increased intracellular Ca^{2+} may be a pathological link between the generation of the trichloromethyl radical and the damage induced at a distance (Waller et al., 1983). Studies carried out by Berger et al (1987) suggested that CCl_4 exerts injury on hepatocytes in the absence of lipid peroxidation by a direct action of the compound on cell membranes. However these studies were carried out *in vitro* and they may therefore not be relevant to an *in vivo* situation.

Kupffer cells are the resident macrophages of the liver and when activated they may release cytokines, which can contribute to hepatocyte death (Birmelin and Decker, 1983; Decker et al., 1989). The process of lipid peroxidation induces cell damage, which may stimulate the perisinusoidal cells to release extracellular matrix proteins leading to hepatic fibrogenesis.

The hepatotoxic effects of CCl_4 depend not only on the metabolism by the P450 system but also on the regenerative capacity of the hepatocytes (Mehendale, 1990). Following treatment with CCl_4 the P450 activity is greatly decreased and its return to control levels coincides with regeneration of the liver cells (Cockerill et al., 1983). Pre-treatment of animals with a P450 inducing agent such as phenobarbital (Noguchi et al., 1982a; 1982b), or ethanol renders the animal more susceptible to the toxic effects of CCl_4 and therefore lipid peroxidation is increased. In the same way, P450 inhibitors decrease the CCl_4 toxicity. Studies have shown that the administration of garlic and vitamins inhibits cytochrome P450 and therefore acts to protect the liver from the toxic effects of CCl_4 (Sheweita et al., 2001). The administration of CCl_4 has been shown to induce hepatocyte growth factor (HGF) in Kupffer cells of damaged livers. HGF stimulates the regeneration of hepatocytes following injury (Noji et al., 1990).

Other tissues have been shown to contain small levels of the cytochrome P450 2E1 enzyme, such as the mucosa of the bronchial tree, the tracheal mucosa, the olfactory and respiratory mucosa and the kidney cortex, therefore these extrahepatic sites also possess the ability to metabolise CCl_4 (Tjälve and Löfberg, 1983).

1.7 Proteomics

Proteomics has been defined as "the use of quantitative protein-level measurements of gene expression to characterize biological processes and decipher the mechanisms of gene expression control" (Anderson and Anderson, 1998). The proteome is a representation of the proteins that are expressed by any particular cell at a particular time, under a particular set of conditions (Beranova-Giorgianni, 2003). The term proteome may also be used to describe the total complement of proteins found in a compartment or tissue or bodily fluid. However in each of these cases the term proteome could reflect a protein complement that has been acquired rather than expressed. Changes in the protein complement of for example the liver, kidney, plasma or urine may reveal toxicity or a disease state. This project aims to analyse the protein complement of urine as a means of assessing hepatotoxicity.

Proteomics has developed rapidly over the past ten years with the increasing need to understand all the biological mechanisms of organisms and the realization that the one gene to one protein concept is not realistic (Stanley, 2002; Leftovits, 2003). It has been estimated that the human genome contains approximately 33,000 genes but as many as 2-300,000 proteins. Genome sequencing projects have produced vast genetic information, but there are many points between transcription and functional protein at which gene products can be modified. Therefore there is poor correlation between the mRNA transcribed and the protein translated (Banks et al., 2000; Yanagida, 2002). Different proteins can be produced by alternative splicing of transcripts and by post-translational modifications; including phosphorylation, glycosylation and acetylation. Other factors that can not be accounted for by genomics include protein-protein interactions and potential protein expression alterations due to the cellular and environmental conditions surrounding the protein e.g. pH and hypoxia (Banks et al., 2000; Pandey and Mann, 2000). Therefore, alterations in protein expression at the cellular level will not be reflected by genomic analysis. In this way the proteomic approach is necessary for providing a more complete picture, since the measurement of proteins reflect the actual state of the system (Wasinger and Corthals 2002).

Proteomics can be divided into three main categories: expression proteomics, bioinformatics, and functional proteomics (Klein and Thongboonkerd, 2004)

Expression proteomics aims to compare the protein expression between different cells or tissues. It is useful for identifying proteins, which are increased or decreased as a result of a disease state; therefore comparative protein expression maps can be useful as diagnostic tools. This type of proteomics is carried out mainly using two-dimensional gel electrophoresis and mass spectrometry. Other methods, which allow quantitative analysis include isotope coded affinity tags (ICAT) and difference gel electrophoresis (DIGE).

Bioinformatic analysis of the identified protein provides additional information including the primary, secondary and tertiary structure of the protein, homology, motifs and domains, potential post-translational modifications and subcellular locations.

Functional proteomics is the study of existing protein-protein interactions, protein complexes and post-translational modifications. Many biological events occur via protein-protein interactions (Wasinger and Corthals, 2002); therefore the identification of these interactions is of extreme importance. Co-immunoprecipitation techniques followed by 2-D electrophoresis, affinity chromatography, protein chip analysis and tandem affinity purification (TAP) are some of the methods used to analyse interactions between proteins (Klein and Thongboonkerd, 2004). TAP analysis involves the use of affinity tags that are attached to the protein; the protein can then be purified and separated by SDS-PAGE.

This project is concerned with detecting changes in protein composition of rat urine following exposure to a toxic compound. The current methods used for this purpose include gel electrophoresis and mass spectrometry, isotope-coded affinity tagging (ICAT) and surface enhanced laser desorption ionisation protein chip technology (SELDI).

1.7.1 Gel electrophoresis and mass spectrometry

1.7.1.1 Gel electrophoresis

Gel electrophoresis is perhaps the most widely used technique for the analysis of protein expression. SDS-PAGE is a method of electrophoresis that separates proteins according

to their mass. This type of gel electrophoresis involves denaturing the proteins with sodium dodecyl sulphate (SDS), which carries a negative charge. Most proteins will bind the SDS in a constant weight ratio (1.4 g of SDS per g of polypeptide), which masks the intrinsic charge of the polypeptide chains (Reynolds and Tanford, 1970). This means that when SDS binds to the proteins they acquire a net negative charge. When an electric field is applied the proteins move through a gel matrix towards a positive electrode. The use of a discontinuous system, whereby a separate stacking gel is poured on to the top of the resolving gel, increases the resolution of complex protein mixtures (Chiou and Wu, 1999).

The smallest proteins migrate fastest since they easily flow through the matrix. Protein standards are run alongside the protein so that a standard curve can be constructed. By comparing the molecular size of the protein standards, the mass of the proteins in the gel can be estimated. However, many proteins (including glycoproteins and extremely basic or acidic proteins) behave anomalously and produce artefactually high molecular mass estimates (Hames and Rickwood, 1990).

The proteins in the gel can be visualised by staining with a dye such as Coomassie blue, or silver stain. The gels are saturated with a dye containing Coomassie blue R-250 dissolved in an aqueous solution containing methanol and acetic acid, followed by a de-staining step in a similar solution minus the Coomassie blue. Coomassie blue R-250 is a non-polar, sulphated aromatic dye. In an acidic environment, ionic interactions occur between the dye and basic amino acid moieties of the protein, as well as binding due to hydrogen bonding, Van der Waals interactions and hydrophobic bonding. The proteins have a higher affinity for the dye than the gel, ensuring that the protein bands are stained against a clear background. The limit of detection for the Coomassie blue dye is 8-10 ng protein.

The method of silver staining is more sensitive than Coomassie blue and can detect as little as 1 ng of protein. The gel is saturated with a silver nitrate solution so that the silver ions bind to the protein and the less tightly bound ions are washed out of the gel, the protein-bound metal ions are then reduced to form metallic silver. Most proteins stain monochromatically with silver yielding brown or black bands, although some proteins e.g. lipoproteins and glycoproteins stain blue and red, respectively (Goldman et al., 1980). Silver stains are more complex and time-consuming to use than Coomassie

blue stain. Also, some proteins stain very poorly or not at all, appearing as negatively stained bands against a darker background (Wirth and Romano, 1995).

Other less commonly used dyes for protein gels include amido black and fluorescent-based stains such as SYPRO red and SYPRO orange. SYPRO dyes can be visualised using a UV transilluminator since they all have a UV excitation peak of approximately 300 nm. SYPRO red and orange are also excited by visible light at approximately 470 and 550 nm, respectively and have an emission maximum at 570 and 630 nm, respectively (Patton, 2002). Therefore SYPRO orange and red dyed gels can also be visualised using a laser-based fluorescence imager. SYPRO Ruby dye is more sensitive than the other SYPRO dyes and can be excited by a 300 nm UV transilluminator. Alternatively, under visible light SYPRO Ruby dye has an excitation maximum at 450 nm and an emission maximum at 610 nm. SYPRO Ruby dye can detect as little as 1 ng of protein and therefore is as equally sensitive as silver stain. However, SYPRO Ruby is compatible with mass spectrometry whereas silver stain is not.

SDS-PAGE gels can also be visualised by reverse staining techniques, using for example zinc or copper, whereby the proteins are detected as transparent bands when viewed against a black background (Patton et al., 2002).

SDS-PAGE can be used to separate all proteins (Chiou and Wu, 1999). It is a simple, quick and reproducible technique enabling the molecular size of a protein to be determined.

Two-dimensional gel electrophoresis is a high-resolution electrophoretic method in which the proteins are separated first by charge and then by molecular size. This allows for separation of proteins, which migrate as closely spaced protein bands on one-dimensional SDS-PAGE. 2D electrophoresis has the ability to resolve several thousand proteins in a single sample (Lilley et al., 2002). It is used for the separation of complex protein samples, for the comparison of protein expression profiles and for looking at protein responses to certain conditions, e.g. disease states or toxic response to chemicals (Barany et al., 1998; Celis and Gromov, 1999). In proteomics, 2D electrophoresis is still the major separation technique applied to complex mixtures of proteins. Although it is time consuming it has been used as a method for the discovery of biomarkers (Celis and Gromov, 2000).

The protein sample is firstly solubilized in a buffer solution containing a non-ionic detergent, urea and a reducing agent, and then salts and other contaminants are removed. The urea is used to disrupt hydrogen-bonded structures in the sample proteins (Garfin, 2003). Non-ionic or zwitterions detergents (e.g. CHAPS) disrupt the hydrophobic interactions and the reducing agent is added to reduce disulphide bonds. The use of the reducing agent tributyl phosphine (TBP) is preferred over dithiothreitol (DTT) since it is uncharged and therefore will not migrate through the pH gradient.

In the first dimension the sample proteins are separated by isoelectric focussing (IEF), a technique in which the proteins migrate in the presence of a continuous pH gradient until they reach their isoelectric point (pI). At their pI the proteins migrate at a steady state and become focussed into narrow zones. The use of immobilized pH gradients (IPG's) has become widespread. These are standardized acrylamide strips, which have a pH gradient copolymerised within the gel matrix and are supplied as dried gel strips requiring rehydration before use. The IPG strips are made with acrylamide buffers each consisting of a single acidic or basic group that are copolymerised with acrylamide and bis-acrylamide, and therefore the range of the pH gradient is defined by the concentration of the individual acrylamide buffers in the gel. The IPG's are cast on to plastic sheets and remain stable over extended run times at high voltages (Garfin, 2003). The sample protein can be loaded on to the IPG strip during the rehydration step enabling higher protein loading. IEF is best carried out at the highest voltage possible.

The use of IPG strips overcomes some of the problems that existed with the old method of generating pH gradients using carrier ampholytes, such as cathionic drift. IPG strips are generally more reproducible and have increased resolution (Celis and Gromov, 1999). They also allow for the use of either narrow or wide pH ranges, which enables the pH range to be individually chosen for each sample type.

The second step in 2D gel electrophoresis involves SDS-PAGE; the IPG strip is placed on the top of a SDS gel and sealed in with agarose. The focussed proteins are then separated according to their molecular size.

However, after the proteins have been focussed and prior to the SDS-PAGE step, they need to be equilibrated, this is carried out in order to saturate the proteins with SDS and to keep sulphydryl groups reduced and prevent their reoxidation. The IPG strips are

equilibrated in two SDS buffers, the first buffer contains DTT as a reducing agent and in the second buffer the DTT is replaced by iodoacetamide. Iodoacetamide is an alkylating agent, it coverts sulphydryls to carboxyamidecysteine residues, thereby preventing them from recombining to form disulphides (Garfin, 2003).

After the second dimension the proteins can be visualised using Coomassie blue or silver stain. There are numerous image analyses software available, which analyse the proteins in a gel by scanning and then comparing the proteins in one gel with those of other gels. Image-analysis software is able to compare the relative intensities of spots in a gel. By comparison of the presence or absence of a spot in a gel when compared to a control gel, it is possible to determine a cellular response to a particular treatment (Hunter et al., 2002). Spot-excision robots exist which can automatically cut a spot out of a gel for further analysis (Garfin, 2003).

Two-dimensional gel electrophoresis has been used for over 25 years to profile complex protein mixtures (Mahmoud and Righettu, 2003), and has become much more powerful recently due to the combination of 2D gel electrophoresis with mass spectrometry in proteomic studies. This combination allows for the separation of proteins in a mixture, then visualization of these proteins followed by identification (Ong and Pandey, 2001). 2D electrophoresis offers the easiest way to look for changes in protein expression. Post-translational modifications can also be determined by identifying the same protein in two different locations within one gel.

However, the method of 2D electrophoresis is time-consuming and requires the removal of all salts from the sample before analysis. Most staining techniques reveal only moderate to high abundance proteins in a protein sample (Hamdan and Righetti, 2003). It is also not effective at separating membrane proteins, high molecular weight proteins and proteins that are either very basic or very acidic. 2D electrophoresis is mainly a qualitative technique requiring further processing to produce quantitative results.

1.7.1.2 Difference gel electrophoresis (DIGE)

Although 2D gel electrophoresis is useful for comparing the protein expression profile for two different samples, often there are experimental variations between the two gels.

If the two samples can be compared on the same gel then this problem can be overcome; this approach can be applied using DIGE. This technique uses fluorescent cyanine dyes, Cy2, Cy3 and Cy5 to label as many as three different samples. The Cy-dyes have a succinimidyl ester group, which can covalently attach via an amide bond to the amino group of lysine groups in the proteins. The technique has been designed so that only approximately 1-2 % of the lysine residues in the protein are labelled so as to maintain the solubility of the labelled proteins during electrophoresis (Unlu et al., 1997). The labelled samples can then be mixed together and run on the same 2D gel. After electrophoresis the gel is scanned at the three different excitatory wavelengths of the Cy-dyes. The images are overlayed and the ratio of the different fluorescent signals can be used to compare the differences between the protein samples (Patton et al., 2002). Cy-dyes are compatible with mass spectrometry and are as sensitive as both SYPRO Ruby and silver stain.

1.7.1.3 Mass spectrometry

Mass spectrometry has become an important tool in protein identification (Yates, 1998). With the development of electrospray ionisation (ESI) and matrix assisted laser desorption/ionisation (MALDI) techniques, mass spectrometry has become accepted as the method of choice for the identification of low abundance proteins. The major breakthrough in proteomics has been the ability to identify proteins that have been separated on gels, by mass spectrometry (Pandey and Mann, 2000). Mass spectrometry relies on separating charged peptide ions according to their mass to charge ratio (m/z). Mass spectrometry is faster, simpler and more sensitive than traditional sequencing techniques such as Edman degradation (Larsen and Roepstorff, 2000).

MALDI is a useful analytical technique for protein identification because of its high throughput, sensitivity and high mass accuracy (Beranova-Giorgianni, 2003). It is a pulsed ionisation technique, which usually uses a time of flight mass analyser (MALDI-TOF). The idea is based upon the use of a laser, typically a nitrogen laser at 337 nm, which uses pulses of energy rather than a continuous beam of ions to bring about desorption of the sample molecules. The sample is mixed with a matrix; these are small UV-absorbing molecules that are solid at room temperature, but sublime easily when heated with the laser (Figure 1.10). The most commonly used matrices are sinapinic

acid or ferulic acid dissolved in acetonitrile and trifluoroacetic acid (TFA). The sample and matrix are applied to a target plate and allowed to dry so that the matrix crystallizes. The laser uses pulses of energy and can be focussed to submicrometer diameters allowing precise control (Nguyen et al., 1995). The laser strikes the sample and matrix molecules absorb the photon energy provided from the laser. This energy causes rapid heating of the crystals resulting in sublimation of the matrix crystals (Yates, 1998).

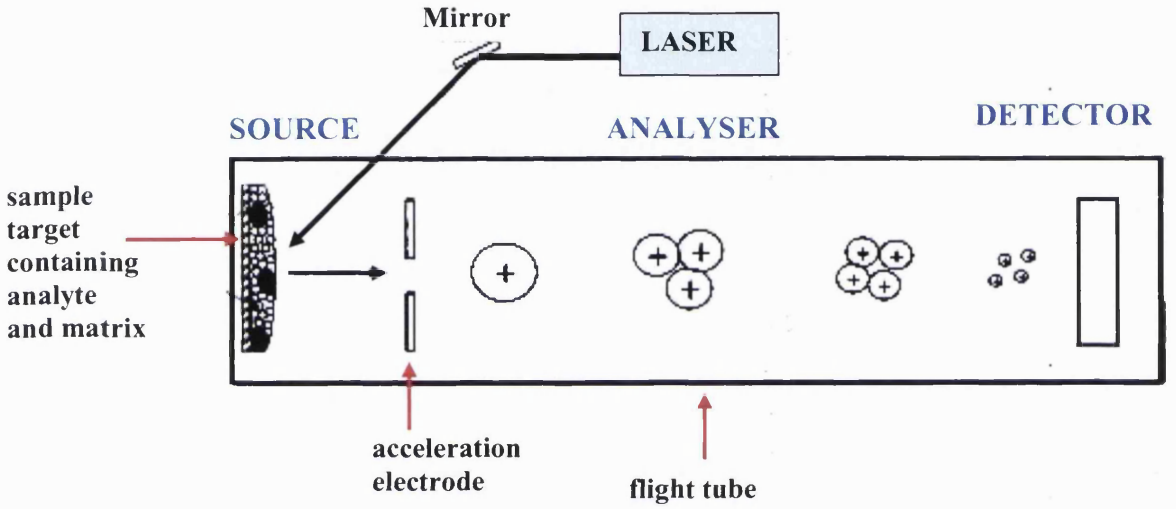


Figure 1.10 Diagram showing the principles of MALDI (Adapted from Yates, 1998). A laser is fired at a sample mixed with matrix. The matrix absorbs the energy from the laser resulting in ionisation of the matrix, which is transferred to the analyte. The desorbed ions can then be analysed using a time of flight mass spectrometer.

It is thought that ions are formed in this vapourised material by proton transfer from the matrix to the sample (van Baar, 2000). But other methods of ionisation have been proposed, such as ion-molecule reactions, disproportionation, gas phase photoionisation, energy pooling, thermal ionisation and desorption of preformed ions (Marvin, 2003). The desorbed ions are then analysed using a time of flight mass spectrometer in either the linear or reflectron mode. The use of an ion reflector overcomes the problems of saturation of the ion detector by large numbers of low molecular weight ions. This would result in less detection of high molecular weight ions (Gevaert and Vandekerckhove, 2000). MALDI creates mainly singly charged ions and is less sensitive to salts in the sample than ESI.

ESI was introduced by Yamashita and Fenn in 1984, and is the method of choice for determining the molecular weight of intact proteins (Kuster and Mann, 1998). ESI is a technique that creates highly charged ions without fragmentation (Yates, 1998).

ESI produces ions as a result of a potential difference, which exists between a capillary needle and the inlet to the mass spectrometer (Figure 1.11). Liquid flowing from the capillary needle acquires a highly charged surface due to the electric field. This high electric field near the tip of the capillary needle results in the capillary operating in the positive ion mode (i.e. it becomes the positive electrode). This means that the positive ions move towards the surface of the liquid, whereas the negative ions move away from the surface leaving a net charge. The positive ions at the surface of the liquid repel each other and this breaks the surface tension of the liquid, forming a cone-jet of highly charged droplets accelerating towards the negative collector electrode (Smyth, 1999).

The highly charged droplets evaporate before they reach the collector; this decreases the droplet size, thereby increasing the surface charge. When the surface charge exceeds the surface tension, the droplet falls apart and evaporates with the production of charged gas phase ions. Nitrogen gas is delivered in the opposite direction to the sample flow; this excludes large droplets and declusters the ions (Nguyen, 1995). The charged ions result from protonation of basic sites or attachment of metal ions to the acidic sites in the molecules. The distribution of these charged ions is therefore represented by the mass spectrum (Nguyen et al., 1995).

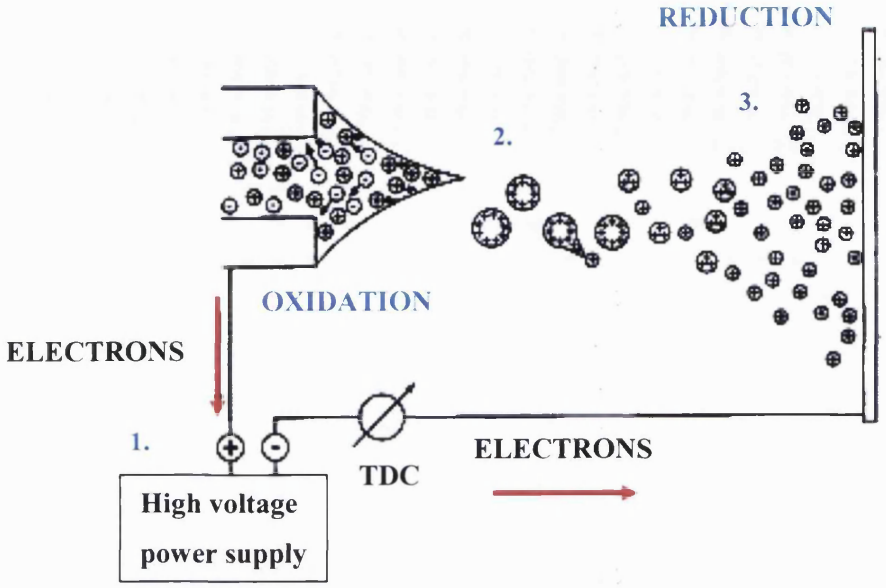


Figure 1.11 The formation of highly charged droplets during the process of electrospray ionisation (Adapted from Smyth, 1999). A voltage is applied to the capillary resulting in the production of highly charged droplets (1). The droplets shrink due to solvent evaporation (2) followed by the production of charged ions (3).

The use of low solvent flow rates (nanoelectrospray) results in the formation of smaller droplets, which allows the bulk of the spray to be directed into the mass spectrometer inlet (Lin et al., 2003).

ESI produces multiply charged ions, which lowers the m/z values for higher molecular weight compounds. The charge state is indicated by the m/z separation, whereby adjacent peaks differ by only one charge and the charge is due to the protonation or addition of another ion to the molecular ion.

Both MALDI and ESI are described as soft ionisation techniques due to the mimimal extent of analyte fragmentation during both processes (Aebersold, 2003).

The most commonly used mass analysers include time of flight (TOF), ion trap, quadrupole and fourier transform ion cyclotron (FT-MS) (Aebersold and Mann, 2003). The time of flight (TOF) analyser provides high-sensitivity molecular mass measurements (Cotter et al., 1994). The TOF analyser determines the mass to charge ratio of ions by measuring the time it takes for them to travel through a field free tube.

The ions are pulsed down a flight tube; smaller mass ions have a greater velocity than ions of a larger mass. The ions therefore reach the detector at different times and the arrival time is related to mass (Willoughby et al., 1998). The use of an ion reflector at the end of the flight tube can compensate for minor kinetic energy differences between ions of the same m/z value (Yates, 1998).

Ion traps consist of two end traps and a ring; the ions are trapped in a three-dimensional electric field. Helium gas is introduced to the trap, which collides with the ions causing them to move towards and become focussed in the centre of the trap (Willoughby et al., 1998). Mass spectrum is then obtained by causing instability in the trajectories of the ions, which forces them to be ejected into a detector one at a time (Yates, 1998). The ion trap can be manipulated to eject ions of a particular m/z ratio to the detector.

The FT-MS analyser also works as a trapping mass spectrometer, it consists of a cell contained within a high vacuum chamber centred in a very high magnetic field. Ions are trapped in the cell and rotate around the magnetic field with a frequency related to their m/z ratio. The frequency of ion rotation is measured via the induction of an electric current by detector plates. The frequency of rotation can be converted to mass through a fourier transformer (Willoughby et al., 1998).

The simplest method for identifying proteins, which have been separated by electrophoresis is by peptide mass fingerprinting (PMF) (Nyman, 2001). The protein spot or band of interest is digested in-gel with a proteolytic enzyme, e.g. trypsin, and the resulting peptides extracted from the gel and the molecular mass of the peptides measured. Trypsin is the most commonly used enzyme for in-gel digests since it cleaves proteins specifically after arginine and lysine residues. Proteolytic digestion produces a series of peptide masses that are unique for a specific protein. These molecular masses are then compared with theoretical masses obtained from a protein database (Nguyen et al., 1995). The method can be carried out using either MALDI or ESI and the interpretation is relatively straightforward since most peptides carry only one charge and are therefore represented by a single peak in the spectrum (Rowley et al., 2000). The peptide map, or fingerprint obtained is compared to theoretical maps obtained from the protein database.

However, the protein sequence must be present in the database for successful identification and proteins with many translational modifications may be difficult to identify (Larsen and Roepstorff, 2000; Henzel et al., 2003).

Post source decay (PSD) describes a technique, which can be carried out for peptide sequencing. This is based on the knowledge that metastable ions are formed during the MALDI ionisation process (Gevaert and Vandekerckhove, 2000). Analyte ions travelling through the time of flight instrument collide with matrix ions resulting in fragmentation. These fragment ions travel with almost the same velocity as the precursor ions but with lower kinetic energy (Nguyen et al., 1995). By replacing the detector with a reflector, which reverses the flight of the ions, the fragment ions can be separated from their precursors. The fragment ions have lower kinetic energy and do not travel as deep into the reflector. Therefore, they leave the reflector earlier and arrive at the detector sooner than the precursor ions and appear at lower apparent mass in the spectra. The reflectron field has to be calibrated using known fragment masses of synthetic peptides. It has been suggested that the PSD is due mainly to the result of amide bond cleavage (Gevaert and Vandekerckhove, 2000). However, due to the high complexity of the fragmentation patterns, and a low mass accuracy of high mass fragments MALDI-PSD is rarely used for protein identification.

Peptide sequence information can also be obtained by electrospray tandem mass spectrometry (ESI-MS/MS). The protein sample is proteolytically digested and the peptide masses separated. Then one of the peptides is selected by the first mass analyser and refragmented by collision with a neutral gas such as argon or helium to produce fragment ions. This process is referred to as collision-induced dissociation (CID) (Nguyen et al., 1995). Peptides generally fragment at the amide bond to generate N-terminal (b-type) ions and/or C-terminal (y-type) ions (Larsen and Roepstorff, 2000). Each of the fragment ions represents part of the amino acid sequence. Subtracting the masses of adjacent fragment ions identifies the amino acid by which they differ and reveals the sequence of the selected peptide. The identity of the whole protein suggested by the peptide mass fingerprinting can now be confirmed from the database by searching for the peptide sequence determined. This dual approach increases the probability of identifying the protein.

Proteomic studies using mass spectrometry as the analytical tool have been successful in the analysis of rat liver proteins that may be of interest in toxicological studies (Fountoulakis and Suter, 2002; Thome-Kromer et al., 2003). The use of high performance liquid chromatography (HPLC) coupled to mass spectrometry (LC-MS) has become widespread as a method of removing contaminants and to separate complex mixtures of peptides before analysis by mass spectrometry (Yates, 1998; Wolters et al., 2001; Hunter et al., 2002; Mazza et al., 2003). LC-MS has been used in the analysis of the male human urinary proteome (Spahr et al., 2001). Andersen et al. (2002) described a study on the proteomic analysis of the human nucleoli using mass spectrometry combined with sequence database searching.

A combination of 2D gel electrophoresis and MALDI-TOF has been used for the discovery of tumour markers, several proteins linked to Alzheimers disease and the identification of autoantibodies involved in rheumatoid arthritis (Marvin et al., 2003).

Mass spectrometry has played a useful role in the field of biochemical genetics and newborn screening (Carpenter and Wiley, 2002). It has been used to detect protein variants in blood and tissue in the diagnosis of diseases such as amyotrophic lateral sclerosis (Shimizu et al., 2002).

1.7.2 Isotope-coded affinity tagging (ICAT) and mass spectrometry

Recent developments in mass spectrometry include the use of isotope-coded affinity tagging (ICAT) for protein quantitation (Steen and Pandey, 2002). The ICAT reagents consist of a protein-reactive group, a linker region and a biotin tag and are used to specifically label the cysteine residues in a protein. A reduced protein sample is coupled with an isotopically heavy version of the ICAT reagent while a second protein sample is coupled to a light version. The heavy version (the d8 reagent) differs from the light version (the d0 reagent) only in that eight hydrogen atoms have been replaced with deuterium atoms (Wasinger and Corthals, 2002). The two samples are then combined and digested with a protease e.g. trypsin. The cysteine-containing peptides are then separated from the protein sample using avidin affinity chromatography (Patton et al., 2002). Avidin binds to biotin so the biotin tagged peptides will be purified. The light and heavy versions of the ICAT reagent differ only by 8 Da and therefore mass

spectrometry can be used to determine the relative amounts of each protein in the sample.

However there are limitations to the technique in that proteins that do not contain cysteine residues can not be detected by ICAT. The ICAT molecule is relatively large and may interfere with the ionisation of some smaller peptides therefore leading to difficulties when interpreting mass spectra.

1.7.3 Surface Enhanced Laser Desorption/Ionisation ProteinChip technology (SELDI)

Surface Enhanced Laser Desorption/Ionisation ProteinChip technology (SELDI) combines chromatography and mass spectrometry to provide a rapid protein expression profile (Issaq et al., 2002). It was originally described in 1993 by Hutchens and Yip and since then has undergone remarkable progress as an analytical technique (Tang et al., 2004). Proteins are applied to an array surface, which has been chemically or biologically modified for the retention of proteins of interest from biological samples and the bound proteins are then analysed directly by mass spectrometry.

The most important elements in the construction of a protein array include the generation of a large number of recognition molecules to which the protein of interest will interact, the methods by which the protein will become immobilised to the array, and the detection of the proteins which have interacted with the protein array (Jenkins and Pennington, 2001). Ideally a protein array would bind only those proteins of interest and have very low non-specific binding (Tang et al., 2004). Also, the mass spectrum should contain no array-mediated noise.

Array based proteomics has developed following the success of many genome projects resulting in the necessity to create parallel high-throughout analysis of proteins (Albala, 2001). Each protein array has at least eight spots to which sample can be applied, this coupled with the fact that the array can be modified for a particular sample type makes protein arrays ideal for high-throughout screening using robotic, imaging or analytical methods (Emili and Cagney, 2000).

SELDI allows the preparation and purification of the sample to be carried out directly on the protein array thereby eliminating any sample losses and enabling the analysis of small sample amounts. Standard methods of sample purification prior to mass spectrometry such as liquid chromatography, membrane dialysis and immunoprecipitation are not only time-consuming but require greater sample volume and may result in sample dilution or sample loss (Merchant and Weinberger, 2000).

The SELDI ProteinChip system is most effective at analysing low molecular weight proteins (i.e. < 20 kDa) and requires less sample than 2D-PAGE (Issaq et al., 2002). SELDI is used to analyse proteins direct from crude biological samples e.g. serum, urine or tissue homogenates with limited sample preparation.

Prior to the development of the SELDI ProteinChip system, protein arrays had been designed using a variety of surfaces including nitrocellulose, polyvinylidene fluoride (PVDF), silicone, glass and plastic. But these are all flexible surfaces and much care was required when using them (Albala, 2001). Several types of protein chips have been developed using glass slides, porous gel pads and microwells (Figeys and Pinto, 2001; Zhu and Snyder, 2001 and 2003; Glokler and Angenendt, 2003).

SELDI-TOF technology consists of three major components: the ProteinChip Array, the system reader and the software. The ProteinChip arrays are made of aluminium and contain spots up to 1 mm in diameter formatted into 8 to 24 distinct regions on the array. Each of the spots has a chemically (anionic, cationic, hydrophobic, hydrophilic etc.) or biologically modified surface (antibody, receptor, enzyme etc.) for the retention of proteins of interest. The surface of the chip therefore allows for sample fractionation and purification of the sample (Tang et al., 2004)

A crude sample is applied to the spot and left to incubate for a few minutes so that proteins with specific affinity for the binding sites on the chip will bind to the spots. Then non-specific binding proteins and interfering substances are removed by washing, a process known as "retentate mapping". By increasing the stringency of the wash conditions, for example increasing the pH or salt concentration, different proteins will be retained on the chip surface (Chapman 2002; Weinberger et al., 2002). An EAM solution is applied to the ProteinChip array and the chip is inserted into the ProteinChip reader.

The ProteinChip reader is a laser desorption ionisation time of flight mass spectrometer equipped with a pulsing nitrogen laser. When the laser is fired at the spot the matrix solution transfers laser energy into kinetic energy and becomes irradiated and the energy is emitted in the form of heat. This rapid heating causes the matrix and sample to detach from the protein array (desorption) and release gaseous ions from the ProteinChip arrays (Fetsch et al., 2002). The gaseous ions then travel through a vaccum chamber until they reach a detector. The detector measures the mass to charge (m/z) ratio of each protein based on the velocity through the ion chamber (Issaq et al., 2002). Time lag focussing is used to increase the mass accuracy of the final output. Signal processing is promoted by a high-speed analog-to-digital converter (A/D), which is linked to a computer (Merchant and Weinberger, 2000). The detected proteins are then displayed as a series of peaks (protein fingerprint) on graphs of mass (x-axis) versus abundance or intensity (y-axis) (Fetsch et al., 2002). The results can be converted using the software from peak trace graphs into simulated 1D gel electrophoresis displays (Figure 1.12).

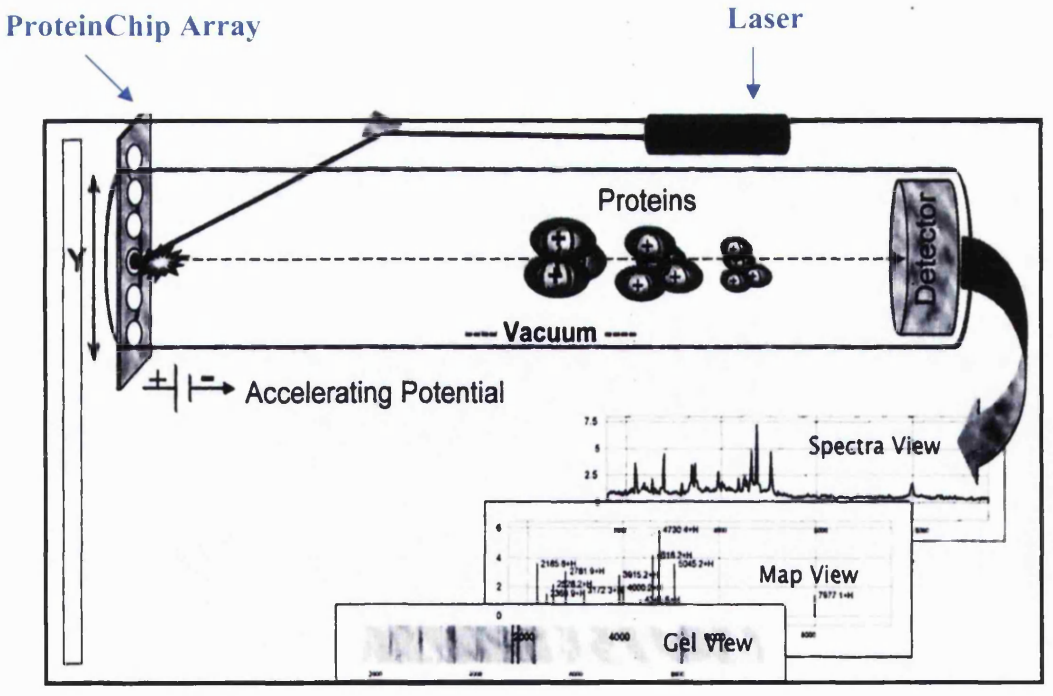


Figure 1.12 A schematic diagram of the Ciphergen Surface Enhanced Laser Desorption/Ionisation Mass Spectrometer (SELDI).

Chemically modified chip surfaces are used to capture a group of proteins with specific chemical/physical properties, whereas chips with biologically modified surfaces are used to isolate a specific protein of interest. The different types of ProteinChip surfaces available include:

1. **Ionic Chips:** Ionic chips have either a cationic or anionic surfaces and bind proteins through an electrostatic interaction:

   a) Cationic Exchanger: The surface of these chips has carboxylate groups and therefore they have an overall anionic charge. Proteins containing a net positive charge at the pH used will bind to the surface. The optimum binding conditions are a low pH and low salt concentrations.

   b) Anionic Exchanger: These chips have a cationic surface as a result of the ammonium groups present on the surface. Anionic chips will bind proteins containing a net negative charge at the pH used. Binding requires high pH and low salt concentrations.

2. **Hydrophobic Chips:** Hydrophobic groups are present on the chip surface. The chip will bind proteins containing hydrophobic residues; e.g. alanine, valine and leucine via a hydrophobic interaction. High salt concentrations and aqueous conditions are necessary for binding.

3. **Normal Phase Chip:** Normal Phase Chips have a silicon dioxide (SiO_2) surface. Proteins bind to these chips through hydrophilic and charged residues on their surfaces via electrostatic interactions or hydrogen bonds.

4. **Immobilised Metal Affinity Capture (IMAC):** IMAC chips have a nitrilotriacetic acid surface. The chips will bind proteins or peptides that have an affinity for metal, e.g. proteins with exposed histidine or tryptophan residues. Optimum binding conditions are high salt concentrations and a pH of 6-8.

5. **Antibody-antigen:** These ProteinChip Arrays are designed to have an antibody attached to the surface, which is specific for a protein of interest in the sample.

6. Receptor-ligand Chip: Cell surface or soluble receptors can be attached to a chip and used to bind ligands.

7. DNA-protein: These chips contain nucleic acid molecules (such as sequence specific DNA), which can be used to bind proteins from complex solutions.

Potential biomarkers of diseases such as Alzheimer's disease have been discovered by SELDI analysis. SELDI has been successfully used to detect prostate cancer at an early stage in patients, and protein fingerprint profiles have been generated of serum samples obtained from patients with breast cancers (Paweletz et al., 2001; Laronga et al., 2003-2004). ProteinChip Arrays have also been used to characterise protein-protein interactions, phosphorylated and glycosylated proteins. SELDI arrays have been applied in the production of recombinant proteins from bench-top to high-volume scales (Tang et al., 2004). Studies have been carried out using SELDI as a method to identify biomarkers in urine samples (Vlahou et al., 2001; Dare et al., 2002). This method of searching for biomarkers is highly desirable due to the non-invasive nature of obtaining the sample

An advantage that SELDI holds over traditional proteomic methods is that once a potential biomarker has been identified it is then possible to apply the particular array surface and conditions to rapidly screen a much larger sample size (Chapman, 2002). In this way the entire process from biomarker discovery, to identification and validation can be carried out on the same SELDI platform (Tang et al., 2004).

SELDI offers a rapid and straightforward means of identifying proteins as a result of on-chip binding and detection, it is capable of comparing hundreds to thousands of proteins in a small sample volume (Bischoff and Luider, 2004). Unfortunately SELDI generates enormous amounts of data that can be difficult to analyse visually and the test platform can not identify specific proteins corresponding to peaks of interest (Fetsch et al., 2002). However, software exists that can deconvolute the large data produced to find potential biomarkers, important information can be extracted through the use of mathematical algorithms, which are applied to differential protein-profile data sets (Tang et al., 2004). It is also possible to generate peptide maps and therefore identify the proteins of interest

by direct on-chip digestion with a proteolytic enzyme (e.g. trypsin) and then the tryptic peptides can be analysed using the SELDI-TOF system.

1.7.4 Protein databases

The use of mass spectrometry in proteomics has been made easier by the development of sensitive and rapid searching algorithms (Rowley et al., 2000). Many database programmes exist for searching data obtained from mass spectrometry such as peptide mass maps and amino acid sequences. Spectra obtained experimentally are matched with theoretically produced fingerprints. Such sequence databases are still growing exponentially ensuring that protein identification will become easier and more reliable.

In this project we used gel electrophoresis and mass spectrometry and SELDI (techniques described in sections 1.7.1 and 1.7.2) to analyse the rat urinary proteome for changes in protein expression in response to CCl_4-induced hepatotoxicity.

1.8 Aim of this project

The objective of this project is to identify novel markers of liver toxicity using non-invasive methods. Chemicals and drugs may induce organ toxicity as unwanted side-effects. It would be useful if these toxic responses could be detected at an early stage and without the need for invasive techniques such as biopsy and serum analysis. In this project liver injury will be induced in Han Wistar rats by the administration of carbon tetrachloride since it is a widely used hepatoxicant producing centrilobular necrosis in acute adminstration and fibrosis/cirrhosis following repeated dosing. Models of necrosis and fibrosis will be developed in the Han Wistar rat. In this project the non-invasive method chosen is urine, since samples are easily collected and this method causes minimal distress to the animal.

Therefore, rat urine samples will be analysed as a means of detecting protein markers for the developing lesions. Urine samples will be analysed initially using 1D gel electrophoresis and by SELDI to look for the appearance of liver specific proteins appearing in the rat urine following hepatic injury.

Proteins that are detected in the urine sample following 1D gel electrophoresis will be identified by mass spectrometry. This would involve carrying out an in-gel trypsin digest of the protein bands and analysing the digest using nano-electrospray tandem mass spectrometry (nano-ES-MS/MS). The MS/MS data obtained would be searched using a protein database.

However, CCl_4 can induce damage in a number of other organs besides the liver and therefore it will be important to ensure that proteins appearing in the urine from rats treated with CCl_4 are liver specific. It will therefore be necessary in this project to measure serum enzymes and carry out histological analysis of the liver tissue in order to determine hepatic injury.

A good protein biomarker should reflect the early changes induced by CCl_4 and should be sensitive to low dose levels. Therefore in this project studies will be carried out to investigate both of these factors in mature animals.

Two-dimensional gel electrophoresis will be used to look for proteins which may not be detected by 1D SDS-PAGE or by SELDI. This method will provide a greater display of the proteins expressed in the urine sample.

Chapter two

Materials and Methods

2.1 Materials

The one-dimensional gel electrophoresis equipment; isoelectric focussing equipment; Trans-Blot semi-dry electrophoretic transfer cell; ReadyStrip IPG strips; 3-(3-cholamidopropyl)dimethylammonio)-1-propanesulfonate (CHAPS); tributylphosphine; Bio-lytes ampholyte buffer; electrode wicks; mineral oil and goat anti-rabbit IgG horseradish peroxidase conjugate, were all supplied by Bio-Rad laboratories, Hemel Hempstead, Hertfordshire, UK.

The Temed, Protogel and Coomassie blue R-250 came from National Diagnostics, Itlings Lane, Hessle, Hull, UK. The Superdex 75 resin; rainbow markers and ECL western blotting reagents came from Amersham BioSciences, Amersham, Buckinghamshire, UK. The PVDF Western blotting membrane came from Perkin Elmer Life Sciences Inc, Boston, USA.

The iodoacetamide, Tween 20, bovine serum albumin, sodium azide, copper/zinc superoxide dismutase, ammonium persulphate, urea, agarose, sodium lauryl sulphate, formaldehyde, 2-mercaptoethanol, cytochrome c, α-lactalbumin, myoglobin, trypsin inhibitor, carbonic anhydrase, glyceraldehye-3-P dehydrogenase, chick egg ovalbumin, trypsinogen and bromophenol blue, soya bean trypsin inhibitor (SBTI), benzamidine, phenylmethanesulfonyl fluoride (PMSF) and Commassie blue were supplied by Sigma-Aldrich company Ltd, Gillingham, Dorset, UK.

The SOD-1 primary antibody came from Santa Cruz Biotechnology, Inc, as supplied by Autogen Bioclear UK Ltd, Wiltshire, UK. The albumin anti-rat antibody came from Biogenesis, Poole, Dorset, UK.

Citric acid and trifluoroacetic acid (TFA) came from Avocado Research Chemicals Ltd, Heysham, Lancs, UK. Acros Organics, Greel, Belgium supplied the silver nitrate. The dithiothreitol (DTT) came from Melford Laboratories, Ipswich, Suffolk, UK. The sequencing grade modified trypsin was supplied by Promega, Southampton, UK.

The Ransod assay kit for measuring the activity of superoxide dismutase came from Randox Laboratories, Antrim, N. Ireland. Multistix® 10 SG reagent strips used for urine analysis were supplied by Bayer plc, Newbury, Berks, UK.

Millipore Corporation, Bedfore, USA supplied the C18 zip tips. The heptafluorobutyric acid was supplied by Lancaster Third Party, via Merck, Lutterworth, Leicester, UK.

Rats were supplied by B and K Universal Ltd, Grimston, Aldbrough, Hull, UK; the metabolism cages were manufactured by Techniplast, Kettering, Northants, UK. The animal diet came from SDS Ltd, Wiltham, Essex, UK. The pentobarbitone sodium (Sagatal) used during the post mortems came from Rhone Merieux, Harlow, Essex, UK. Carbon tetrachloride was supplied by Fluka Chemicals, New Road, Gillingham, Dorset, UK. Serum separator tubes were supplied by Beckton and Dickinson and Co., Franklin Lakes, NJ, USA. Reagents for ALP, ALT and AST, GLDH, bilirubin, urea and creatinine were supplied by Roche Diagnostics Ltd, Lewes, Sussex, UK.

All other chemicals and standards used in this project were obtained from BDH, Poole, Dorset, UK.

2.2 Animals and animal husbandry

Female Han Wistar rats of 180 – 210 g were used throughout, caged in groups of five. After arrival the animals were allowed to acclimatise, for at least 7 days before the start of each experiment, in communal cages. When in communal cages, animals had access to diet and water *ad libitum.* The temperature of the room was controlled at 19 - 21° C with a relative humidity of 45 to 65 % and the lighting was fixed to give a regular 12-hour light and 12-hour dark cycle, (lights on at 7 am). Animals were weighed before dosing and at appropriate times and were observed for signs of ill health throughout each experiment. All animal procedures were carried out under local Ethical Committee guidelines and approval and followed the Home Office (1986) "Code of Practice for the Housing and Care of Animals Used in Scientific Procedures". When urine was being collected, the animals were placed individually in metabolism cages, which are designed to separate urine from faeces. In the metabolism cages the animals were allowed water, but not diet. Animals were weighed before being placed in metabolism cages and when removed and at other appropriate times. Urine was collected over ice to minimize the growth of bacteria.

2.3 Administration of carbon tetrachloride (CCl_4)

CCl_4 was dissolved in vegetable oil and administered by gavage; the concentration of CCl_4 was adjusted so that the maximum volume received by all rats was 2 ml. Control rats received 2 ml of vegetable oil by the same route. Animals were starved overnight prior to dosing.

2.4 Post mortem procedure

Animals were killed by intraperitoneal injection of pentobarbitone sodium (Sagatal) and exsanguinated from the abdominal aorta into Microtainers (Becton and Dickinson and Co. Rutherford N.J. USA) for the separation of serum. After 2 – 3 hours the Microtainers were centrifuged at 5000 rpm for 5 minutes at room temperature. The serum was removed and stored at – 80 ° C for later analysis. Observations were made both of the external and internal condition of the animals and these were recorded. The liver and kidneys were removed and weighed; liver and kidney weights were expressed as weight in grams per kilogram of body weight (relative weight). Sections of the liver lobes and both kidneys were placed in 10.5 % phosphate buffered formalin, processed and sectioned before staining with either haematoxylin and eosin or Sirius red. Tissues to be homogenised were removed from the rat and freeze-clamped in liquid nitrogen. The tissues were stored at – 80 ° C until processed.

2.5 Serum analysis

Serum samples were analysed spectrophotometrically at 37 °C on a Boehringer-Hitachi 917 automated clinical chemistry analyser (Roche Diagnostics Ltd, Lewes, Sussex, UK. The following serum parameters were measured; alanine aminotransferase (ALT), asparate aminotransferase (AST) alkaline phosphatase (ALP), glutamate dehydrogenase (GLDH), lactate dehydrogenase (LDH), creatine kinase (CK), bilirubin, bile acids, haptoglobin, albumin, creatinine and urea.

The assays used to measure the activity of ALT and AST were optimised and followed the International Federation of Clinical Chemistry and Laboratory Medicine (IFCC) method. Pyridoxal-5'-phosphate was added as a co-enzyme in both reactions. The ALT assay is based on the following reaction:

$$\text{L-alanine} + \alpha\text{-oxoglutarate} \xleftrightarrow[\text{P-5'-P}]{\text{ALT}} \text{pyruvate} + \text{L-glutamate}$$

$$\text{pyruvate} + \text{NADH} + \text{H}^+ \xrightarrow{\text{LDH}} \text{L-lactate} + \text{NAD}^+ + \text{H}_2\text{O}$$

Whereby LDH acts as a catalyst for the conversion of pyruvate to L-lactate. The AST assay is based on the following reactions:

$$\text{L-asparate} + \alpha\text{-oxoglutarate} \xleftrightarrow[\text{P-5'-P}]{\text{AST}} \text{oxalacetate} + \text{L-glutarate}$$

$$\text{oxaloacetate} + \text{NADH} + \text{H}^+ \xrightarrow{\text{MDH}} \text{L-malate} + \text{NAD} + \text{H}_2\text{O}$$

In the AST assay malate dehydrogenase (MDH) catalyses the second reaction. For both assays the oxidation of NADH to NAD^+ can be measured as a decrease in absorbance at 340 nm and this is directly proportional to enzyme activity.

The GLDH assay follows the kinetic Deutsche Gesellschaft Für Klinische Chemie (DGKC) method. The reaction that occurs is as follows:

$$\text{L-glutamate} + \text{NAD}^+ \xleftrightarrow{\text{GLDH}} \text{2-oxoglutarate} + \text{NADH} + \text{H}^+$$

The assay conditions favour the reverse reaction and the decrease in absorbance at 340 nm can be measured as before.

The ALP assay is based upon the following reaction:

$$\text{4-nitrophenol phosphate} \xrightarrow{\text{ALP}} \underset{\text{(colourless benzenoid form)}}{\text{4-nitrophenoxide}} \xleftrightarrow[\text{at alkaline pH}]{\text{rearranges}} \underset{\text{Yellow quinoid form}}{\text{4-nitrophenoxide}}$$

The assay is carried out at alkaline pH and therefore the reaction can be followed by observing the rate of formation of the yellow colour. This assay uses diethanolamine (DEA) as a buffer since it enhances the rate of phosphatase action.
In the LDH assay LDH acts as a catalyst in the following reaction:

$$\text{L - lactate} + NAD^+ \underset{\text{pH 7.4 – 7.8}}{\overset{\text{LDH}}{\xleftarrow{\text{pH 8.8 - 9.8}}}} \text{pyruvate} + NADH + H^+$$

Under the conditions of the assay the reverse reaction is favoured and therefore the assay measures the change in absorbance at 340 nm produced by the oxidation of NADH.

The method used for the determination of CK is based on the following reactions:

$$\text{creatine} + ATP \xrightarrow[\text{pH 9.0}]{\text{CK}} CrP + ADP$$

$$ADP + \text{phosphoenolpyruvate} \xrightarrow{\text{PK}} ATP + \text{pyruvate}$$

$$\text{pyruvate} + NADH + H^+ \xleftrightarrow{\text{LDH}} \text{lactate} + NAD^+$$

In this assay method CK activity is directly related to the oxidation of NADH and this is measured as a change in absorbance at 340 nm.

The amount of urea in the serum samples was determined by measuring the decrease in absorbance at 340 nm following the oxidation of NADH. Urease and GLDH are employed as catalysts in the following reactions:

$$\text{Urea} + H_2O \xrightarrow{\text{urease}} 2NH_4^+ + CO_2$$

$$\alpha\text{-ketoglutarate} + NH_4^+ + NADH \xrightarrow{\text{GLDH}} \text{L - glutamate} + NAD^+ + H_2O$$

The creatinine assay employed in this project followed the Jaffe reaction using alkaline picrate. The assay is based on the fact that a red/orange colour develops when alkaline picrate is added to creatinine. The difference in colour intensity is then measured at 500

nm and this difference is directly proportional to creatinine concentration. The reaction is as follows:

$$\text{creatinine} + \underset{\text{alkaline}}{\text{picric acid}} \longrightarrow \underset{\text{red colour}}{\text{creatinine picrate acid}}$$

Serum bilirubin concentration was measured using the Jerdrassik-Graf method in which conjugated bilirubin is coupled with a diazotised sulphanilic acid to form a coloured compound. The colour change is measured at 540 nm.

$$\text{bilirubin} + \text{diazotized sulphanilic acid} \longrightarrow \underset{\text{Red/purple colour}}{\text{azobilirubin}}$$

The reaction uses a caffeine-benzoate solution as an accelerator.

The method applied to the measurement of bile acids in the serum samples also used the oxidation of NADH as a means of measuring the rate of reaction. In the presence of thio-NAD the enzyme 3-α-hydroxysteroid dehydrogenase converts bile acids to 3-keto bile acids and thio-NADH. Under the conditions of the assay the reverse of the equilibrium reaction is favoured.

$$\text{bile acid} + \text{thio-NAD}^+ \xleftrightarrow{3\text{-}\alpha\text{-HSD}} \text{3-keto bile acid} + \text{thio-NADH} + \text{H}^+$$

The oxidation of NADH is then determined by measuring the decrease in absorbance at 340 nm.

The amount of albumin in serum samples was determined using the bromocresol green (BCG) method during which albumin binds to bromocresol green to form a blue colour as follows:

$$\text{albumin} + \text{bromocresol green} \longrightarrow \underset{\text{Blue colour}}{\text{albumin-BCG complex}}$$

The formation of the blue albumin-BCG complex is measured as an endpoint reaction at 596 nm.

The basis behind the haptoglobin assay is that free haemoglobin has peroxidase activity, which is inhibited at low pH. Haptoglobin is able to bind to the haemoglobin and at low pH results in the preservation of the peroxidase activity. Therefore the preservation of the peroxidase activity of haemoglobin is directly proportional to the amount of haptoglobin present in the sample.

2.6 Histopathology

Organs were removed, weighed and cut into smaller pieces using a razor blade, then placed in fixative; 10.5 % phosphate buffered formaldehyde pH 7.2 until processing. Tissues were fixed for at least 48 hours. Tissues were dehydrated in increasing alcohol concentrations, from 50 % to absolute alcohol. Tissues were cleared in xylene. The tissues were placed in molten paraffin at 60 °C for 2-4 days and the block of molten wax allowed to cool and solidify. The wax block was mounted on a microtome and sections cut, at 4-7 μm. Sections were placed in a drop of water on a glass microscope slide and heated to 60 °C.

Tissues were rehydrated and stained in haematoxylin and eosin (H&E) or Sirius red for the identification of collagen.

2.7 Tissue homogenisation

The frozen tissues were ground under liquid nitrogen to a fine powder using a pestle and mortar. The frozen powdered tissue was weighed and 0.2 g was added to 2 ml of homogenisation buffer (0.25 M sucrose, 50 mM Tris HCl, pH 7.2 at 4 ° C, 1 mM EDTA, 100 mM NaCl, 1 mM DTT, 1 mM PMSF, 1 mM benzamidine, 2 μg/ml SBTI) in an ice-cold glass homogeniser. The tissue was homogenised using 30 strokes of the pestle, the homogenate was then frozen in liquid nitrogen and stored at – 80 °C for later

analysis. Homogenates could then be assayed for protein concentration and samples prepared to be run on SDS-PAGE.

2.8 Urine dipstick analysis

Urine samples were semi-quantitatively analysed using Multistix® 10 SG reagent strips. One drop of urine was applied to each test spot and the colour obtained after 1-2 minutes was compared to a colour chart. Reagent strips test urine for glucose, ketones, specific gravity, blood, pH, protein, urinobilinogen, nitrite and leucocytes.

2.9 Trichloroacetic acid (TCA) precipitation

TCA precipitation of urine, serum and liver homogenates was carried out to precipitate the protein and therefore remove excess salts. Excess salts in the sample can cause horizontal streaking in the second dimension when the sample is analysed using 2D electrophoresis and also interferes with the isoelectric focussing step. Samples to be TCA precipitated were centrifuged at 13,000 rpm for 3 minutes. Ice-cold TCA (20 %) and sample were mixed together in a ratio of one volume sample to four volumes of TCA and incubated on ice for at least one hour. The mixture was centrifuged at 13,000 rpm for 10 minutes to pellet the precipitate, the TCA was removed and the pellet washed with 300 µl of cold acetone. TCA is highly acidic and should be removed before electrophoresis therefore acetone is used to wash the pellet. The samples were then centrifuged at 13,000 rpm for a further 10 minutes, before removing the acetone. The pellet was then allowed to air dry. The protein pellet was re-suspended directly into SDS sample buffer for SDS gel electrophoresis or with rehydration buffer for two-dimensional gel electrophoresis.

2.10 Assay for determining protein concentration

The protein concentration of tissue homogenates was determined before running on SDS-PAGE using the Bradford assay (Bradford, 1976). The binding of basic and aromatic residues of proteins to Coomassie Brilliant Blue G-250 converts the dye from the red form at absorbance peak at 470 nm to the blue form with a peak at 595 nm.

Bradford reagent was prepared by dissolving 30 mg of Coomassie Brilliant Blue G-250 in 100 ml of absolute ethanol, 50 ml of concentrated phosphoric acid was added and the volume made up to 1 litre with water. The reagent was filtered to remove any undissolved dye and then stored in a light protective bottle at 4 °C.

To obtain a standard calibration curve a stock solution of bovine serum albumin (BSA) 200 μg/ml was prepared, this solution should have an absorbance of 0.13 at 280 nm. From this BSA stock solution a set of 100 μl standards ranging from 0 to 6 μg BSA (i.e. 1 μg/100 μl), were prepared. To each solution 1 ml of Bradford reagent was added and mixed. A blank solution containing only reagent was used to zero the UV spectrometer, which was set to 595 nm. The absorbance of the standards was read after 5 minutes and a standard curve plotted. Samples with an unknown protein concentration were diluted with water, so that the absorbance fell within the linear region of the calibration curve, to a final volume of 100 μl and 1 ml of reagent added. The absorbance of each sample was measured at 595 nm and the protein concentration, taking into account any dilution factors, determined from the calibration curve where:

$$\frac{A_{595} \text{ of unknown sample}}{\text{gradient from calibration curve}} \times \text{dilution factor} = \text{protein concentration (mg/ml)}$$

2.11 One-dimensional sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

Electrophoresis describes the movement of proteins after an electric force is applied. A matrix or gel is used to separate the proteins according to size via a sieving action; smaller molecules readily pass through the matrix whereas larger molecules will be restricted by the pore size of the gel. Proteins are separated on polyacrylamide gels, which are poured using acrylamide and a cross-linker; N, N'-methylene bisacrylamide. The concentration of both the acrylamide and bisacrylamide determines the pore size of the gel.

SDS gels were poured according to the method of Laemmili (1970). Discontinuous gels were poured with a 15 % running gel and a 5 % stacking gel. The running gel: 37.5 mM Tris-HCl, pH 8.8 at 25 °C, 50 % Protogel (30 % Acrylamide, 0.8 %

Bisacrylamide), 0.1 % SDS was poured between two vertical glass plates assembled using standard gel casting equipment. Gels were polymerised by the addition of 10 µl of N, N, N', N'-tetramethylethylenediamine (TEMED) and 100 µl of 10 % ammonium persulphate, the ammonium persulphate acts as a catalyst for the polymerisation reaction. The running gel was poured to 5 cm in height and overlaid with isobutanol to form a flat surface and exclude oxygen from the surface. The gels were left to polymerise at room temperature for approximately one hour, after which the isobutanol was poured off and the gel plates rinsed with distilled water.

A 5 % stacking gel was poured: 12.5 mM Tris-HCl pH 6.8 at 25 °C, 1.7 % Protogel (30 % Acrylamide, 0.8 % Bis-acrylamide), 0.1 % SDS on top of the resolving gel and a comb inserted which forms the loading wells. The gel was left to polymerise at room temperature for at least 30 minutes. The comb was removed carefully. The wells were rinsed with running buffer; 25 mM Tris, 0.19 M glycine, 0.1 % SDS and were ready for sample loading. The use of a stacking gel allows greater separation of the proteins. The stacking gel has a low pH, a low ionic strength and low polyacrylamide concentration. The glycine in the tank buffer has a low mobility at the pH used in the stacking gel whereas the chloride ions in the stacking gel buffer have a high mobility at this pH. The proteins in the sample have a mobility, which is intermediate between the two. This causes the proteins to move through the stacking gel and concentrate into a very thin stack in order of electrophoretic mobility. The proteins run at a faster rate through the stacking gel because of the large pores in the gel. On reaching the resolving gel the pH changes as does the polyacrylamide concentration therefore the proteins unstack as they separate.

Samples to be run were pre-treated with SDS sample buffer containing 62.5 mM Tris HCl, pH 6.8 at 25 °C, 10 % glycerol, 2 % SDS, 5 % 2-mercaptoethanol, 0.05 mg/ml bromophenol blue. The samples were boiled for 3 minutes before loading into gel wells. SDS sample buffer contains bromophenol blue which colours the samples allowing the migration of the solvent front through the gel to be observed. Sodium dodecyl sulphate (SDS) is an anionic detergent, which denatures the protein by disrupting nearly all the noncovalent bonds. SDS binds to the main chain of the protein at a ratio of approximately one SDS molecule for every two amino acids, this confers a negative charge, which is greater than the charge on the protein. Therefore, when the

electric field is applied the negatively charged proteins move through the gel towards the positive electrode and protein separation depends entirely on size. Mercaptoethanol is a reducing agent that is added to reduce the disulphide bonds.

Protein standards of known molecular weight were applied to one of the gel lanes, the following protein standards were used: bovine serum albumin (molecular weight 66,000 Da), ovalbumin (45,000 Da), glyceraldehyde-3-phosphate dehydrogenase (36,000 Da), carbonic anhydrase (29,000 Da) trypsinogen (24,000 Da), trypsin inhibitor (20,000 Da), myoglobin (17,000 Da), cytochrome c (12,300 Da). A stock solution of the protein standards was made up so that each protein standard has a final concentration of 0.29 mg/ml and therefore when 3.5 µl of the stock solution was loaded on to a gel each band represented 1 µg of protein. A blank solution containing SDS sample buffer and mercaptoethanol was loaded into any unused wells (5 µl). The running buffer was added to the upper and lower chambers of the gel running apparatus and a constant voltage of 200 V was applied for one hour or until the dye front had reached the bottom of the resolving gel. The gels were either stained in Coomassie blue stain or further processed for western blotting.

2.12 Coomassie blue staining

On completion of the electrophoresis step proteins were visualised by Coomassie blue staining. Gels were incubated in 0.6 % Coomassie blue R-250 in 50 % methanol, 10 % acetic acid for one hour, destained in 30 % methanol, 10 % acetic acid until bands were visible with at least 3 changes of the destain solution. The molecular weight of the protein bands in the gel could be estimated by comparison with the molecular weight standards. Coomassie blue stain can be used to visualise as little as 0.1 µg of protein.

In an acidic environment ionic interactions occur between the dye and basic amino acid moieties of the protein, as well as binding due to hydrogen bonding, Van der Waals interactions and hydrophobic bonding. The proteins have a higher affinity for the dye than the gel, ensuring that the protein bands are stained against a clear background.

2.13 Silver staining

Silver stain was used to visualise much lower protein quantities than could be detected by Coomassie blue stain. Gels were stained with Coomassie blue first as described above to increase the sensitivity. Following de-staining in 30 % methanol, 10 % acetic acid the gels were washed in 50 % methanol for 10 minutes followed by a 10 minute wash in 5 % methanol. The next step was a reduction step, the gels were incubated in 4 % DTT for 30 minutes followed by a brief 2 second wash with de-ionised water to remove traces of DTT. Gels were incubated for 30 minutes in 0.2 % silver nitrate solution, followed by 3 brief washes with water to remove any silver deposits. Gels were developed in 3 % sodium carbonate and 0.025 % formaldehyde until the bands were visible. Bands were usually visible within 2-3 minutes. The developing reaction was stopped by the addition of anhydrous citric acid until the fizzing stopped. Gels were washed with water and stored in 35 % ethanol, 2 % glycerol until drying. The silver ions in the silver nitrate solution bind to the protein and the protein-bound metal ions are then reduced using formaldehyde to form metallic silver. All gels were dried between two sheets of cellophane.

2.14 Western blotting

After SDS gel electrophoresis the gels were equilibrated in transfer buffer (48 mM Tris, 39 mM glycine, 20 % methanol, pH 9.2), to remove the electrophoresis buffer salts, three washes each of 5 minute duration were carried out. PVDF membrane was cut to the size of the gel and washed in methanol for 2 minutes followed by a brief wash in de-ionized water to remove the methanol. The membrane was equilibrated in the transfer buffer for 10 minutes to allow complete wetting of the membrane. Filter paper was cut to size and saturated in the transfer buffer. The semi-dry transfer method was applied; gels were transferred for 20 minutes at 10 Volts. To assemble the semi-dry unit for transfer a piece of thick filter paper was placed on top of a horizontal platinum anode and air bubbles were excluded. The pre-wetted PVDF membrane was placed on top of the filter paper and any air bubbles were removed. The equilibrated gel was laid on top of the transfer membrane and air bubbles rolled out. Finally a second piece of pre-soaked thick filter paper was placed on top of the gel. The stainless steel cathode was placed on top of the stack and the unit connected to the power supply.

Following transfer PVDF membrane was stored in blocking solution; 1 % marvel in High salt Tween buffer (HST), (20 mM Tris HCl pH 7.4; 0.5 M NaCl; 0.5 % Tween 20) overnight at 4 °C. The blocking solution prevents the non-specific binding of the antibody to the membrane. The blots were probed with primary antibody. The polyclonal rabbit SOD-1 antibody was diluted 1:300 in 1 % marvel in HST and each blot was incubated in 15 ml of the antibody solution over night at 4 °C. Blots were washed with HST buffer (three 10 minute washes) followed by incubation in the secondary antibody for one hour at room temperature on an orbital shaker. The secondary antibody was a goat anti-rabbit IgG horseradish peroxidase conjugate, diluted 1:3000 in 1 % marvel in HST buffer. The membranes were washed again with HST buffer (five 10 minute washes) before detection using enhanced chemiluminescence (ECL) detection reagents.

The ECL detection method is based on the principle that HRP oxidises luminol to an excited state which then decays to ground state via a light-emitting pathway. The light produced by this ECL reaction peaks after 5-20 minutes. For detection the membrane was transferred to a clear plastic sheet and the membrane surface covered with a mixture of 500 µl of detection solution 1 and 500 µl of solution 2 then covered with plastic sheeting. The membrane was then transferred to a Synergy GeneGnome detector and images taken at 1-minute intervals, the final image recorded was that taken after five minutes exposure time.

2.15 Western blotting with an anti-albumin antibody

Urine samples were not loaded on to SDS gels according to equal concentration, but instead they were normalised for the intensity of the albumin band in each sample. Therefore, after Western blotting with the SOD-1 antibody the blots were stripped using the following buffer: 65 mM Tris HCl pH 6.8, 2 % SDS, 0.75 % 2-mercaptoethanol. Blots were incubated in 15 ml of this solution at 50 °C for one hour. The blots were washed with distilled water to remove the 2-mercaptoethanol (approximately four washes). The blots were transferred to blocking solution (1 % marvel in HST buffer) for at least one hour at room temperature or overnight at 4 ° C.

The polyclonal rabbit albumin antibody was reconstituted with 1 ml of 20 mM Tris HCl pH 7.4, 135 mM NaCl, 0.1 % BSA, 0.01 % sodium azide. The antibody was diluted 1:300 with 1 % marvel in HST buffer and 15 ml was applied to each blot. Blots were incubated in the primary antibody for four hours on a shaker at 37 ° C. The albumin antibody was removed and the Western blotting procedure continued as described above.

2.16 In-gel trypsin digest and nano-electrospray tandem mass spectrometry

2.16.1 Preparation of gel slices for trypsin digest

Gel slices were cut out from a Coomassie blue stained gel using a fine razor blade taking care to remove as little of the surrounding gel as possible. The slices were washed with 100 µl water repeatedly until the pH of the solution became neutral as tested using pH paper. The slices were de-stained using a rapid destain solution; 50 mM NH_4HCO_3 in 40 % ethanol, pH 7.8 at room temperature. Six changes of 200 µl were used to completely remove the Coomassie blue stain. Gel slices were chopped into very fine pieces using a clean razor blade. The chopped pieces were placed in an Eppendorf tube, washed with 50 µl acetonitrile, vortexed briefly and spun down. After a 15-minute incubation the acetonitrile was carefully pipetted off and discarded, a fresh 50 µl acetonitrile was added and the process repeated until the gel pieces felt brittle. The acetonitrile was removed and the gel pieces dried down in a Centrivap (Labconco Centrivap Console, Genetic Research Instrumentation Ltd., Essex, UK.) for 30 minutes.

2.16.2 Trypsin digest of gel slices

The dried down gel pieces were broken up into smaller pieces using a syringe needle working through holes punched in the Eppendorf tube lid, this creates a greater surface area of the gel pieces and allows greater absorption of the trypsin. Sequencing grade modified trypsin was made up in 100 µl of 25 mM NH_4HCO_3 and stored as 5 µl aliquots at –20 °C (0.2 µg/µl). The trypsin was diluted to 75 ng/µl by adding NH_4HCO_3 just prior to use to avoid autolysis of the trypsin. Then 9 µl of the diluted trypsin

solution was added to the gel pieces; the protein to trypsin ratio was approximately 10:1. The gel pieces absorbed the trypsin solution and they became translucent. To allow the trypsin to be fully absorbed the samples were kept on ice for 30 minutes. 25 µl of 25 mM NH_4 HCO_3 was added to completely cover the gel pieces and the samples incubated at 37 °C overnight to allow the trypsin to digest the protein. Trypsin hydrolyses the peptide bonds on the carboxyl side of lysine and arginine residues.

2.16.3 Extracting the digested peptides from the gel pieces

To extract the peptides from the gel slices 30 µl of 5 % trifluoroacetic acid (TFA), 50 % acetonitrile was added to each digest and mixed. The gel pieces were sonicated for 6 minutes to extract the peptides. The samples were centrifuged at 13,000 rpm for 3 minutes to pellet the gel pieces and the supernatant containing the digested peptides was removed and transferred to a clean Eppendorf tube. This process was repeated and the extracts pooled and dried down using the Centrivap.

2.16.4 Desalting the digest for nanospray mass spectrometry

The digest must be desalted prior to mass spectrometry since the presence of salts and detergents such as SDS interfere with the electrospray signal. The dried down digest was rehydrated by the addition of 10 µl water and 0.2 µl heptafluorobutyric acid and sonicated for 15 minutes. A C18 zip tip was used to desalt the digest. The zip tip is a 10 µl pipette tip with a silica bed fixed at the end of it; the silica has a particle size of 15 µm and pore size of 200 Å. The peptides bind to the reverse phase zip tip in an aqueous mobile phase and are eluted with an organic mobile phase. The C18 zip tip is used for smaller proteins and peptides since it has a long hydrophobic chain length and therefore is able to bind the smaller peptides.

The C18 zip tip was wet with 10 µl of 50 % acetonitrile and then with 10 µl methanol, each wetting step was repeated three times. The zip tip was equilibrated with 10 µl freshly prepared 0.05 % TFA in water. The sample was loaded on to the zip tip by pipetting in at the top using a gel-loading tip. The sample was expelled from the zip tip

and collected and then re-loaded on to the pipette, this was repeated 10 times to ensure as much of the sample as possible bound to the zip tip. The sample loaded tip was washed 7 times with 10 µl of 0.05 % TFA. Then peptides were eluted from the zip tip using 20 µl of 70 % methanol, 0.5 % formic acid and were collected and stored.

2.16.5 Nano-electrospray tandem mass spectrometry (Nano-ES-ms/ms)

Nano-ES-ms/ms was carried out using an LCQ^{duo} mass spectrometer fitted with a nanospray source (ThermoElectron, Hemel Hempstead, U.K). The sample was loaded (10 µl) into a capillary glass PicoTip^{TM} emitter (New Objective, Inc. Woburn, MA, USA) using a Gel-Saver pipette tip (Fisher). The PicoTip^{TM} emitter was loaded into the nanospray probe. The mass spectrometer was operated in the positive ion mode with the following settings: capillary temperature 180 °C; spray voltage 1.00 to 1.10 kV. The acquisition mass range in the full scan mode was m/z 400-2000. Centroid data was collected automatically over a period of 60 minutes, and the following four scan events were repeated:

1. Full scan of *m/z* 400-2000, consisting of eight averaged "microscans" with a maximum injection time of 200 ms.
2. Data dependent MS/MS on the most intense ion from 1.
3. Data dependent MS/MS on the second most intense ion from 1.
4. Data dependent MS/MS on the third most intense ion from 1.

MS/MS scans consisted of three microscans with a maximum injection time of 200 ms. The isolation width for the parent ion in MS/MS scans was m/z 3.0 and the normalised collision energy was set to 35%. The minimum signal required to trigger MS/MS data collection was 100,000. If an ion appeared as one of the top three most intense ions for more than 5 full scans in a time period of thirty minutes, it was put on an exclusion list for 30 minutes; during this time, no MS/MS scans would be performed on that ion, whatever its intensity. The exclusion mass width was m/z 2.0.

2.16.6 Identification of peptides using Sequest software

In order to identify peptides, MS/MS data was searched using Sequest software (ThermoElectron) against a rat protein database (Finnigan Xcalibur, Revision 1.0 P/N XCALI-64012, July 2000).

The Sequest approach finds the amino acid sequence in the database that, when fragmented, would give a spectrum that correlates most closely with the experimentally obtained MS/MS spectrum. Candidate sequences are found in the database on the basis of intact peptide mass, and the theoretical spectra predicted to result from the fragmentation of these candidate peptides are generated and compared to the experimental MS/MS spectrum. The final score assigned to each candidate amino acid sequence is the Xcorr, a measure of how well the theoretical spectrum cross-correlates to the observed spectrum. Proteins that were matched by two or more peptides with Xcorr values ≥ 2.5 and ΔC_n values (the difference in the Xcorr values for the top two candidate amino acid sequences) ≥ 0.1 were considered identified, provided that those peptides were unique to the protein in the database.

The following parameters were used when creating data files from raw data files to be used in the Sequest search: precursor mass tolerance 1.4 *m/z*; minimum number of different ions 15; maximum number of intermediate scans 25; minimum number of grouped scans 1; minimum total ion count 5×10^5, parent ion mass range 400-4000 Da. The following parameters were used when searching data files using Sequest: fragment ion tolerance 0.00 *m/z* (fragment ion mass values are rounded to the nearest Da, therefore an automatic fragment ion tolerance of $\pm$ 0.5 Da is included in this parameter); peptide mass tolerance 1.00 *m/z*; average mass; maximum number of internal cleavage sites 2.

2.17 Surface Enhanced Laser Desorption/Ionisation ProteinChip Technology

SELDI provides a rapid and sensitive method of identification of protein biomarkers of toxicity. ProteinChip arrays are made of aluminium and contain chemically or biologically treated spot surfaces for specific binding of proteins of interest. The

sample is applied to the spot and will bind specifically to the modified surfaces while the non-specifically bound proteins and other contaminants can be washed away, a process known as "retentate mapping". An Energy Absorbing Molecule (EAM) solution is applied to the spot and allowed to dry. On drying the EAM forms crystals containing the protein of interest and the EAM. The ProteinChip Array is inserted into the ProteinChip Reader, which operates as a linear time-of-flight mass spectrometer. The ProteinChip Reader has a 337 nm nitrogen laser, which is operated in the positive-ion mode; the laser is focussed on the spot causing the protein embedded in the EAM crystals to desorb and ionise. An accelerating electric field causes the released ions to fly through a vacuum tube, towards an ion dectector. An accurate mass of the protein can be determined based on time of flight.

Urine samples were centrifuged at 10,000 g for 10 minutes. Normal phase ProteinChip Arrays (NP-1) were used to analyse the urine samples. NP-1 ProteinChip Arrays have a silicon dioxide (SiO_2) surface. Proteins bind to these chips through hydrophilic and charged residues on their surfaces via electrostatic interactions or hydrogen bonds.

3 µl of the sample was applied directly to the spot and incubated in a humidity chamber for 10 minutes. The sample was removed and the spot washed with 3 µl of deionised water. The chip was allowed to air dry on the bench. Then 0.5 µl of sinapinic acid (EAM solution) was applied, this was left to air dry and the chip was inserted into the ProteinChip reader and analysed.

2.18 Assay for superoxide dismutase activity (SOD)

The method uses xanthine and xanthine oxidase to generate superoxide free radicals. The superoxide free radicals react with 2-(4-iodophenyl)-3-(4-nitrophenol)-5-phenyltetrazolium chloride (I.N.T) to form a red formazan dye.

$$\text{Xanthine} \xrightarrow{\text{XOD}} \text{Uric acid} + O_2^{\bullet}$$

$$\text{I.N.T.} \xrightarrow{O_2^{\bullet}} \text{formazan dye}$$

The production of the red formazan dye is measured spectrophotometrically at 505 nm at 37 °C. SOD activity is measured as its ability to remove the free radical by the degree of inhibition of this reaction. One unit of SOD activity causes a 50 % inhibition of the rate of reaction of INT. The activity of SOD was determined using a Ransod assay kit. The assay kit consists of:

Contents	Initial Concentration of Solutions
1. Mixed Substrate	
Xanthine	0.05 mmol/l
I.N.T	0.025 mmol/l
2. Buffer	
CAPS	40 mmol/l, pH 10.2
EDTA	0.94 mmol/l
3. Xanthine Oxidase	80 U/L
4. Standard	5.00 U/ml

The mixed substrate was reconstituted with 20 ml of the buffer whereas the standard (S6) and Xanthine oxidase were reconstituted with distilled water.

A standard curve was produced by diluting the standard (S6) 1:2 with a Ransod sample diluent to give sample S5, then carrying out subsequent 1:2 dilutions of each of the samples to produce S4, S3 and S2. For the procedure 0.05 ml of standard was mixed with 1.7 ml of the mixed substrate and 0.25 ml of xanthine oxidase was added, the solution mixed and transferred to a cuvette and an initial absorbance reading (A_1) taken at 30 seconds. The final absorbance (A_2) is read after 3 minutes.

To calculate the % inhibition of the reaction:

$$\frac{A_2 - A_1}{3} = \Delta A/\text{min of standard or sampl}$$

Where sample diluent rate (S1 rate) = rate of uninhibited reaction = 100%. Therefore all the standard rates and sample rates are expressed as a percentage of the sample diluent:

$$100 - \frac{(\Delta A_{std/min} \times 100)}{(\Delta A_{S1/min})} = \% \text{ inhibition}$$

Or:

$$100 - \frac{(\Delta A_{sample/min} \times 100)}{(\Delta A_{S1/min})} = \% \text{ inhibition}$$

A standard curve was then constructed by plotting the percentage inhibition for each standard against $\log_{10}$ (standard concentration in SOD units/ml).
The assay method was carried out for the urine samples as for the standards. Urine samples did not need to be diluted in order for the % inhibition of the reaction to fall on the standard curve. The percentage inhibition of each of the samples was calculated as above and using the standard curve the units of SOD per ml of sample could be determined.

A Ransod control sample was assayed each day as a quality control. The control was bovine lyophilised blood, which was reconstituted with 1 ml of redistilled water. After reconstitution the control was diluted 1:200 with Ransod sample diluent and the assay carried out as for all other samples.

2.19 Two-dimensional gel electrophoresis

Two-dimensional gel electrophoresis combines an isoelectric focussing (IEF) step with SDS-PAGE. The IEF step separates proteins according to their isoelectric points (pI); this is the specific pH at which the net charge of the protein is zero. The amino acid residues of a protein can be positively or negatively charged or partially polarised depending on the functional group on the side-chain. The sum of these charges is the net charge of a protein. IEF depends upon the presence of a pH gradient so that when an electric field is applied the protein will move towards the pH at which its net charge

is zero. Positively charged proteins will move towards the cathode whereas negatively charged proteins migrate towards the anode. The proteins become less charged as they move towards their pI and concentrate at the position at which pI is equal to pH. If the protein diffuses away from its pI it immediately acquires a net charge and migrates back.

The IEF step was carried out using pre-cast immobilized pH gradient (IPG) gel strips, these strips are made with acrylamide buffers each consisting of a single acidic or basic group that are copolymerised with acrylamide and bis-acrylamide. Therefore the pH gradient is pre-cast into the gel. The range of the pH gradient is defined by the concentration of the individual acrylamide buffers in the gel. The IPG gels are cast onto a plastic backing and dried down.

Prior to carrying out the IEF step the IPG strips must be rehydrated. The rehydration solution contains urea, non-ionic and/or zwitterionic detergents, carrier ampholytes and a reducing agent.

Urea is added to the rehydration buffer in order to solubilize and denature the proteins; a very high concentration of urea is required for complete sample solubilization. The detergent should have a zero net charge and is used to solubilze hydrophobic proteins and to prevent protein aggregation. Tributylphosphine (TBP) is added as a reducing agent to cleave the disulphide bonds between proteins. TBP has the advantage over other reducing agents e.g. dithiothreitol (DTT) because it is not charged and therefore will not migrate through the pH gradient when an electric field is applied, whereas DTT migrates to its pI (9.5) during IEF resulting in a loss of solubility of some proteins. Carrier ampholytes are a complex mixture of amphoteric molecules, which form a pH gradient during electrophoresis. They migrate towards and focus at their isoelectric point when an electric field is applied and as a result of their buffering capacity are able to create a hydrogen plateau around the isoelectric point. Therefore the presence of a great number of these molecules with evenly distributed isoelectric points will result in an overlap of the hydrogen plateau and therefore create a continuous pH gradient. In this way they maintain the already established pH gradient. The carrier ampholytes also enhance sample solubility and minimize protein aggregation.

2.19.1 Rehydration of IPG strips and loading of protein sample

All samples were isofocussed on 7 cm IPG strips. Samples for IEF were centrifuged at 13,000 rpm for 3 minutes and the required volume was TCA precipitated as described above. The protein pellet formed as a result of precipitation was re-suspended in rehydration buffer; 8 M urea, 1 % CHAPS, 2 mM TBP, 0.2 % Bio-Lytes pH 3-10. The TBP and Bio-lytes were added just prior to use. Rehydration took place in disposable rehydration trays; 200 µl of the buffer was pipetted as a line along the back edge of the whole length of the channel, taking care not to introduce any bubbles. The plastic coversheet was peeled off the IPG strip using forceps and the strip gently placed gel side down on to the sample rehydration buffer. Any bubbles that were introduced were removed by lifting the IPG strip up and down until the air bubbles were expelled. The strips were left in the buffer and sample for one hour to allow most of the sample to be absorbed by the strip; the IPG strip was overlayed with mineral oil (2-3 ml) to prevent evaporation during the rehydration process. The IPG strips were left at room temperature overnight (12-16 hours) to rehydrate and absorb the sample.

2.19.2 Isoelectric focussing step (IEF)

The IEF step was carried out using focussing trays, which have an electrode at either end and these are covered with a paper electrode wick during the IEF step. The electrode wicks were wetted with 8 µl of de-ionised water and then placed at both ends of the channel in the isofocussing tray covering each of the electrodes. The rehydrated IPG strips were removed from the rehydration tray and the tip of the strip blotted on to filter paper to remove the mineral oil. By removing the excess mineral oil the unabsorbed sample protein is also removed which could cause streaking during the focussing step. The strips were transferred to the focussing tray with gel side down. The strips have a side marked "+" and this side was positioned at the end of the tray marked "+". Each IPG strip was covered with a fresh 2-3 ml of mineral oil, removing any air bubbles as before. The lid was placed on the tray ensuring good contact between the IPG strips and the electrodes by positioning the lid pressure tabs on the strips directly over the electrodes. Isofocussing was carried out according to the manufacturers instructions. The first step was a conditioning step (S1), when a 250

voltage was applied for 15 minutes. This low voltage step was applied to remove salt ions and charged contaminants. The S2 step was a voltage ramping step; a linear voltage ramping method was chosen, this increases the voltage to a maximum in a linear fashion. This step is carried out for 2 hours at a maximum of 4000 volts. The final focussing step (S3) is carried out at a maximum of 4000 volts for 20,000 volt-hours (vhours). Volt-hours is defined as the number of hours elapsed at the voltage attained. For all IPG strips the current should not exceed 50 μA/strip and the temperature was fixed at 20 °C.

2.19.3 Second dimension-SDS gel electrophoresis

The second dimension of two-dimensional gel electrophoresis involves separating the isofocussed proteins according to mass on SDS-PAGE. The SDS gels are poured as described above according to Laemmili (1970). The IPG strips need to be equilibrated in a buffer containing SDS and a reducing agent prior to running the second dimension. The excess mineral oil was drained off the IPG strips and then they were equilibrated in buffer 1; 6 M urea, 2 % SDS, 0.375 M Tris-HCl pH 8.8, 20 % glycerol and 130 mM DTT for 20 minutes. The strips are transferred to a second equilibration buffer containing 6 M urea, 2 % SDS, 0.375 M Tris-HCl pH 8.8, 20 % glycerol and 135 mM iodoacetamide for a further 20 minutes. The second equilibration buffer is necessary since the iodoacetamide prevents the re-oxidation of the proteins, which have been reduced by DTT, during electrophoresis. The IPG strips were removed from this buffer and dipped briefly into a cylinder containing electrophoresis running buffer, before being placed with gel side facing out onto the back plate of the SDS-PAGE gel. The strip was pushed down so that it was in direct contact with the second dimension gel and the strip overlayed with 1 % agarose in 0.5 M Tris HCl pH 6.8. The agarose was allowed to solidify for 5 minutes before the electrophoresis was started. The gels were set up as for one-dimensional SDS-PAGE and electrophoresis carried out at 200 V for approximately 40 minutes or until the bromophenol blue dye front reached the bottom of the gel. After the second dimension the gels were stained as for one-dimensional gels in Coomassie blue and then silver stained as above in order to visualise more protein spots.

2.20 Gel filtration to remove albumin from serum sample.

Gel filtration was carried using a Superdex 75 column on FPLC with the flow rate set to 0.5 ml/min and a chart recorder speed of 0.25 cm/ml. The column was equilibrated with buffer; 200 mM NaCl, 10 mM Tris HCl (pH 7.2 at room temperature), 1 mM EDTA. Protein standards bovine serum albumin (BSA) (66,000 Da), cytochrome C (12,300 Da) and ∝-lactalbumin (14,200 Da) were run first and the elution volume of each determined and a calibration curve constructed.

The serum samples were filtered and 200 µl of sample was loaded on to the column and 0.3 ml fractions were collected between elution volume of 10 and 20 ml. The same buffer as described above was used to wash the column during the runs. The fractions from the same elution volume for each sample were pooled in groups of two and concentrated down to 100 µl samples using a Centrivap. The concentrated samples were prepared for SDS-PAGE.

2.21 Statistical analysis

Treated and control groups were compared using Student's t-test for unpaired samples using Microsoft Excel (Microsoft Corporation).

Chapter three

Urinary protein markers in acute carbon tetrachloride (CCl_4)-induced hepatotoxicity

3.1 Introduction

Carbon tetrachloride (CCl_4) was chosen as the hepatotoxic compound for use in the present studies since it induces well-described toxic effects in the liver. CCl_4 is metabolised in the liver by the cytochrome P450 system and the metabolites from this reaction induce the hepatic injury. CCl_4 is a very lipid soluble compound and is therefore readily distributed throughout the body. Therefore, it is not solely a hepatotoxicant and damage may be induced in other organs, such as the kidney, lungs and brain. However, the liver is the main site of injury since the metabolising cytochrome P450 enzymes are expressed mainly in the liver. The products of the metabolism are highly reactive free radicals with short half-lives, therefore the injury is usually localised to the site of origin, which tends to be the centrilobular region of the liver. The centrilobular region is mostly affected since the cytochrome (CYP) genes are expressed predominantly in a zonated pattern (Oinonen and Lindros, 1998).

Although CCl_4 is used widely as a model hepatotoxicant it was necessary to determine a dose that induced hepatic injury in the strain of rats (Wistar Han) used. It was also necessary to establish the age groups of rats to use throughout since the activity of cytochrome P450 enzymes in the rat is dependent on the age of the animals. As toxic effects of CCl_4 result from free radical generation, the relative activity of the cytochrome P450 enzyme in the strain, sex and age of the rats used for these studies was very important. Newly born rats have less of the P450 metabolising enzymes and therefore are more resistant to the toxic effects of CCl_4. The highest activity of P450 enzyme is found in young adult rats and this activity diminishes as the rat ages (Rikans and Notley, 1982; Kamataki et al., 1985; Kitagawa et al., 1985; Rikans, 1989). Rikans et al (1999) proposed that age-associated changes in cytokines such as IL-6 could also affect the severity of liver damage to CCl_4.

The first objective was to identify the dose of CCl_4 that would cause maximal injury to the liver without either damage to other organs or mortality. Once this dose had been determined it would then be necessary to decide on the time period after dosing at which to collect samples. CCl_4 induces a toxic response within minutes of administration; lipid peroxidation induced by CCl_4 has been detected within five minutes of dosing (Recknagel and Glende, 1973). Therefore it was important to determine the optimal dose of CCl_4 and the time period at which CCl_4 toxicity and liver

injury were at their height before the liver starts to regenerate and repair the damage induced. Using the results from both a dose response and time course study a CCl_4 toxicity model would be generated accordingly.

It is important to be able to identify hepatotoxicity once it has been induced and the methods of identification currently used apply invasive techniques. Observations can be made during a post mortem as to changes in the liver weight or colour. As a result of a toxic insult e.g. repeated administration of CCl_4; the liver may change in colour from the normal dark red colour to a much paler yellow-tan colour. The liver can also appear more nodular and swollen and there may be an increase in the weight (Evans and Lake 1998).

Histological analysis of liver tissue can be used to determine the type and severity of a lesion produced by a particular hepatic toxicant. Various staining techniques can be used to enable the extent of damage to be assessed using light or electron microscopy.

Another invasive method used for the identification of liver injury includes serum biochemistry analysis. Blood is removed and serum prepared to analyse for serum enzyme activity. Serum enzymes are important as markers of organ toxicity. When tissues are damaged cytoplasmic enzymes may leak from the cells into the plasma. Some intracellular enzymes are expressed specifically in certain tissues and therefore by comparing the levels of different enzymes in the serum it is possible to determine the nature and origin of damage.

However, the main purpose of these studies was to identify a protein biomarker of hepatocellular damage using non-invasive means. A useful biomarker should reflect the early changes induced by CCl_4, and should be highly specific. The relationship between exposure, the level of damage induced and the response of the biomarker should be well established and easily measured. The ability to measure a biomarker using non-invasive sampling techniques makes a biomarker more ideal in a clinical setting (de Zwart et al., 1998). The samples can be collected with greater ease and without the need for trained phlebotomists; also patients often express fear of invasive methods such as biopsy as a means of sample collection. Therefore an obvious non-invasive source for possible biomarkers is urine.

Urine has already been widely analysed using different techniques as a source of biomarkers. Winterkamp et al. (2002) measured the urinary excretion of the histamine metabolite, N-methylhistamine by radioimmunoassay. They found high levels correlated with disease activity in inflammatory bowel disease. The levels of 8-epi-prostaglandin $F_{2\alpha}$ (isoprotane) have been measured using LC-MS (Murai et al., 2000) and by gas chromatography mass spectrometry (Obata et al., 2000). Murai and colleagues found increased levels in the urine of diabetics whereas Obata et al. (2000) discovered increased levels in smokers. In the study by Murai et al. (2000) three of the patients were smokers and although the urine samples of these patients had relatively high levels of isoprostane this difference was not statistically significant. Hermanns et al. (1998) applied HPLC and gas chromatography to the analysis of urine samples after the induction of oxidative stress to the kidney by the administration of ferric nitrilotriacetate. They reported increases in coproporphyrin III, 8-hydroxy-2-deoxyguanosine, acetone, propanol, pentanal, hexanal and malondialdehyde, all of which may be good biomarkers of renal oxidative stress. A study by Poór et al. (2003) measured urinary steroid metabolites using gas chromatography in patients with unipolar recurrent major depression. Dare and colleagues (2002) identified parvalbumin as a marker of skeletal muscle toxicity in rats after the analysis of urine samples from rats treated with TMPD using SELDI. In this project SELDI and gel electrophoresis with mass spectrometry will be used as a means of identifying urinary markers of liver injury.

Therefore the objective of these studies was firstly to develop a reproducible model of hepatotoxicity. Since CCl_4 also induces nephrotoxicity we need to ensure that kidney structure and function was not affected. Injury to the kidney would have an effect on the samples collected and in particular the urine samples would have higher protein content, as impaired kidney function could result in many more proteins passing through the filtration barriers. This would mean that our urine samples could contain proteins resulting from both liver and kidney injury. During these studies we determined if kidney damage had been induced. The kidneys were weighed and serum creatinine and urea levels were measured and urine samples were analysed using reagent strips as described in Section 2.8. Our objective therefore was to develop a model of hepatotoxicity without nephrotoxicity. On achieving the model of hepatotoxicity the final goal was to identify a urinary marker for this model.

3.2. Animal experimental design

Two studies were carried out, a dose response study and a time course study. The dose response study allowed us to identify the dose of CCl_4 that caused significant injury to the liver without either mortality or injury to other organs. Once this dose had been determined it was then necessary to decide on the time period after dosing at which to collect urine, blood and tissue samples for analysis. A time course study showed an increase in liver injury up to a maximum followed by a decrease as the liver regenerates and repairs the damage induced. Using the results from both the dose response and time course studies our CCl_4 toxicity model was generated accordingly.

3.2.1 Dose response study

In the dose response study 40 female rats (mean body weight 201.9 g ± 13.5 g) were divided into 8 dose level groups of 5 animals each. The rats were dosed by gavage, with a single dose of vegetable oil (control) or CCl_4 dissolved in vegetable oil at 0.4, 0.8, 1.2, 1.6, 2.0, 2.4 and 2.8 ml/kg. After dosing the rats were placed individually in metabolism cages with water but without diet and urine collected over ice for 24 hours. Urine samples were stored at –80 °C for later analysis.

At 24 hours the animals were removed from the metabolism cages sacrificed and a post mortem carried out as described in Section 2.4. Blood was taken from the abdominal aorta for serum preparation. Serum was stored at –80 °C until analysis. Kidneys and livers were weighed and sections placed in 10.5 % phosphate buffered formalin.

3.2.2 Time course study

In the time course study 105 rats (mean body weight 182.3 g ± 13.8 g) were divided into 21 groups of 5 rats each. At 0 hours, 50 rats were treated with vegetable oil (control), and 50 animals with CCl_4 (0.8 ml/kg). At 0 hours, 5 control and 5 CCl_4-treated animals were placed individually in metabolism cages and urine collected over ice for 12 hours. At 12 hours these rats were removed and autopsied and the next group of 5 control and 5 CCl_4-treated rats were placed in the metabolism cages to collect urine over the next 12

hour period (12 to 24 hours after treatment). At 24 hours these rats were removed and sampled and the next group of 5 control and 5 CCl_4-treated rats placed in the metabolism cages. This process was repeated every 12 hours for the first three days of the experiment i.e. at 12, 24, 36, 48, 60 and 72 hours and then on days 4, 7, 10 and 17. On days 4, 7, 10 and 17, animals were placed in metabolism cages and urine collected over a 24 hour period. When rats were not in metabolism cages they were housed in groups of 5 and had access to food and water *ad libitum*. An additional 12-hour pre-dose urine collection was carried using 5 untreated control animals.

At each autopsy blood was taken for serum preparation, livers and kidneys were removed, weighed and placed in 10.5 % phosphate buffered formalin. Serum and urine samples were stored at – 80 ° C until analysis.

3.3 Dose response study

For experimental details see Section 3.2.1, page 106.

3.3.1 Observations during the dose response study

At 24 hours post CCl_4 dosing the animals in the control and lower dose CCl_4-treated groups (i.e. 0.4 and 0.8 ml/kg) appeared to be healthy. Animals in the 1.2 and 1.6 ml/kg groups also appeared healthy but the urine from rats at 1.2 ml/kg and above was red in colour and tested positive for blood using reagent strips as described in Section 2.8. In the 2.0 ml/kg group two rats were judged to be very unwell and one of these rats had bloody urogenital soiling. One rat in the 2.4 ml/kg group had died and all the rats in this group had urogenital soiling. In the 2.8 ml/kg group one rat had died and two other rats in the group were cold and unresponsive with bloody urogenital soiling. At autopsy the livers from CCl_4-treated rats were very pale and swollen.

3.3.2 Reagent strips

Urine samples were analysed using reagent strips as described in Section 2.6. Urine from the dose response study was tested (rats were treated with CCl_4 at 0, 0.4, 0.8, 1.2, 1.6, 2.0, 2.4 and 2.8 ml/kg and urine collected for 24 hours after dosing). Urine from rats in the lower dose groups (0.4 and 0.8 ml/kg) did not show any differences in comparison with control urine. The higher dose groups 1.6, 2.0, 2.4 and 2.8 ml/kg had positive readings for blood. Control and low dose groups (0.4, 0.8 and 1.2 ml/kg) had trace protein levels whereas in the higher dose groups the readings for protein levels were 0.3, 1.0 and 3 g/L.

3.3.3 Liver weights

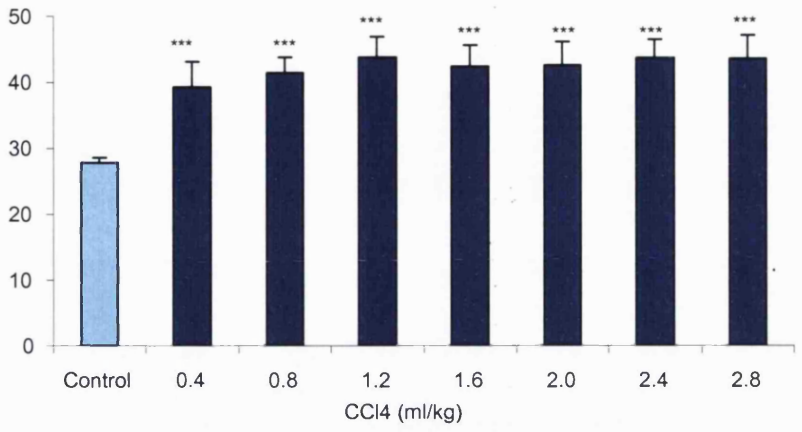
At post mortem both the absolute and relative liver weights were increased in the CCl_4-treated rats in a dose-related fashion. Figure 3.1A shows the mean relative liver weights for each treatment group. The liver weight for rats treated with CCl_4 was significantly increased at all dose levels compared to control. In the 0.4 ml/kg group the relative

liver weights were 1.41 times greater than control whereas in the highest dose group the liver weights were 1.5 times greater.

3.3.4 Kidney weights

At autopsy the kidneys were removed, weighed and placed in formalin. The kidneys from all CCl_4-treated rats appeared normal. Kidney weight was expressed as a mean of the left and right kidney. Absolute and relative kidney weights for rats treated at 0.4 and 0.8 ml/kg CCl_4 were similar to control. However at 1.2 ml/kg and above the absolute and relative kidney weights were increased. Figure 3.1B shows the mean relative kidney weights for rats in this study. The relative kidney weight was significantly increased ($P < 0.05$) in rats treated with 1.2 ml/kg CCl_4, and at higher dose levels. Above 1.2 ml/kg the relative kidney weight increased with increasing dose level of CCl_4 until in the 2.8 ml/kg group the kidney weights were 1.47 times greater than control.

A.



B.

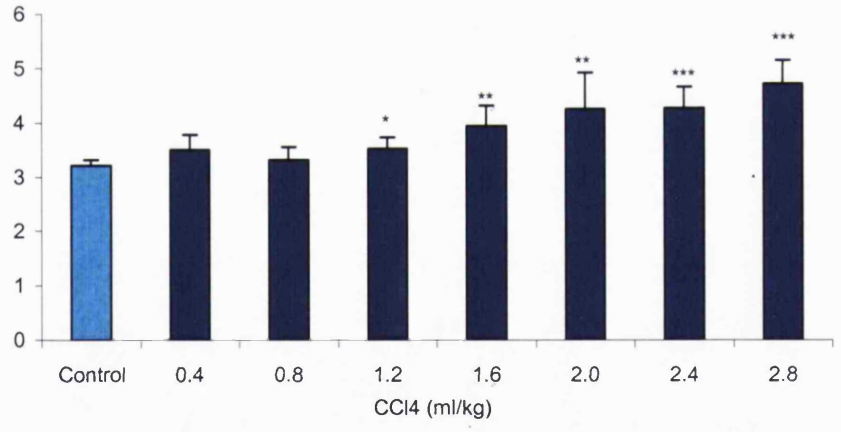


Figure 3.1 Relative liver (A) and kidney (B) weights of rats treated with CCl_4 at dose levels from 0.4 to 2.8 ml/kg and autopsied at 24 hours post-dosing. Values are means of 5 animals per dose level group (error bars represent S.D.). For the kidneys, the mean of the left and right organ was taken and the relative weight calculated. Values that differ from control by Student's t-test are shown, * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

3.3.5 Serum clinical biochemistry

Liver injury was confirmed in this study by serum enzyme analysis. Levels of alanine transferase (ALT), asparate transaminase (AST) and glutamate dehydrogenase (GLDH) were determined as indicators of liver injury at each CCl_4 dose (Figure 3.2).

Levels of ALT, AST and GLDH were increased in all the CCl_4-treated groups when compared to control. Furthermore, the enzyme activities increased with increasing dose of CCl_4 indicating that there is a dose response relationship. At the lowest dose of CCl_4 administered (0.4 ml/kg) ALT levels were increased by 50-fold, and there was a 30-fold increase in AST levels in the CCl_4-treated rats, whereas GLDH was increased by 200-fold in the CCl_4-treated rats over control. The ALT and AST levels in the 0.8 ml.kg group were not significantly greater than control. This was a result of the high standard deviation values obtained due to large intragroup variation. In the 1.2 ml/kg CCl_4 group the activity of the three enzymes was further increased; ALT was 74 times greater than in the control, AST was 46 fold greater and GLDH increased to 292 fold greater than control. In the highest dose group (2.8 ml/kg) ALT activity in the CCl_4-treated rats was almost 300 fold greater than control, AST was 158 times greater and GLDH activity increased to 672 times greater than in the control group. Increased serum AST, GLDH and ALT activities indicate liver injury.

Serum levels of LDH and CK were also measured (Table 3.1), however these enzymes are less specific for liver injury. At 0.4 ml/kg CCl_4, LDH levels were increased 5-fold and CK levels were raised 3-fold. There is an overall trend for increases in both enzymes with increasing dose levels of CCl_4. At 2.8 ml/kg the increase in LDH was 31.7-fold greater than control and CK levels were 18.9-fold greater. However the standard deviation values for all dose groups were very high for both LDH and CK and this was due to large intragroup variation.

To assess possible kidney damage, urea and creatinine levels were measured in the serum (Table 3.2). Increased levels of both of these markers indicate kidney damage. Levels were increased in all the CCl_4-treated groups but at the lower dose levels, i.e. 0.4, 0.8 and 1.2 ml/kg this increase was only slight; the urea levels in the 1.2 ml/kg group were 1.22 times greater than control and creatinine levels were 1.25 times greater. At dose levels at 1.6 ml/kg and above the levels were greatly increased. In the 1.6

ml/kg group the urea levels were 2.55 times greater than control and creatinine levels were 2.01 times greater than control. In the highest dose group (2.8 ml/kg) the urea levels were 3.08 times greater than control and creatinine levels were 2.77 times greater. These results together with increased kidney weights above 1.2 ml/kg suggest that the administration of CCl_4 at dose levels greater than 1.2 ml/kg results in nephrotoxicity.

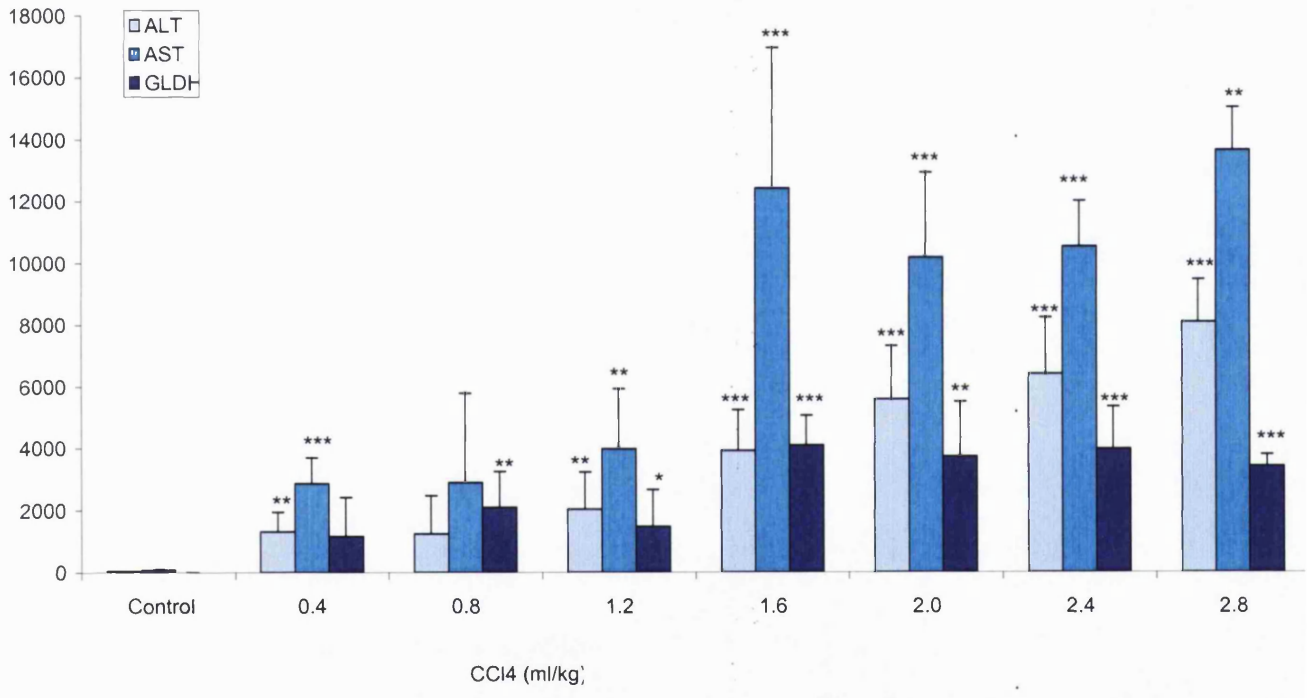


Figure 3.2 Mean serum ALT, AST and GLDH activities for rats treated with increasing dose levels of CCl_4 and sampled at 24 hours post-dosing. Enzyme assays were carried according to methods described in Section 2.5. Values are the means of 5 animals per dose level group (error bars represent S.D.). Values that differ from control by Student's t-test are shown, * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

Table 3.1: Mean (and S.D.) of serum lactate dehydrogenase (LDH) and creatine kinase (CK) levels for rats treated with CCl_4 at increasing dose levels and sampled at 24 hours post-dosing[a]. Assays were carried out according to the methods described in Section 2.5.

CCl4 dose level (ml/kg)	LD (U/L)		CK (U/L)	
	Mean	S.D.	Mean	S.D.
Control	480.80	226.23	241.60	75.31
0.4	2748.00**	1169.31	630.20*	367.03
0.8	2837.20*	2100.51	429.60*	134.27
1.2	2359.40***	674.69	462.20**	120.59
1.6	31438.00	34040.29	1190.40	1220.56
2.0	35457.50*	25658.81	1092.40	841.10
2.4	45771.00*	32180.27	765.75**	259.84
2.8	15242.50	17106.46	4561.25	5397.23

[a]Values are the means of 5 animals per dose level group. Values that differ from control by Student's t-test are shown, * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

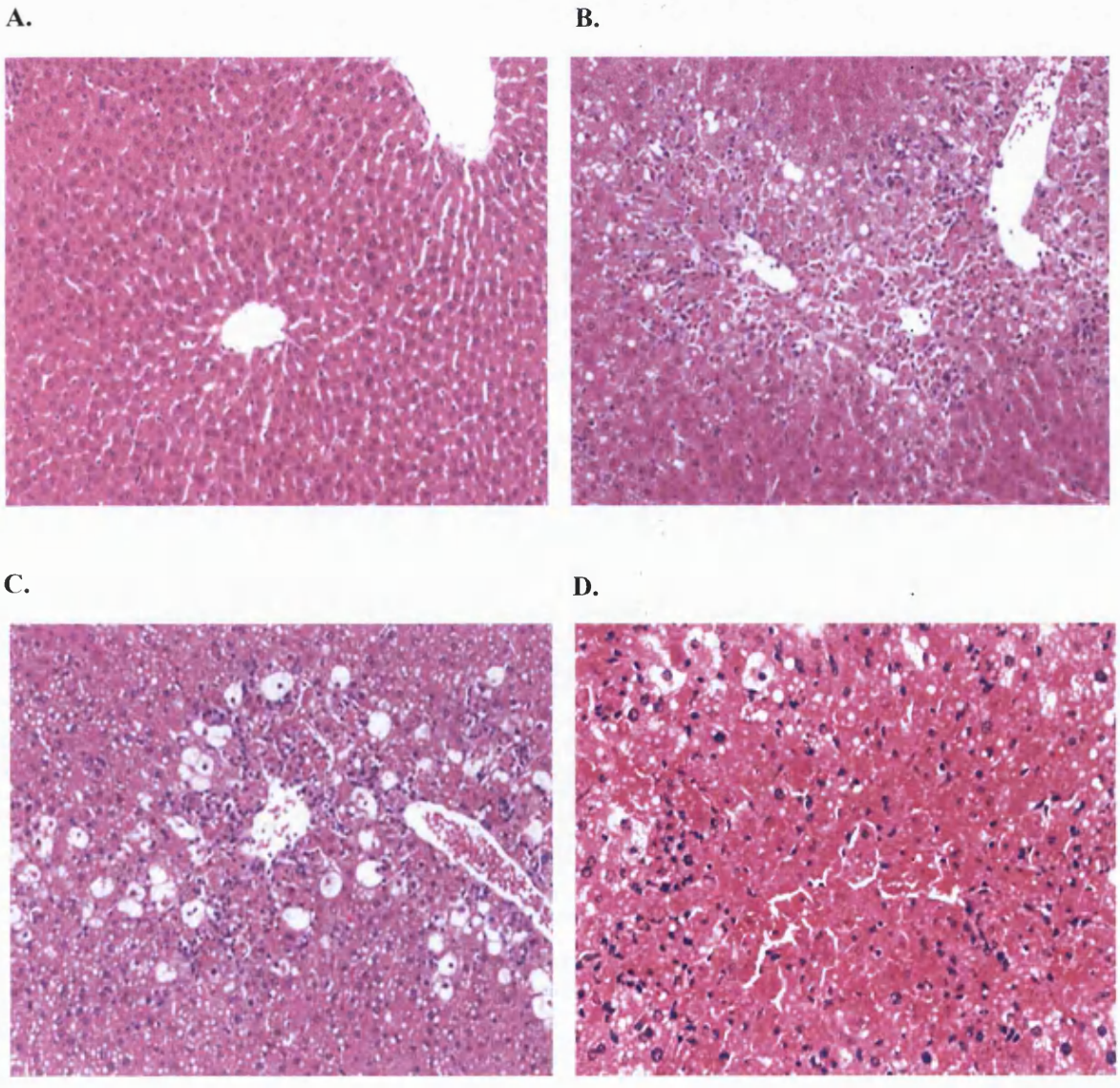
Table 3.2 Mean urea and creatinine levels (and S.D.) in serum from rats treated with increasing doses of CCl_4. Assays were carried out according to the methods described in Section 2.5.

CCl4 (mg/ml)	Urea (mmol/L)		Creatinine (umol/L)	
	Mean	S.D.	Mean	S.D.
Control	6.80	0.70	45.20	5.72
0.4	8.80*	1.14	52.60*	2.30
0.8	8.25	1.67	54.00*	4.58
1.2	8.32*	1.11	56.60**	4.45
1.6	17.35	11.83	90.80	45.62
2.0	27.20***	5.25	112.60***	25.10
2.4	21.76***	5.96	77.25***	11.62
2.8	20.92***	3.34	125.00**	51.34

[a]Values are the means of 5 animals per dose level group. Values that differ from control by Student's t-test are shown, * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

3.3.6 Histopathology

At post mortem, the livers and kidneys were removed from all animals for histopathological examination. In the livers of all CCl_4-treated rats there was hepatocellular degeneration, with vacuolation and necrosis of cells in the centrilobular region. Infiltrating neutrophil polymorphs were also present in the centrilobular region and in many animals there was also evidence of midzonal vacuolar degenerative changes. The severity of lesions was dose-related and more marked in the higher dose groups (Figure 3.3).



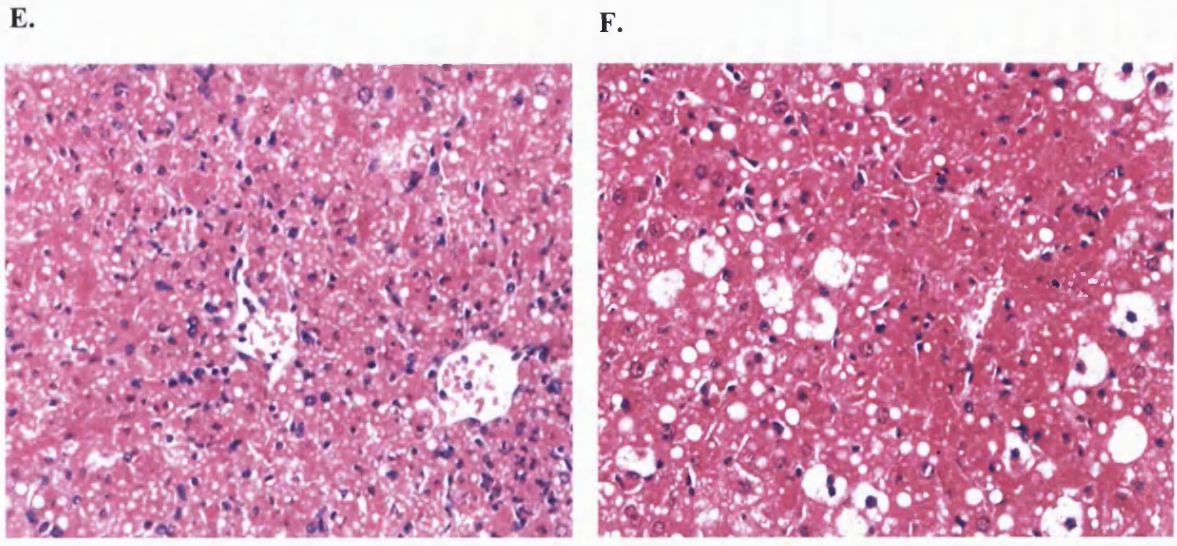


Figure 3.3 Histology of the liver sections from rats treated with increasing dose levels of CCl_4 and sampled at 24 hours post-dosing. Original magnification of all images, x 100; H&E.

A. Liver from a control rat to show the normal appearance and arrangement of hepatocytes in the centrilobular region.

B. Liver from rat treated with CCl_4 at 0.4 ml/kg; there is some swelling and vacuolation of hepatocytes in the centrilobular region and a diffuse infiltration of inflammatory cells present in the perivascular zones.

C. Liver from rat treated with CCl_4 at 0.8 ml/kg; in the centrilobular region there is hepatocellular vacuolation, with inflammatory cells and necrotic hepatocytes in the perivascular area.

D. Liver from rat treated with CCl_4 at 1.6 ml/kg; there is hepatocellular vacuolation with necrotic cells in the perivascular region, and a moderate infiltration of inflammatory cells (polymorph neutrophils) into the area.

E. Liver from rat treated with CCl_4 at 2.0 ml/kg; diffuse, moderate, centrilobular hepatocellular vacuolation with necrotic hepatocytes is evident, with an infiltration of inflammatory cells.

F. Liver from rat treated with CCl_4 at 2.4 ml/kg; there are large numbers of vacuolated and necrotic hepatocytes diffusely distributed in the perivascular zone, with an infiltration of inflammatory cells.

Histology of the kidneys in rats treated with CCl_4 at 1.2 ml/kg and above (Figure 3.4) revealed swelling, degeneration and necrosis of the proximal convoluted tubule cells, with associated tubular casts. The changes were dose-related. The kidneys from rats in

the 0.4 and 0.8 ml/kg CCl_4 groups showed minor background changes (very slight focal tubular basophilia) similar to those seen in control animals.

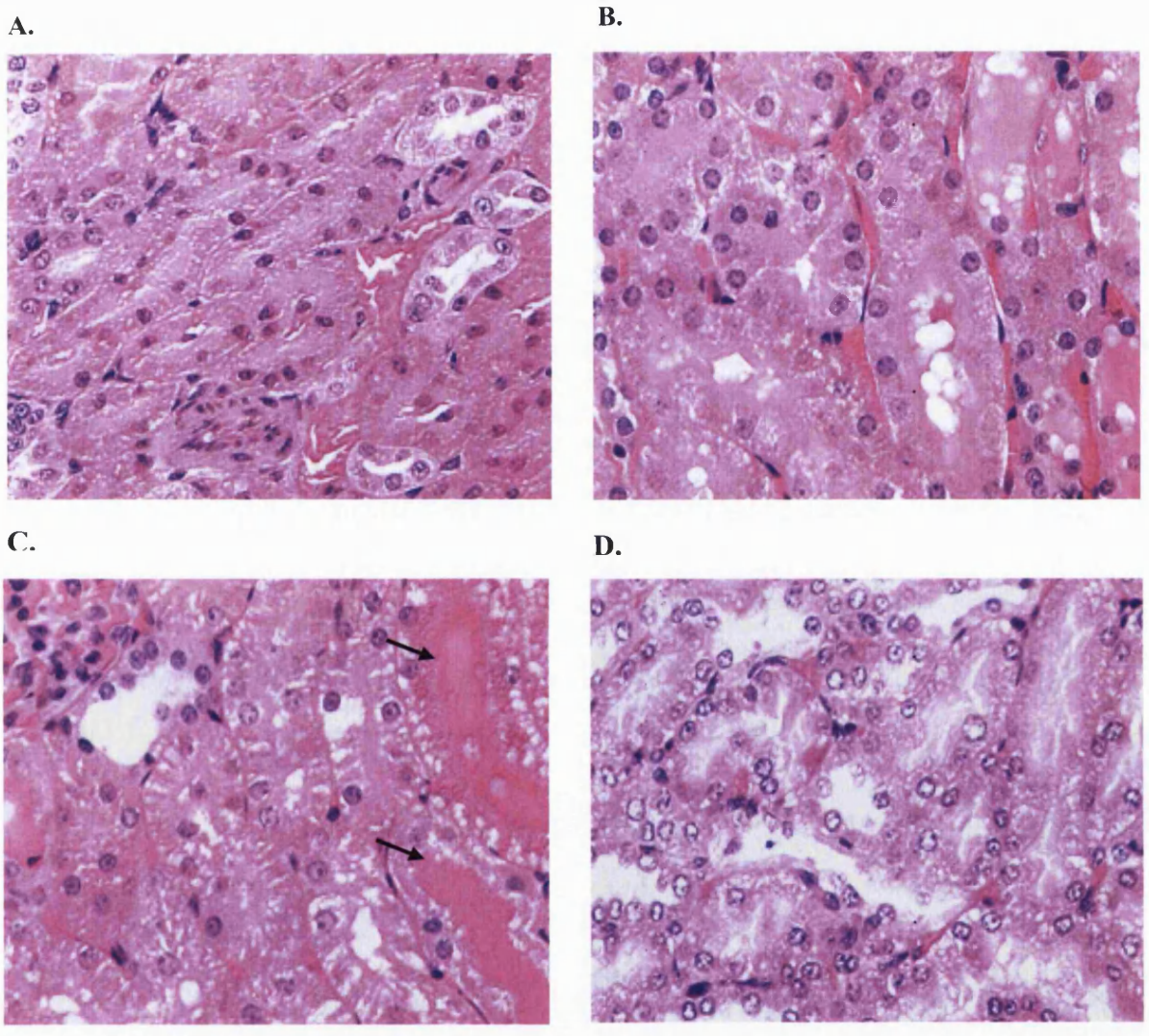


Figure 3.4: Histology of the kidneys from rats treated with increasing dose levels of CCl_4 and sampled at 24 hours post-dosing. Original magnification of all images, x 400; H&E.

A. Kidney from a control rat showing normal appearance of the cortical region

B. Kidney cortex from a rat treated with CCl_4 at 1.6 ml/kg showing swelling and degeneration of the cells of the proximal convoluted tubule.

C. Cortical tissue from a rat treated with CCl_4 at 2.0 ml/kg; swelling and degeneration of the cells of the proximal convoluted tubule and eosinophilic tubular casts (arrows).

D. Kidney cortex from rat treated with CCl_4 at 2.4 ml/kg; degenerating tubular cells are evident.

3.4 Time course study

For experimental details see section 3.2.2, page 106.

3.4.1 Observations during the study

At all time points the animals were all observed to be normal and healthy.

3.4.2 Reagent strips

Urine samples collected during the time course study showed that both the control and CCl_4-treated urine samples contained trace amounts of protein. However all rats tend to have trace levels of protein in their urine sample. All other parameters tested using the strips (as described Section 2.6) showed no differences between control and CCl_4-treated urine samples.

3.4.3 Liver weights

The livers from the CCl_4-treated rats were paler than control liver and slightly enlarged at the 12, 24 and 36-hour time points, indicating liver injury. At all other time points the livers from both the control and CCl_4-treated rats did not differ in appearance or size. Relative liver weights were increased at the first time point (12 hours) but this increase was not statistically significant (Figure 3.5). At 24 hours the liver weights were significantly greater than control ($P < 0.001$) and the weights then remained elevated. By 4 days after dosing the relative liver weights were beginning to drop and return towards normal, although at day 17 the liver weights were still slightly and significantly greater than control ($P < 0.05$). At the 36-48 hour time point the increase in liver weight reached a maximum, being 36 % greater than control weights. These liver weight results suggest that liver injury has been induced by 0.8 ml/kg CCl_4 to a maximum level somewhere between 12 - 48 hours post-dosing. Four days after dosing the liver appears to be undergoing repair as the liver weights return to control values.

3.4.4 Kidney weights

Kidneys were removed from rats at each time point and weighed. The relative kidney weights from rats treated with CCl_4 were not significantly different from the control kidney weights at any time point (data not presented).

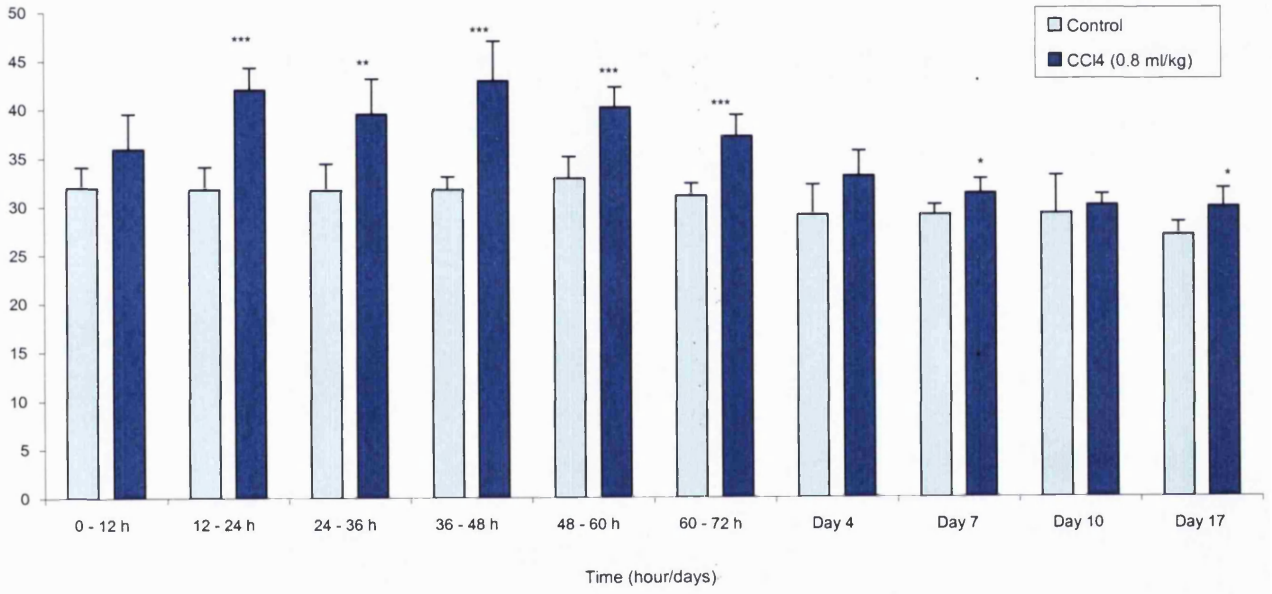


Figure 3.5 Mean relative liver weights of rats treated with CCl_4 at 0.8 ml/kg and sampled at 12, 24, 36, 48, 60, 72 hours and at day 4, 7, 10 and 17 (n=5). Weights are expressed as a relative weight (i.e. g per kg body weight). Values are means of five animals (error bars represent S.D.). Values that differ from control by Student's t-test are shown, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

3.4.5 Serum clinical biochemistry

Increased serum levels of ALT, AST and GLDH were interpreted as indicating hepatotoxicity. Although the dose of CCl_4 administered in this study (0.8 ml/kg) was below the threshold for nephrotoxicity (1.2 ml/kg CCl_4), as determined by the dose response study, the urea and creatinine levels were also measured in the serum at each of the time points to ensure that no kidney damage had been induced.

Mean serum ALT values were significantly increased even at the first time point (12 hours) and remained significantly increased until 48 hours after dosing with CCl_4 ($P < 0.05$). Figure 3.6A shows that at 36 hours post-dosing the ALT values reached a maximum level and were 8 times greater than the control levels. At 60 hours post-dosing the ALT values were still slightly greater than control but this increase was not statistically significant. By day four post-dosing the ALT values had returned to control levels.

AST levels measured in the rat serum samples were significantly increased ($P < 0.05$) at the first time point (12 hours) and increased with time until a maximum level of 11 times greater than control at 36 hours post-dosing (Figure 3.6B). After 36 hours the AST values began to fall and by day four after dosing with CCl_4 the AST levels were back to the control levels.

Mean GLDH levels were increased 3-fold over control values at the 12-hour time point ($P < 0.01$), and this rose to a 60-fold increase over control at 24 hours after dosing (Figure 3.6C). The maximum level of GLDH in the serum was observed at 36 hour post-dosing where GLDH was 100 times greater in the CCl_4-treated rats than in control. By 60 hours the GLDH levels had fallen and the control level was reached by four days after dosing.

The activity of all three enzymes, AST, ALT and GLDH, were all highest at the 24-36 hour time point, indicating that this is the time point after dosing with 0.8 ml/kg CCl_4 that maximum hepatotoxicity was observed. This agrees with the increased liver weights (Figure 3.5). Regeneration occurs following liver injury and therefore by 60 hours post-dosing the activity of the AST, ALT and GLDH were returning to control activity values and by day 4 had done so completely.

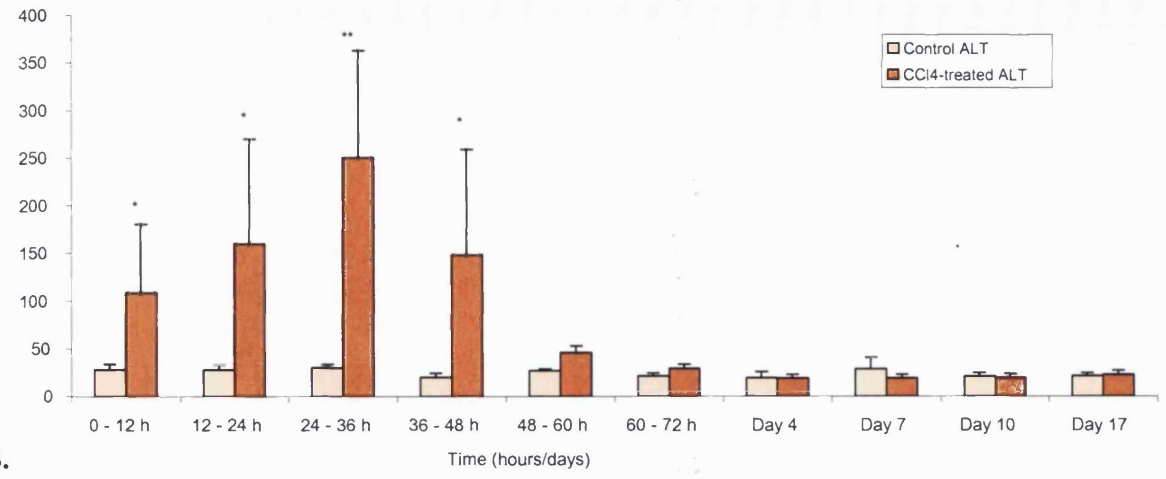
Figure 3.7A shows that serum ALP activity was increased (1.51 times) in serum from rats treated with CCl_4 at the first time point (12 hours), although the increase was not statistically significant (Figure 3.7A). At 24 hours the ALP activity reached a maximum level (2.27 fold greater than control) ($P < 0.001$). The ALP activity in CCl_4-treated rats then began to fall but was greater than control at all time points until 72 hours post-dosing; at day 4 ALP activity in the CCl_4-treated serum was similar to control. ALP is a non-specific enzyme but its activity may be increased following injury to either liver, kidney or bone, therefore its increased activity in these studies compliment the AST, ALT and GLDH results.

The inflammatory protein haptoglobin (HAP-E) was increased from 12 hours post-dosing and was greatest at 48 hours post-dosing ($P < 0.001$) where it was 4.24 fold greater than control values. At 10 days after dosing it had returned to control levels (Figure 3.7B).

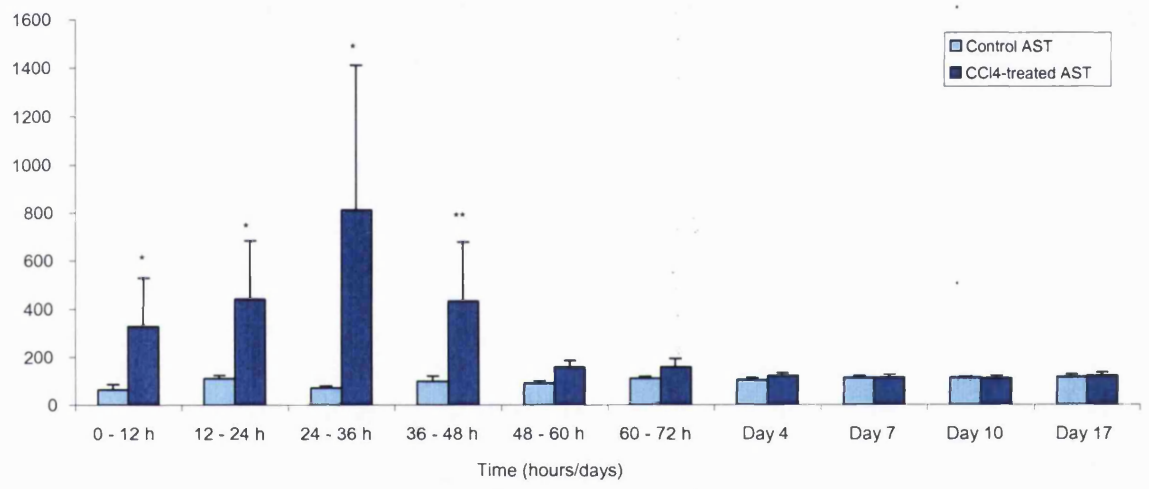
Urea and creatinine levels were measured in the serum samples. Urea values were slightly increased at both the 12-hour and 24-hour sampling points. Although at these times points the urea levels were significantly different this was due to one or two animals in each group having higher than normal levels. (Table 3.3A). The mean creatinine levels in serum samples did not show any significant difference between CCl_4-treated rats and control. (Table 3.3B)

The urea and creatinine levels for this study therefore did not indicate any nephrotoxicity at any of the time points sampled. Therefore we would not expect histopathology analysis to reveal any kidney injury in any of the rats.

A.



B.



C.

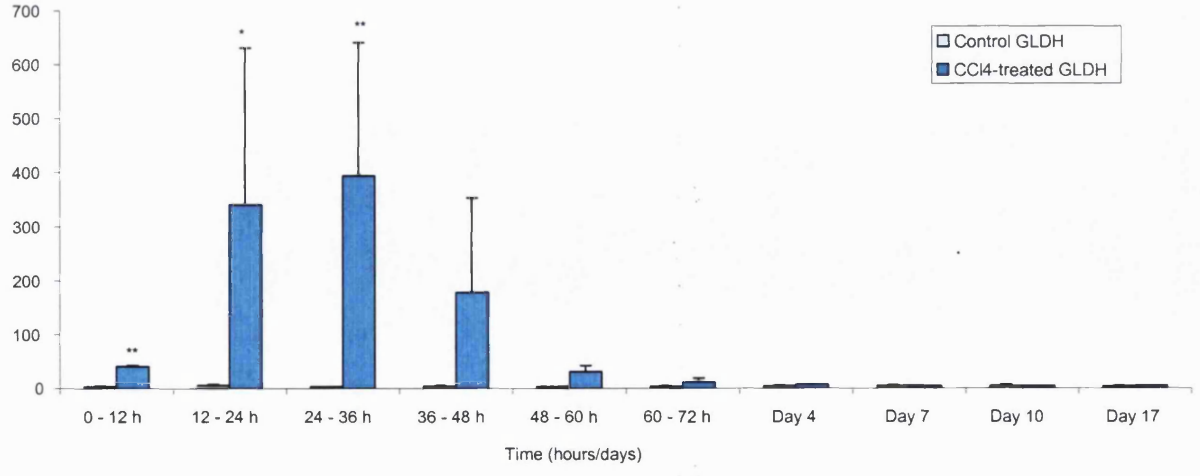
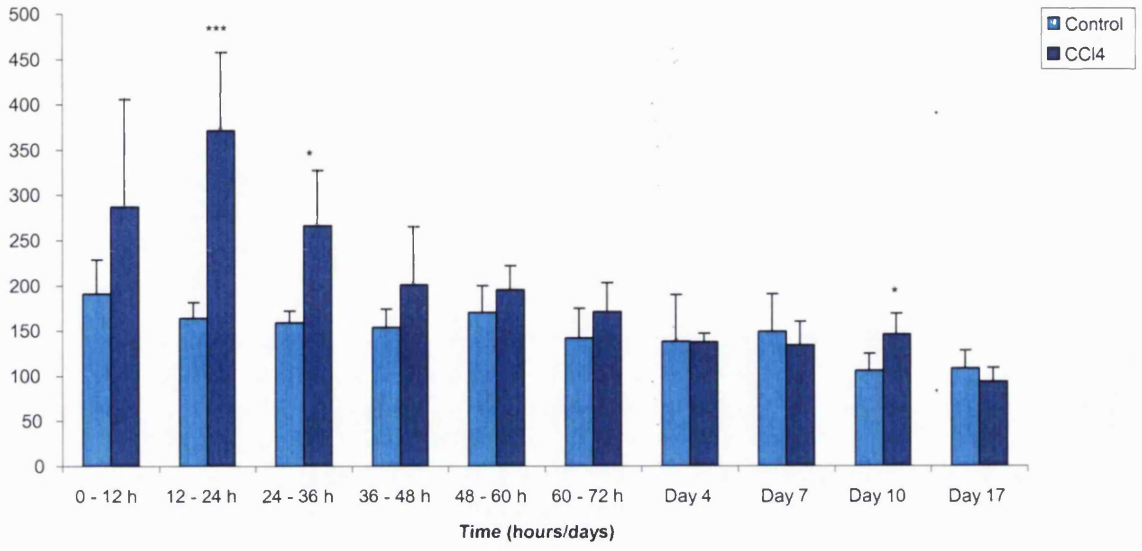


Figure 3.6 Mean ALT (A), AST (B) and GLDH (C) activity measured in serum of rats treated with 0.8 ml/kg CCl_4 and sampled at a series of time points. Enzyme activities were measured according to the methods described in Section 2.5. Each value represents a mean of 5 animals. (error bars represent S.D.) Values that differ from control by Student's t-test are shown, * $P < 0.05$ ** $P < 0.01$.

A.



B.

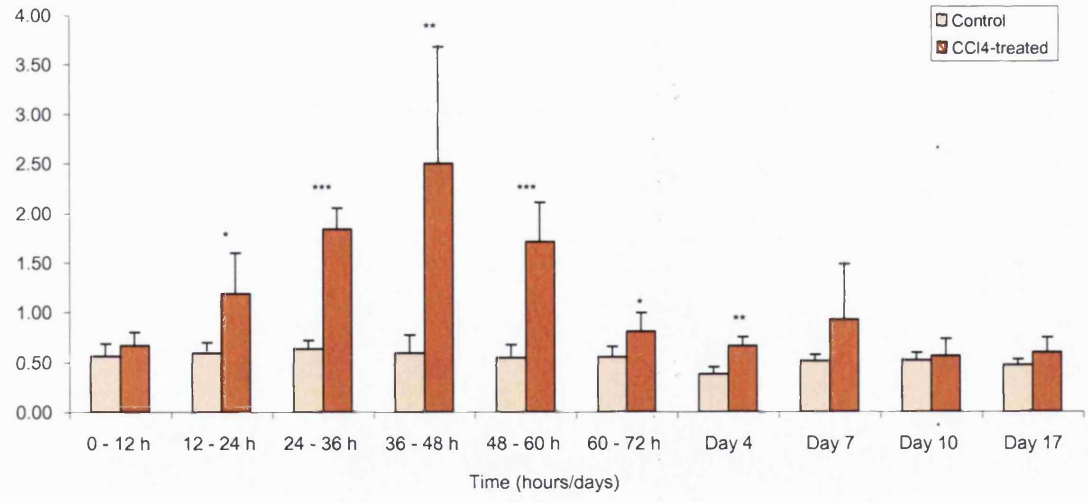


Figure 3.7 Mean ALP (A) and HAP-E (B) activity measured in serum from rats dosed with CCl_4 at 0.8 ml/kg CCl_4 and sampled at a series of time points. Enzyme activities were measured according to the methods described in Section 2.5. Values are means of 5 animals (error bars represent S.D). Values that differ from control by Student's t-test are shown, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

Table 3.3 Mean urea (A) and creatinine (B) levels for rats treated with 0.8 ml/kg CCl_4 and sampled at a series of time points[a]. Assays were carried out according to the methods described in Section 2.5.

A.

	Treatment group			
	Control (mmol/L)		CCl4-treated (mmol/L)	
Time	mean	S.D.	Mean	S.D.
0-12 hour	5.40	0.66	6.36*	0.20
12-24 hour	6.70	0.80	7.71*	0.41
24-36 hour	5.16	0.61	6.96	1.42
36-48 hour	8.06	0.62	7.37	1.76
48-60 hour	5.68	0.93	5.29	0.89
60-72 hour	8.02	1.29	6.94	1.08
Day 4	6.76	0.47	7.34	0.69
Day 7	8.16	1.41	9.59	2.44
Day 10	8.09	0.58	8.01	0.40
Day 17	7.31	0.65	8.01	0.69

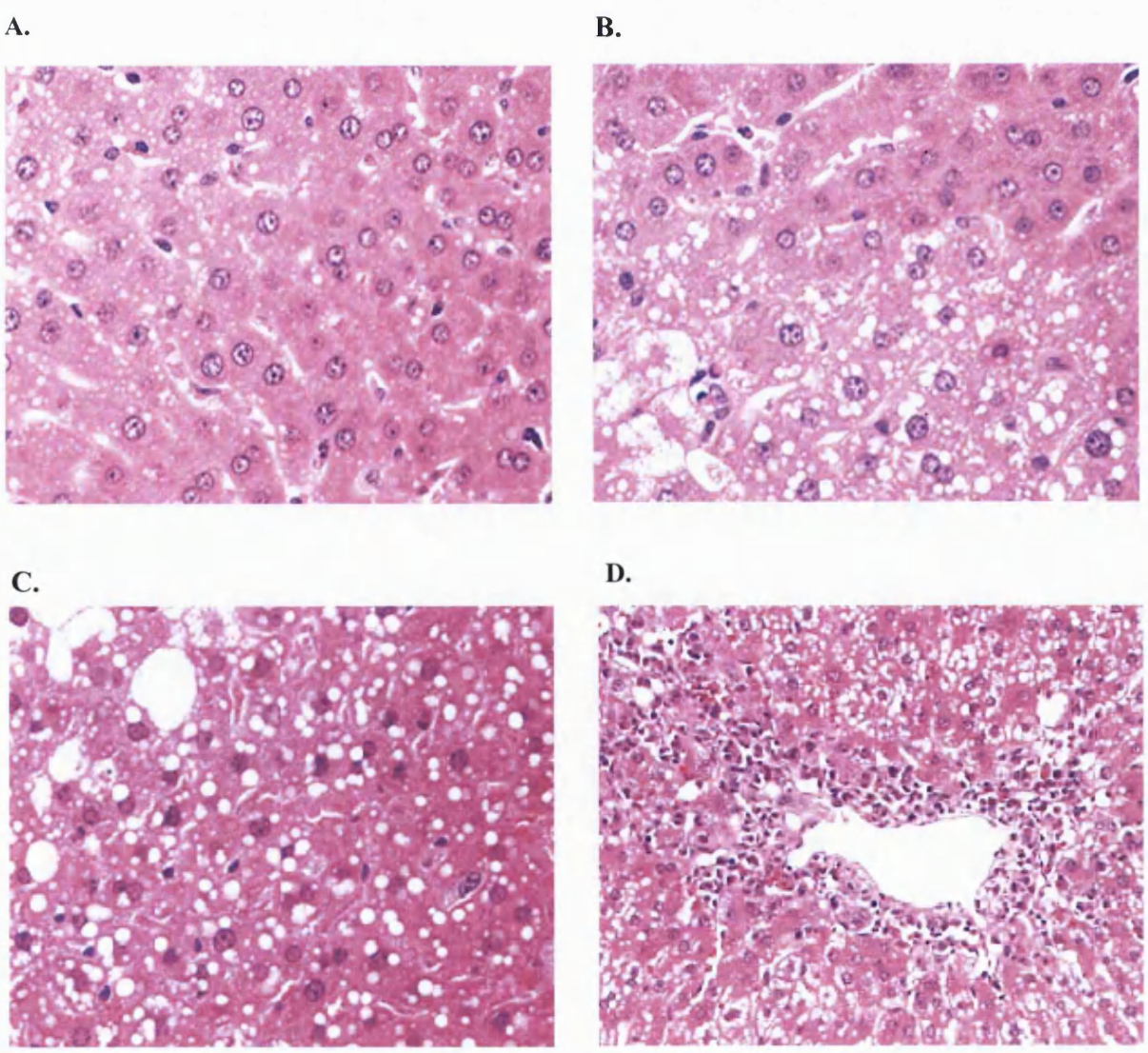
B.

	Treatment group			
	Control (umol/L)		CCl4-treated (umol/L)	
Time	Mean	S.D.	Mean	S.D.
0-12 hour	49.40	3.36	47.00	3.08
12-24 hour	47.40	1.95	53.40	4.72
24-36 hour	50.50	2.08	52.80	6.06
36-48 hour	51.60	1.67	54.00	7.38
48-60 hour	51.00	2.12	49.40	4.56
60-72 hour	56.33	4.93	53.33	0.58
Day 4	51.00	1.00	62.50	6.81
Day 7	57.50	8.39	67.50	8.96
Day 10	56.00	2.94	57.00	2.94
Day 17	60.67	4.19	66.75	4.19

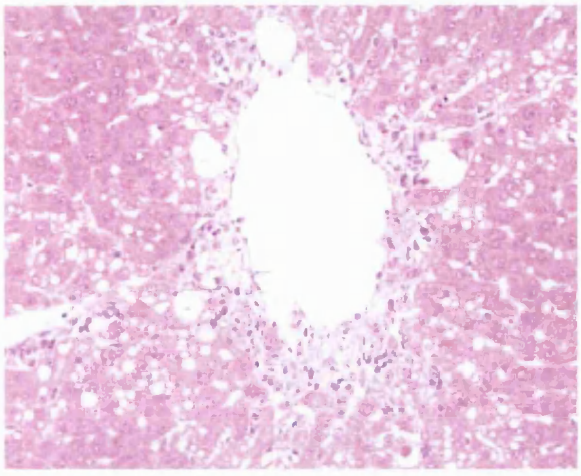
[a]Values are means of five animals (and S.D.). Values that differ from control by Student's t-test are shown, * $P < 0.05$.

3.4.6 Histopathology of liver and kidney sections

Histological examination of the liver and kidneys removed from rats at each time point was carried out (Figure 3.8). In CCl_4-treated livers at 12 hours post-dosing there was centrilobular hepatocellular swelling with an inflammatory cell infiltrate. The hepatocytes then appeared to progress through cytoplasmic vacuolation, cytoplasmic and nuclear degeneration, and finally cell necrosis. Inflammatory cells increased in number, and then waned over this period. By day 7, in most of the livers there was a return to normal pathology. There was no treatment-related pathology in the kidney from rats treated with CCl_4 at any time point.



E.



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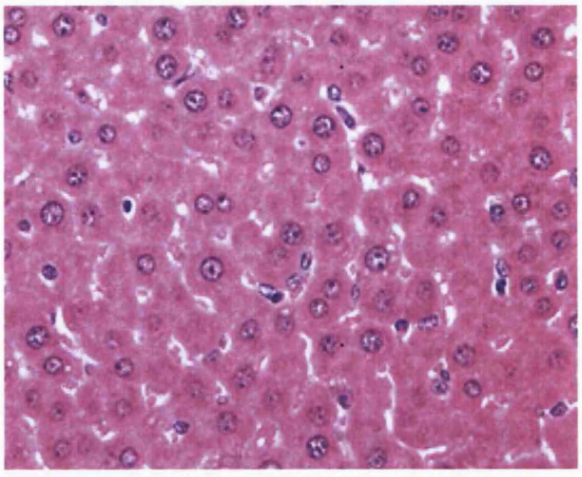


Figure 3.8 Histopathology of the liver from rats treated with CCl_4 at 0.8 ml/kg and sampled at various time points post-dosing. All H&E.

A. Liver from a control rat illustrating the normal appearance of centrilobular hepatocytes and their arrangement. Original magnification of image, x 400.

B. Liver from a rat sampled at 12 hours post-dosing showing hepatocellular swelling in the centrilobular region. Original magnification of image, x 400.

C. Liver from a rat sampled at 24 hours after dosing with CCl_4 showing hepatocellular cytoplasmic vacuolation and necrosis in the perivascular zone. Original magnification of image, x 400.

D. Liver from a rat sampled at 48 hours after dosing with CCl_4; centrilobular region showing hepatocyte vacuolation, degeneration and necrotic cells with inflammatory cell infiltration (polymorph neutrophils) in the perivascular area. Original magnification of image, x 100.

E. Liver from a rat dosed with CCl_4 at 0.8 ml/kg and sampled at 60 hours after dosing; there is hepatocellular vacuolation in the centrilobular region with necrotic cells, and inflammatory cells are also present in the perivascular zone. Original magnification of image, x 100.

F. Liver from a rat dosed with CCl_4 and sampled at 72 hours post-dosing; resolution of the pathological changes has taken place and the normal appearance of the tissues has been restored. Original magnification of image, x 400.

3.5 Evaluation of protein markers of hepatotoxicity

3.5.1 Surface Enhanced Laser Desorption/Ionisation ProteinChip technology (SELDI)

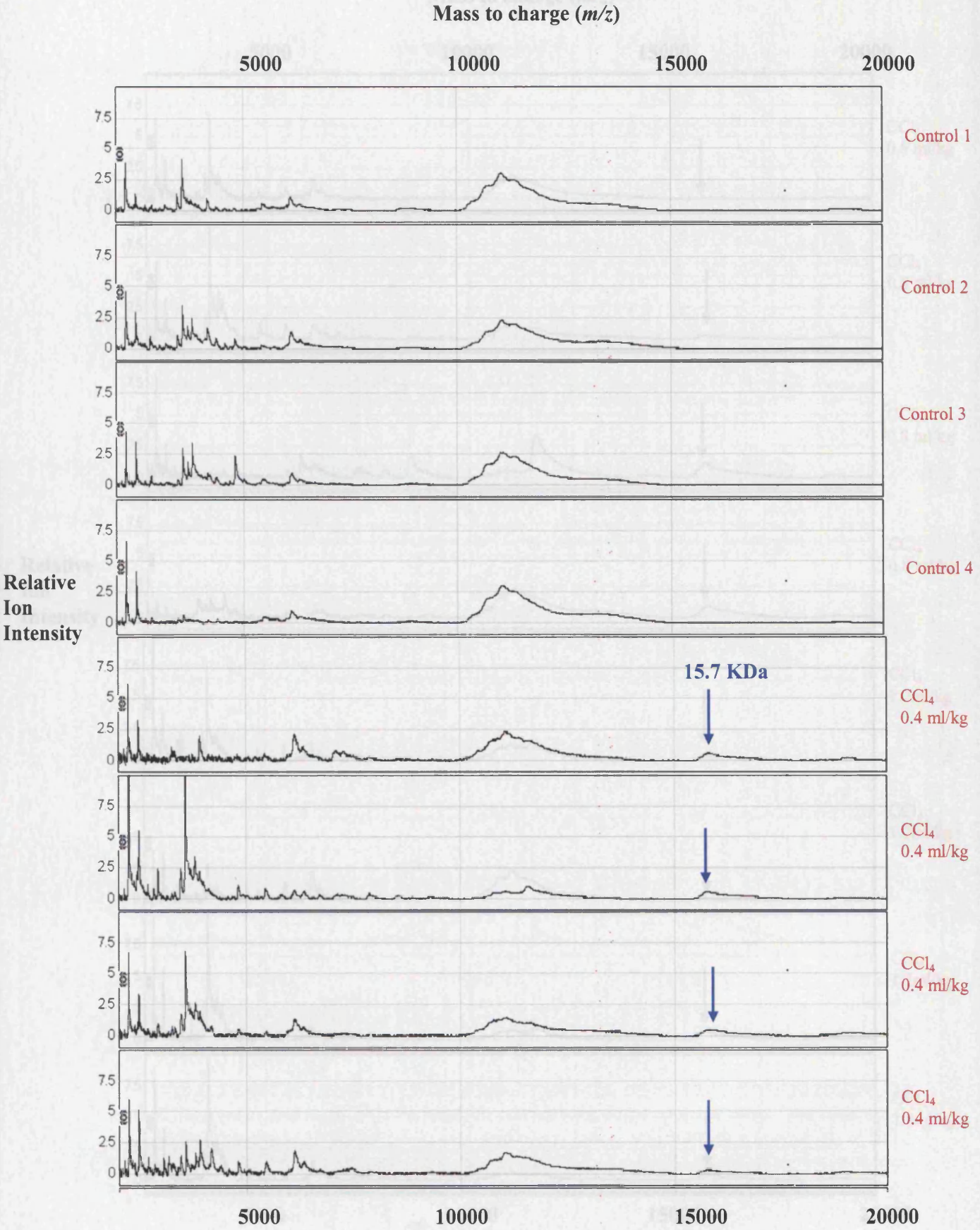
All urine samples collected during both the dose response and time course studies were analysed using SELDI. Urine samples were applied to normal phase (SiO_2) ProteinChip arrays as described in Section 2.17. Proteins in the urine sample will bind to the normal phase chip through hydrophilic residues on their surfaces via electrostatic interactions or hydrogen bonds. Unbound were washed off with de-ionised water before analysis by the ProteinChip reader, therefore the urine sample analysed by SELDI contained a fraction of the total urinary proteome. The fraction of the urinary proteome analysed differs according to the ProteinChip used, for example, an anionic chip binds proteins having a net negative charge at the pH used. The use of an anionic chip would result in the analysis of a different fraction of the total urinary proteome.

Figure 3.9 shows the spectrum obtained after laser desorption and mass spectrometry analysis of urine samples bound to a normal phase chip. Some proteins appear to be common to all urine samples such as the broad peak at 11.6 kDa and the smaller peak at 6.9 kDa. Analysis of the normal phase chips revealed the presence of a peak at approximately 15.7 kDa in the urine samples from the CCl_4-treated rats (Figure 3.9). This peak was not present in any of the control urine samples. The 15.7 kDa peak was detected in the rat urine in response to CCl_4 and as the CCl_4 dose increased the intensity of the peak in general appeared to increase. The urine samples treated at 1.2 ml/kg CCl_4 and above contained blood and the SELDI spectra showed that the urine samples from rats treated with CCl_4 at 1.6 ml/kg and above appeared to have an additional peak alongside the 15.7 kDa peak. This data suggests that the induction of hepatotoxicity by carbon tetrachloride results in the appearance of a protein in the rat urine which does not appear to be present in the control urine. Figure 3.9 shows the spectral view for the mass range 2-20 kDa. All the SELDI spectra has been normalised for total ion current.

The SELDI ProteinChip technique is more effective at analysing low molecular weight proteins (i.e. < 20 kDa) (Issaq et al., 2002) and therefore there is considerable loss of resolution and poor detection of proteins greater than 20 kDa (Figure 3.10). Proteins with a molecular weight greater than approximately 20 kDa would not normally be

found in the urine samples. However the induction of kidney injury or a change in glomerular filtration may allow high molecular weight proteins to pass through the filtration barriers into the urine.

Figure 3.10 shows the spectra for the urine samples collected from CCl_4-treated rats at a mass range between 15 and 100 kDa at a much lower relative ion intensity than that in Figure 3.9. The control urine samples contain two peaks above 15 kDa, the highest at about 25 kDa. However, the urine from rats treated with CCl_4 contains the additional 15.7 kDa protein. This 15.7 kDa protein is present in all the urine samples from rats treated with CCl_4 and is not present in any of the controls. The urine samples from rats treated at 1.2 ml/kg CCl_4 and above contained blood as described in Section 3.3.1. At these high dose levels additional protein peaks appear, for example, the 8.6 kDa peak in the urine from the 1.6 and 2.0 ml/kg groups (Figure 3.9). Figure 3.10 shows additional peaks at the higher mass range in the urine samples from rats treated with CCl_4 at 1.2 ml/kg and higher which are likely to be blood products such as albumin and haemoglobin. These results prove that unless nephrotoxicity has been induced the proteins found in rat urine are generally of small molecule weight i.e. less than 30 kDa.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15000
20000
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5
2.5
0
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Control 2
Control 3
Control 4
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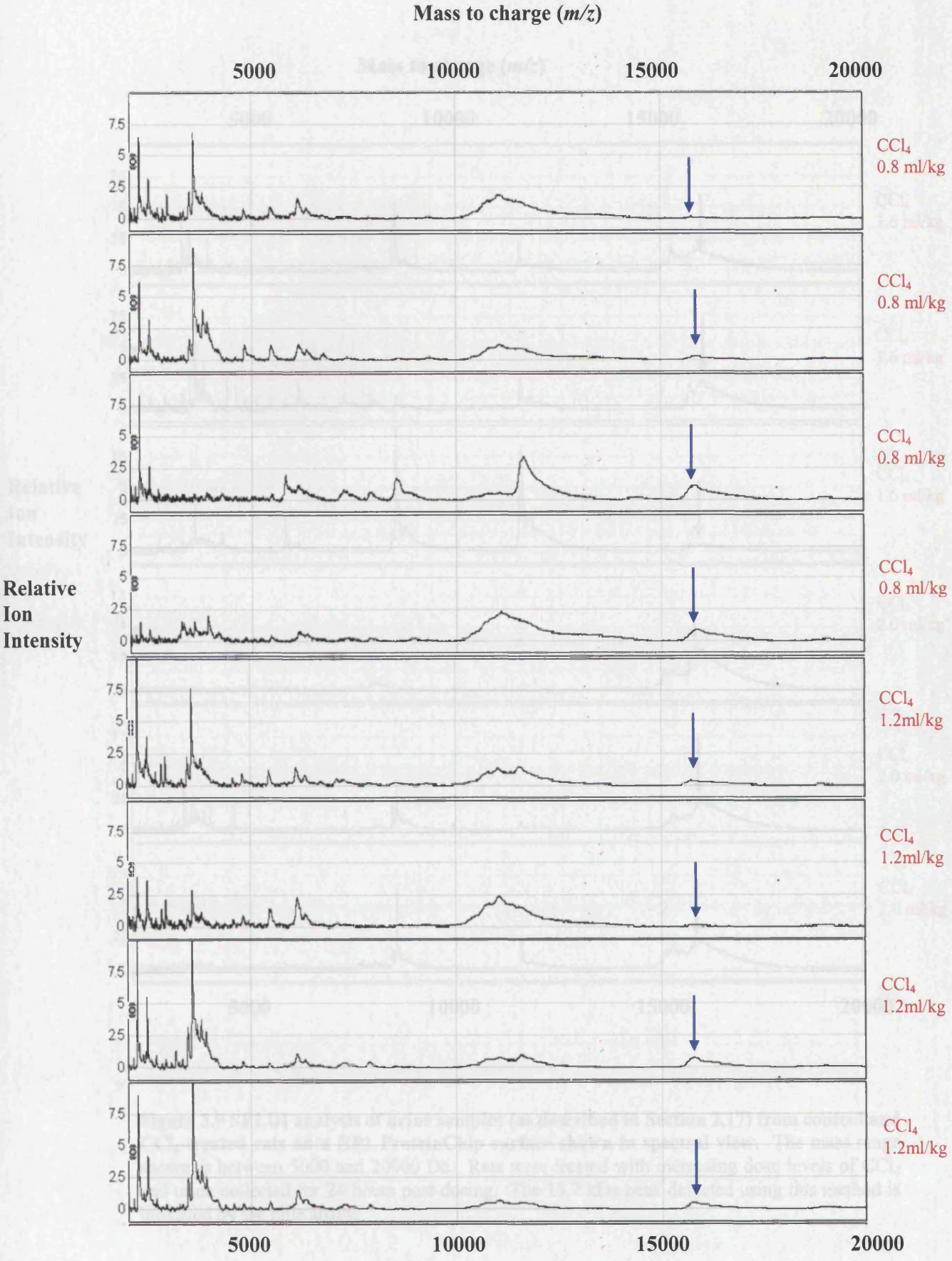
CCl4 0.4 ml/kg
CCl4 0.4 ml/kg
CCl4 0.4 ml/kg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Relative Ion Intensity
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CCl4 0.8 ml/kg
CCl4 0.8 ml/kg
CCl4 0.8 ml/kg
CCl4 1.2ml/kg
CCl4 1.2ml/kg
CCl4 1.2ml/kg
CCl4 1.2ml/kg

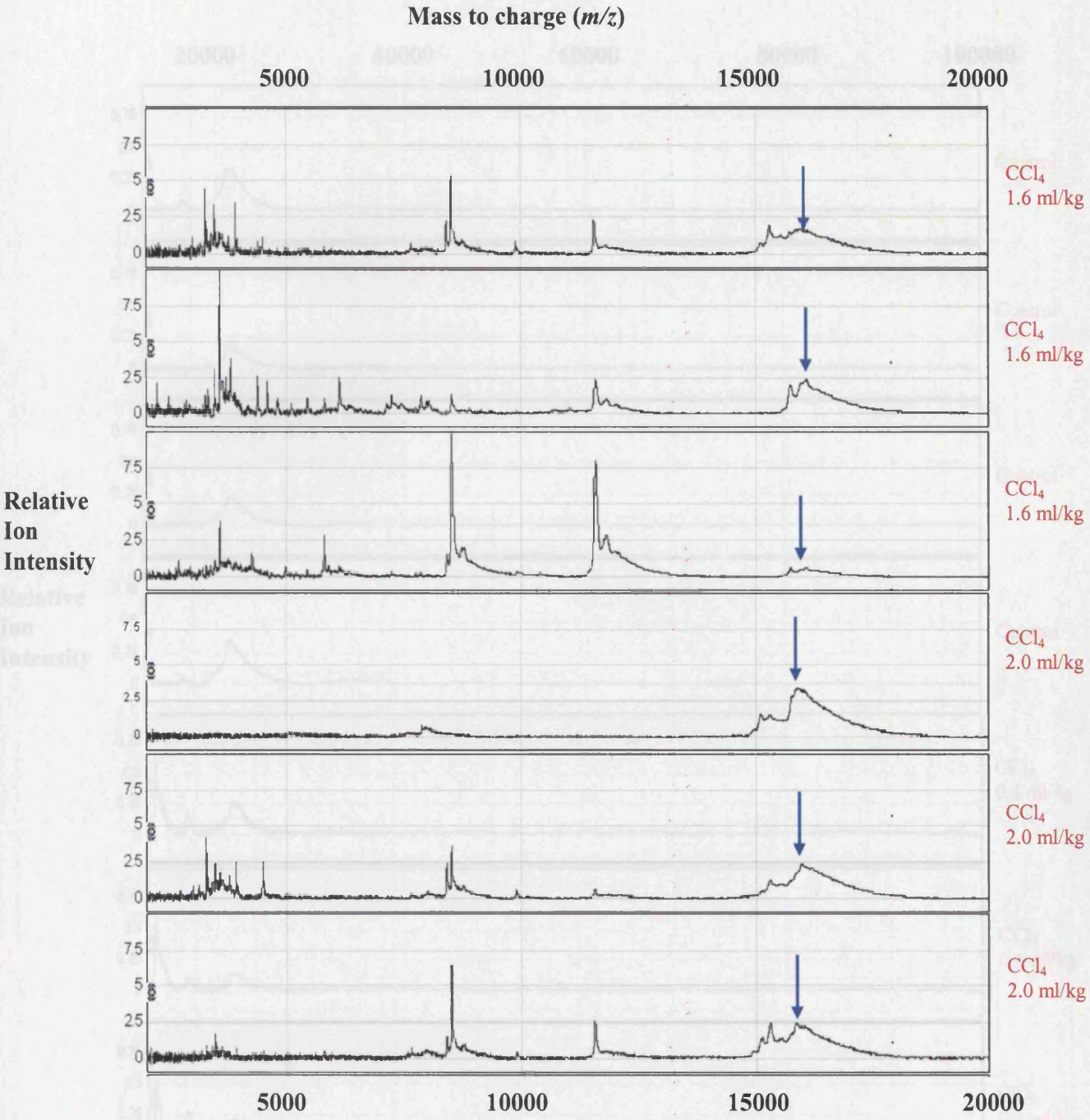
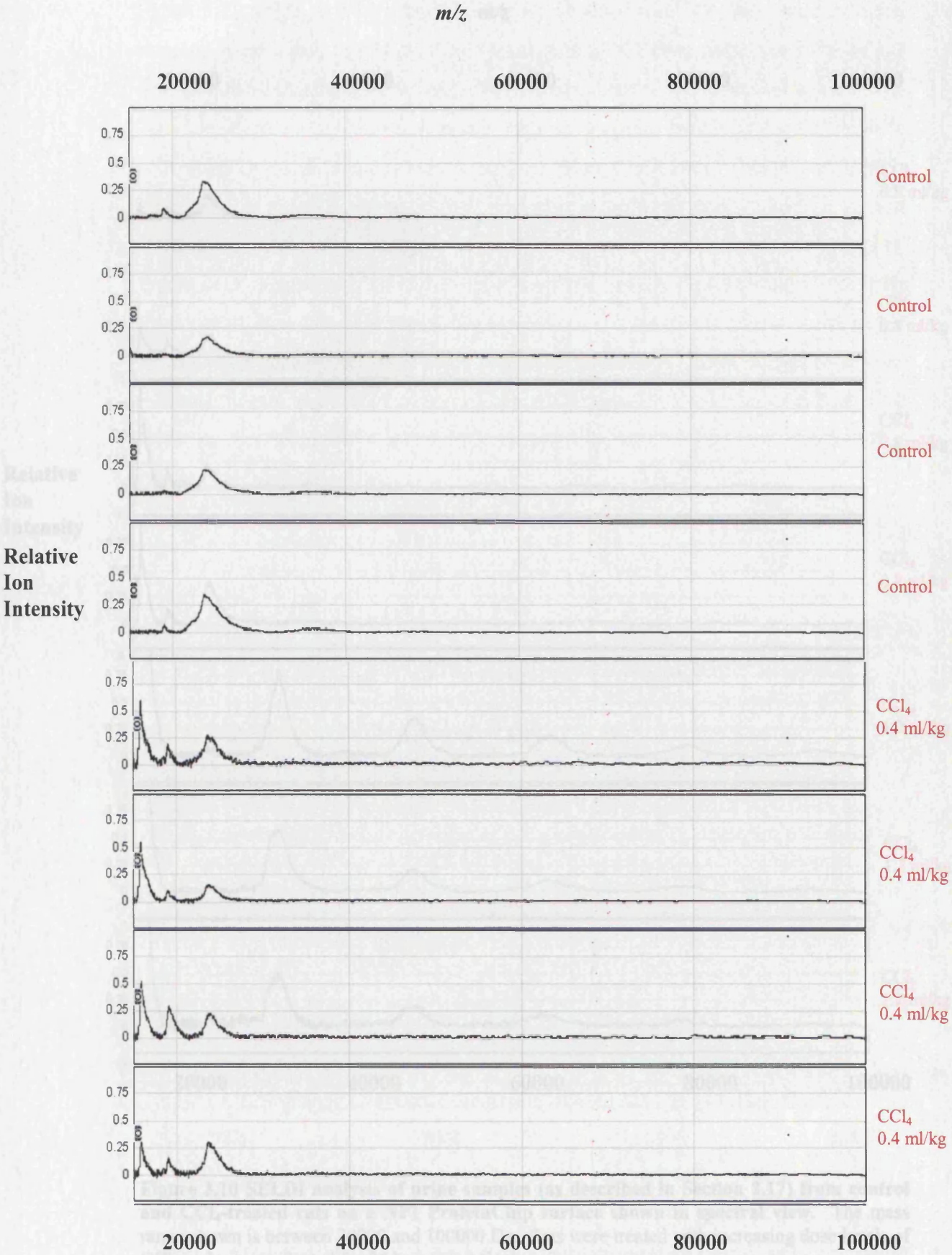


Figure 3.9 SELDI analysis of urine samples (as described in Section 2.17) from control and CCl_4-treated rats on a NP1 ProteinChip surface shown in spectral view. The mass range shown is between 5000 and 20000 Da. Rats were treated with increasing dose levels of CCl_4 and urine collected for 24 hours post-dosing. The 15.7 kDa peak detected using this method is indicated by the blue arrow.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40000
60000
80000
100000
Relative Ion Intensity
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0.5
0.25
0
Control
Control
Control
Control
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CCl4 0.4 ml/kg
CCl4 0.4 ml/kg
CCl4 0.4 ml/kg

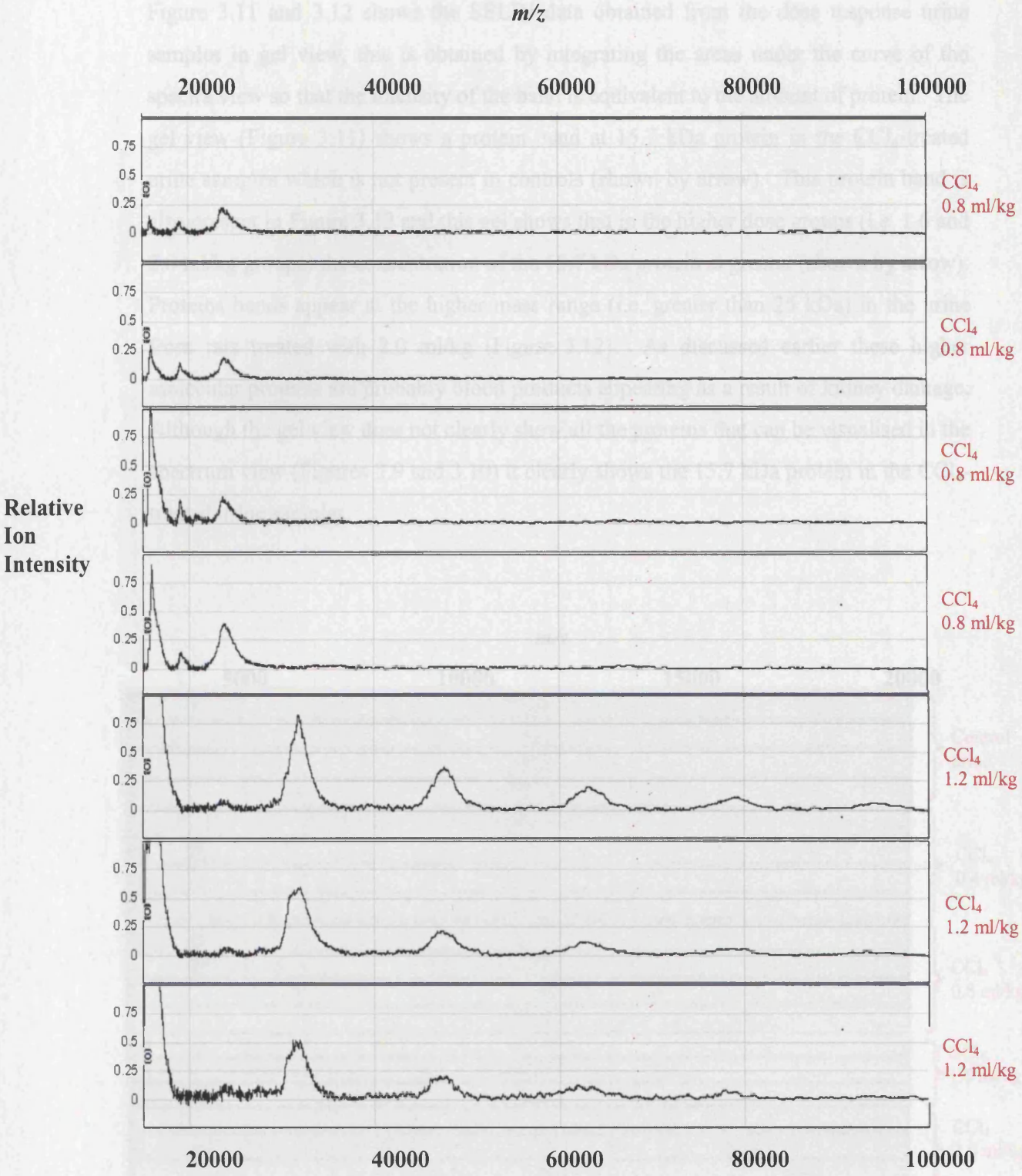


Figure 3.10 SELDI analysis of urine samples (as described in Section 2.17) from control and CCl_4-treated rats on a NP1 ProteinChip surface shown in spectral view. The mass range shown is between 20000 and 100000 Da. Rats were treated with increasing dose levels of CCl_4 and urine collected for 24 hours post-dosing.

Figure 3.11 and 3.12 shows the SELDI data obtained from the dose response urine samples in gel view, this is obtained by integrating the areas under the curve of the spectra view so that the intensity of the band is equivalent to the amount of protein. The gel view (Figure 3.11) shows a protein band at 15.7 kDa protein in the CCl_4-treated urine samples which is not present in controls (shown by arrow). This protein band is also present in Figure 3.12 and this gel shows that in the higher dose groups (i.e. 1.6 and 2.0 ml/kg groups) the concentration of the 15.7 kDa protein is greater (shown by arrow). Proteins bands appear at the higher mass range (i.e. greater than 25 kDa) in the urine from rats treated with 2.0 ml/kg (Figure 3.12). As discussed earlier these higher molecular proteins are probably blood products appearing as a result of kidney damage. Although the gel view does not clearly show all the proteins that can be visualised in the spectrum view (Figures 3.9 and 3.10) it clearly shows the 15.7 kDa protein in the CCl_4-treated urine samples.

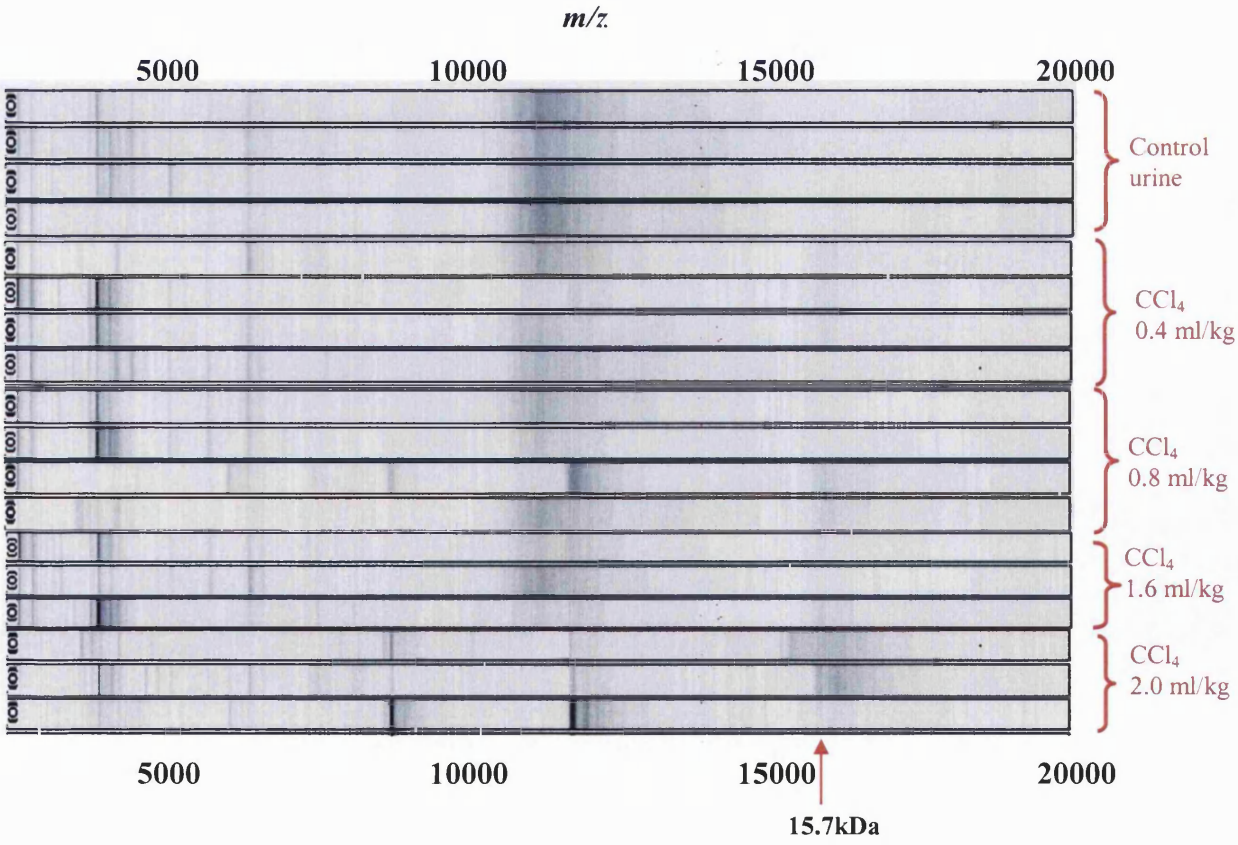


Figure 3.11 Gel view of SELDI spectra from urine samples from control and CCl_4-treated rats on a NP1 ProteinChip surface (as described in Section 2.17). The mass range shown is between 5000 and 20000 Da. Rats were treated with increasing CCl_4 dose levels and urine collected for 24 hours.

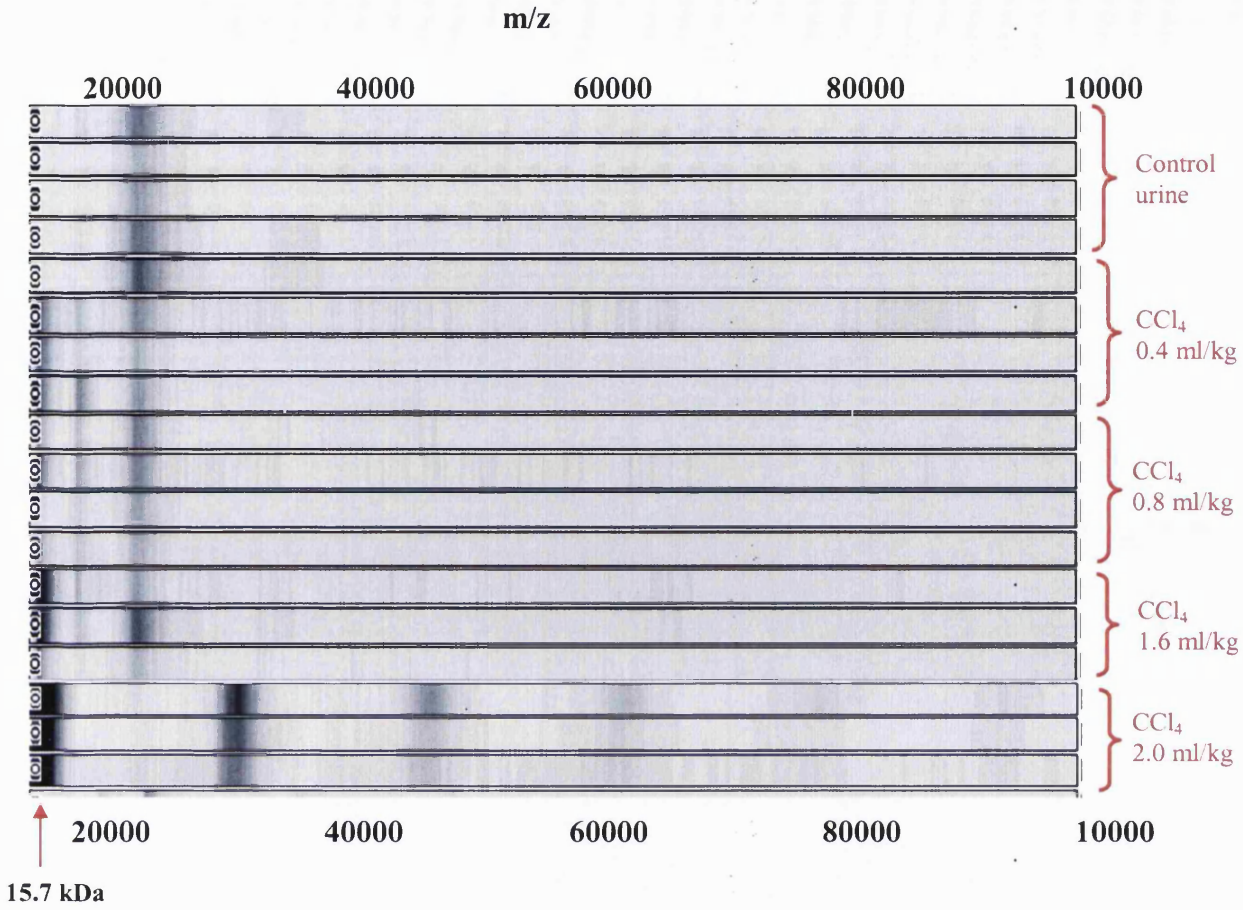
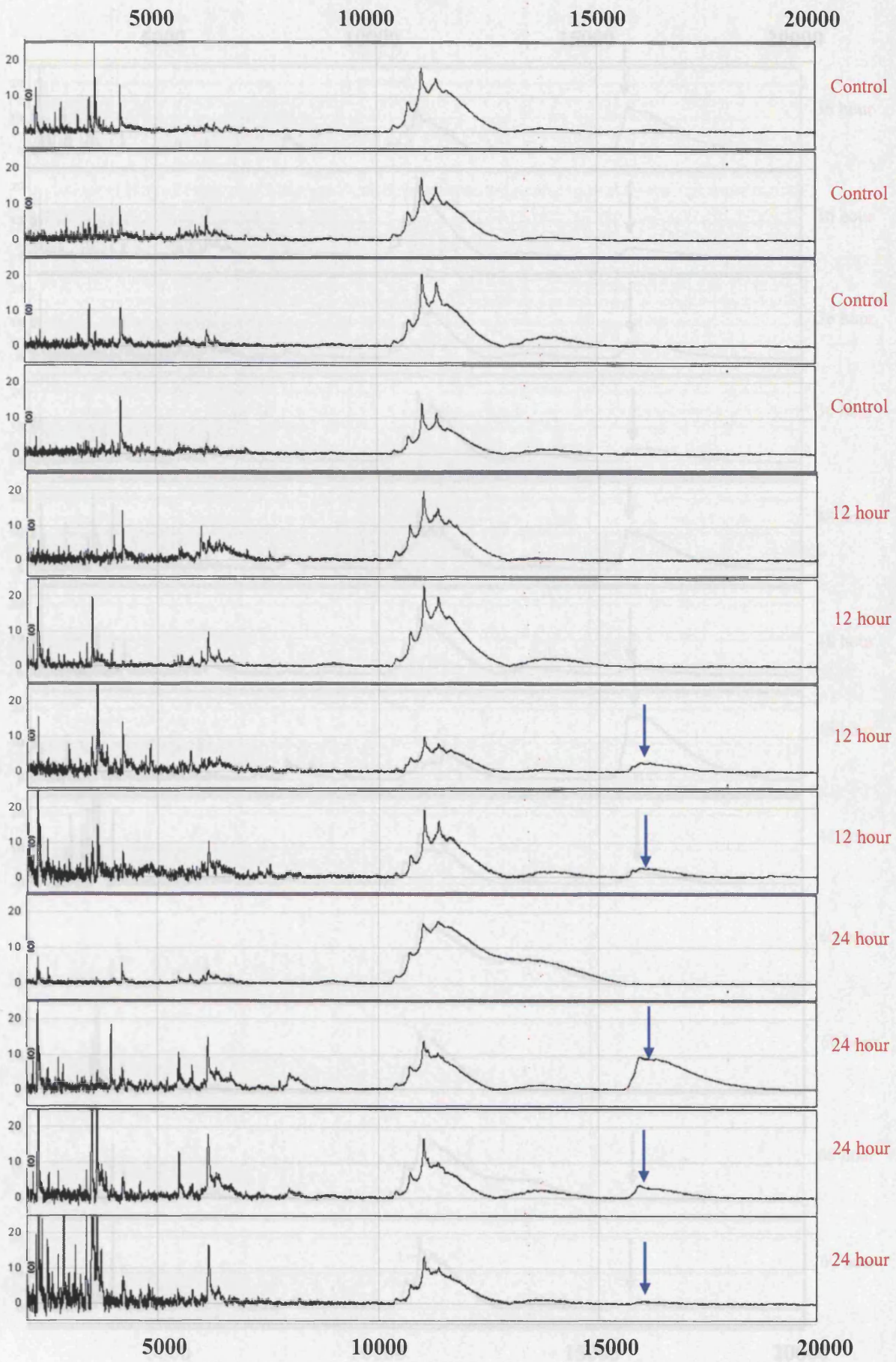


Figure 3.12 Gel view of SELDI spectra from urine samples from control and CCl_4-treated rats on a NP1 ProteinChip surface (as described in Section 2.17). The mass range shown is between 20000 and 100000 kDa. Rats were treated with increasing CCl_4 dose levels and urine collected for 24 hours.

SELDI analysis of urine collected during the time course study revealed the appearance of the same 15.7 kDa protein in response to CCl_4 treatment as in the dose response study. Although, in this study it was not possible to detect the peak in all of the urine samples from CCl_4-treated rats. This is due to intra-group variation as to the degree of injury induced in each animal and the time period at which injury occurs in each individual. The 15.7 kDa peak appeared in three out of the five urine samples collected from CCl_4-treated rats at 12 hours post-dosing (not all data shown). At 24 hours four out of the five rats had the 15.7 kDa peak, and then at both 36 and 48 hours this peak appeared in all five urine samples collected at each time point. At 60 hours post-dosing SELDI detected the 15.7 kDa peak in two out of five of the urine samples collected from CCl_4-treated rats. At all later time points (i.e. 72 hours and greater) SELDI did not detect the 15.7 kDa peak in any of the urine samples (data not shown). SELDI revealed the similar spectra at high mass range (15 – 100 kDa) for these urine samples was similar to those obtained during the dose response study at 0.4 and 0.8 ml/kg as shown in Figure 3.10 (data not shown).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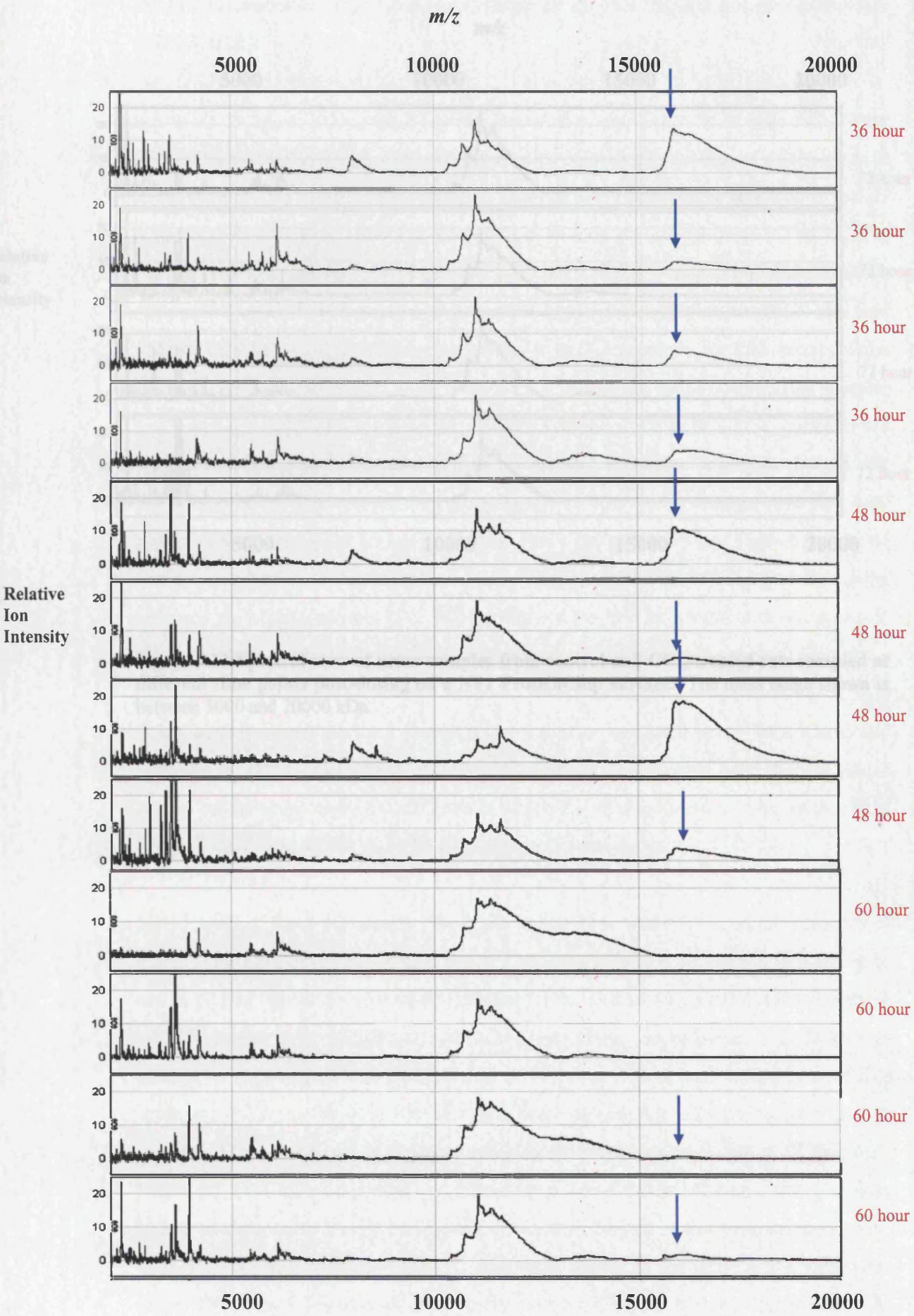
24 hour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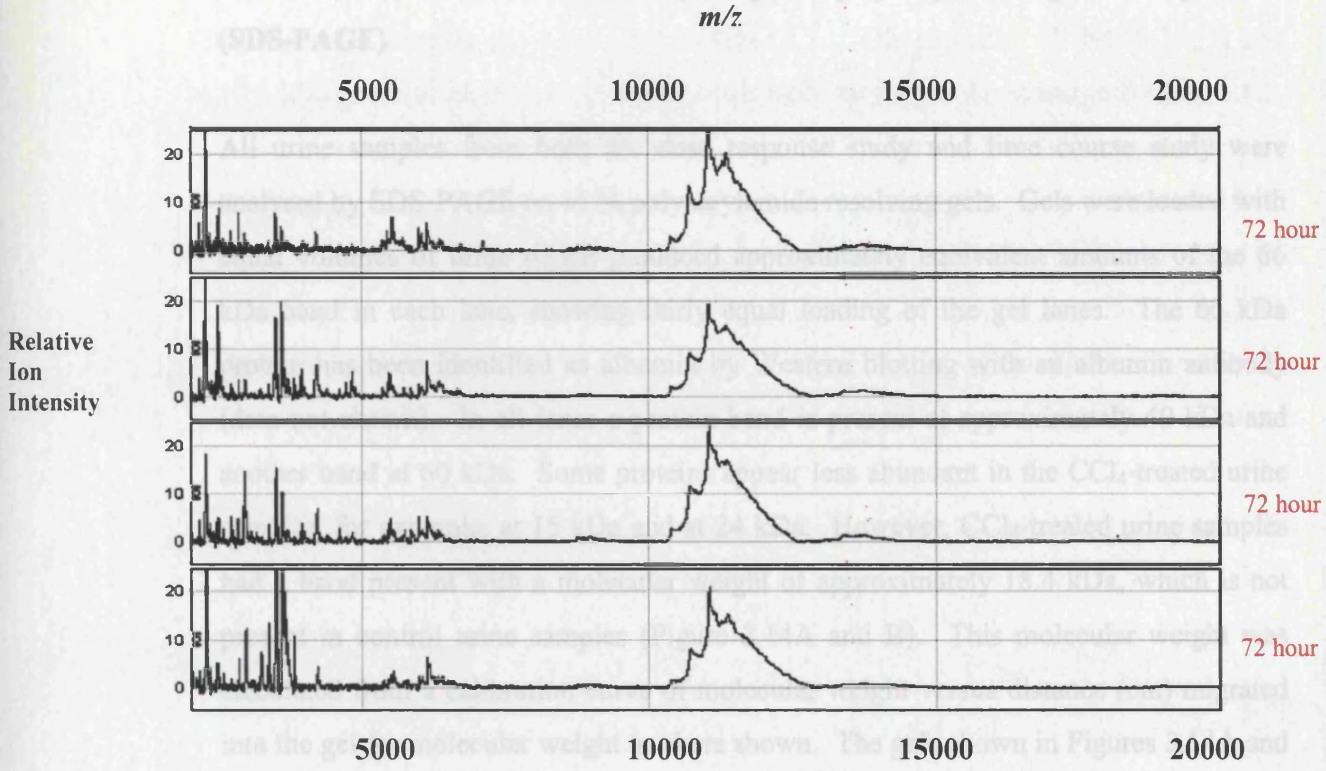


Figure 3.13 Spectral view of urine samples from control and CCl_4-treated rats sampled at different time points post-dosing on a NP1 ProteinChip surface. The mass range shown is between 5000 and 20000 kDa.

3.6 One-dimensional sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

All urine samples from both the dose response study and time course study were analysed by SDS-PAGE on 15 % polyacrylamide resolving gels. Gels were loaded with equal volumes of urine which produced approximately equivalent amounts of the 66 kDa band in each lane, showing fairly equal loading of the gel lanes. The 66 kDa protein has been identified as albumin by Western blotting with an albumin antibody (data not shown). In all lanes a protein band is present at approximately 40 kDa and another band at 60 kDa. Some proteins appear less abundant in the CCl_4-treated urine samples, for example, at 15 kDa and at 24 kDa. However, CCl_4-treated urine samples had a band present with a molecular weight of approximately 18.4 kDa, which is not present in control urine samples (Figure 3.14A and B). This molecular weight was calculated from a calibration curve of molecular weight versus distance (cm) migrated into the gel for molecular weight markers shown. The gels shown in Figures 3.14A and B are two separate studies representative of many carried out showing that the results obtained are highly reproducible. The intensity of the 18.4 kDa band does not appear to be greater in the urine from the 0.8 ml/kg group than in the 0.4 ml/kg group.

Urine samples from the time course study were also analysed using SDS-PAGE and again lanes were loaded with equal volumes of urine. The albumin band in these gels is a good indicator of loading since lanes in which the albumin band is more intense (e.g. Figure 3.15A, lane 4) the 18.4 kDa band appears more intense.

SDS-PAGE of the urine samples from the time course study revealed the presence of the 18.4 kDa band in CCl_4-treated urine in samples from the 12 hour time point up to and including the 60 hour time point (Figure 3.15). The 18.4 kDa band is not present in all CCl_4-treated urine samples at each of the time points, for example, at 12 hours it is present in three out of five samples and at 24 hours it is present in four out of five samples. At 36 and 48 hours the 18.4 kDa band is present in all urine samples in both groups. At 60 hours two of the urine samples show the band and then at all later time points the 18.4 kDa band does not appear to be present (not all data shown). These results correspond to the 15.7 kDa peak detected by SELDI in the same urine samples. Both the 18.4 kDa band and the 15.7 kDa peak appear in the urine at the same time points following CCl_4-induced liver injury, and by 72 hours both are not present. At

both the 36 and 48 hours time points the 18.4 kDa and 15.7 kDa proteins are present in all the urine samples from rats treated with CCl_4. The detection of the 15.7 kDa and 18.4 kDa proteins in the rat urine correlates with the results described in Section 3.4.3, which show that serum enzymes such as ALT, AST and GLDH are increased in rats treated with CCl_4. The enzyme activities increased with time up to a maximum at 36-48 hours post-dosing, and after this time point the enzyme activities began to return to control levels. After the 48 hour time point the 18.4 kDa band was not found in all of the urine samples from rats treated with CCl_4. Figure 3.16 shows the number of urine samples at each time point in which both the 18.4 kDa band and 15.7 kDa peak were detected.

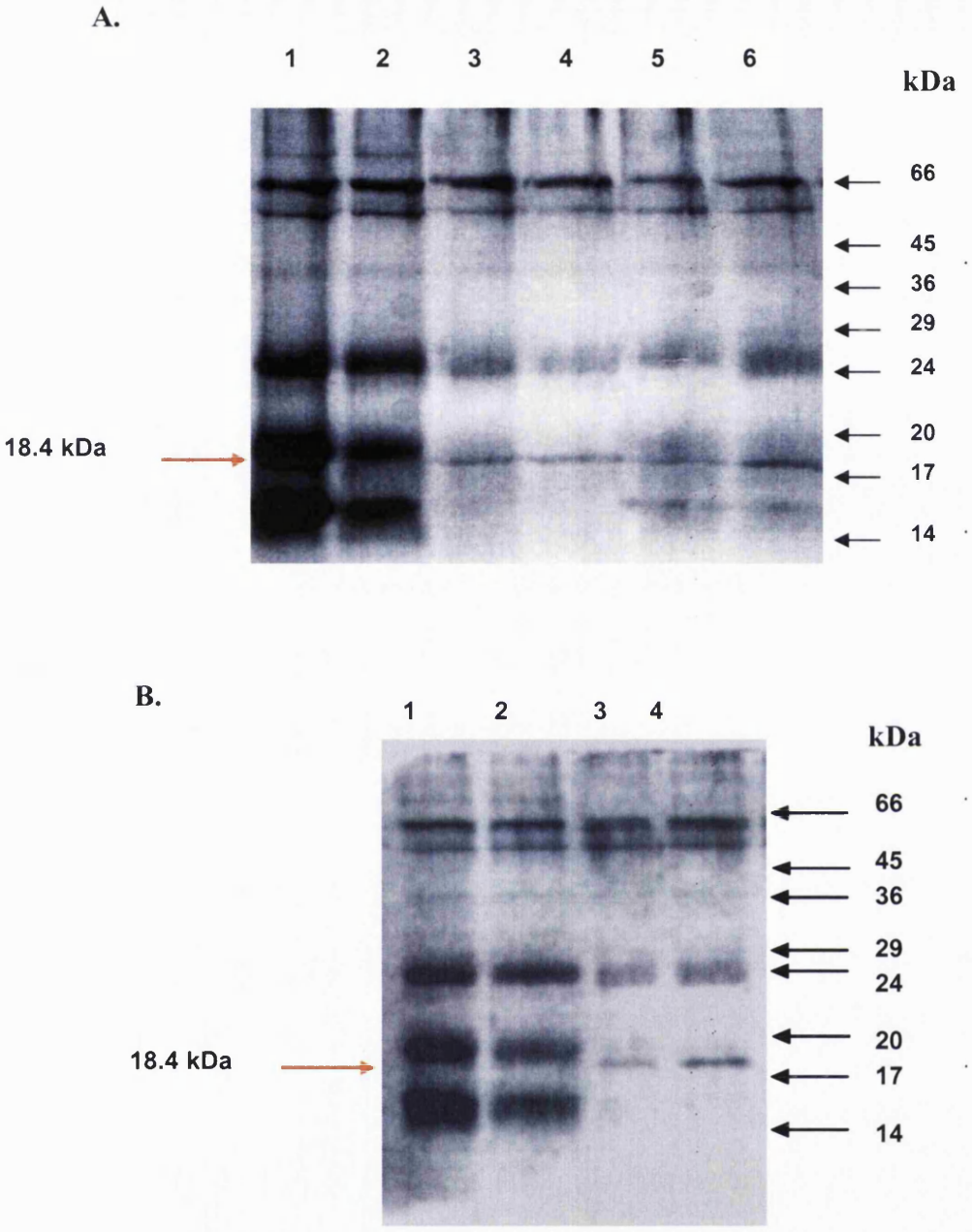
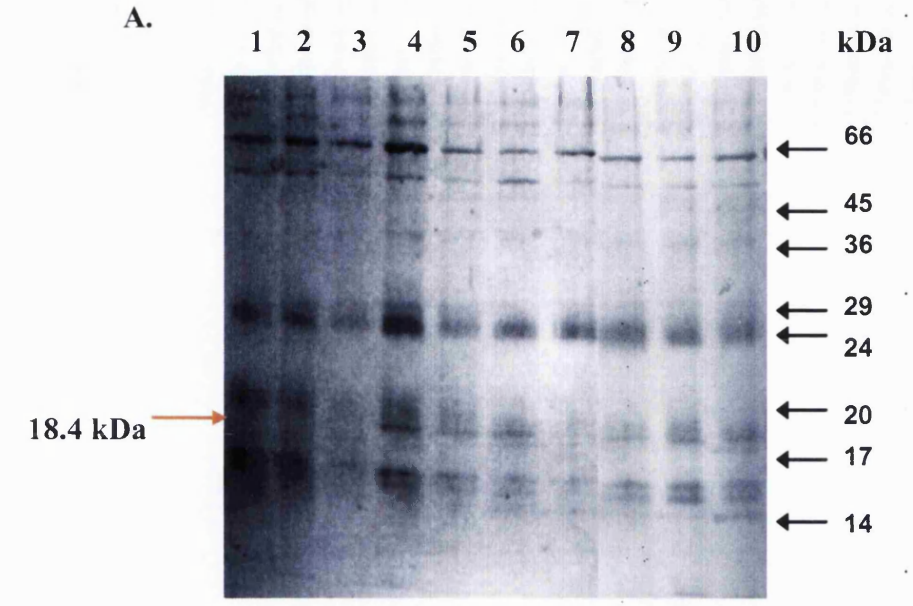
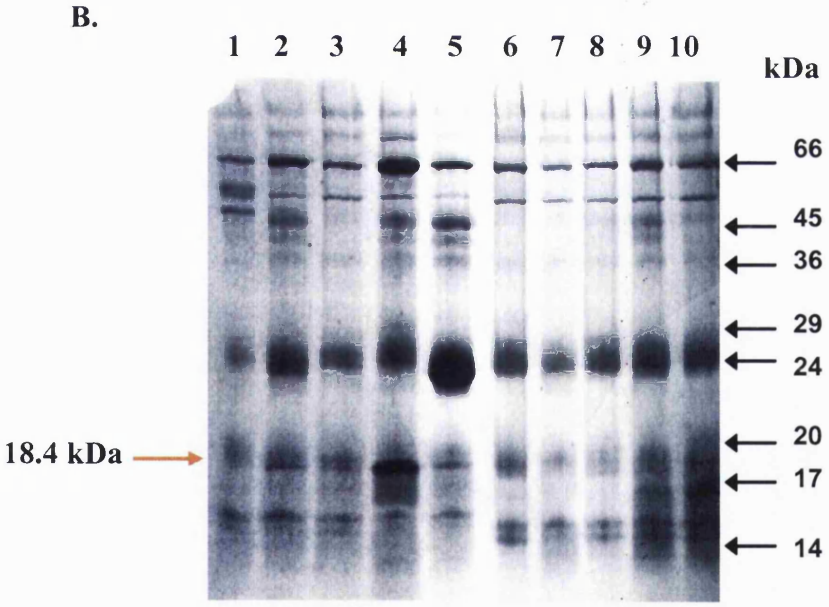


Figure 3.14 SDS PAGE of urine samples from rats treated with CCl_4 and urine collected for 24 hours following dosing. A. Lanes 1 and 2 = control, lanes 3 and 4 = urine from rats treated with CCl_4 at 0.4 ml/kg, lanes 5 and 6 = urine from rats treated with CCl_4 at 0.8 ml/kg. B. Lanes 1 and 2 = control, lanes 3 and 4 = urine from rats treated with CCl_4 at 0.4 ml/kg. Gels were run as described in Section 2.11 and stained with Coomassie blue.


A.
1 2 3 4 5 6 7 8 9 10
kDa
66
45
36
29
24
20
17
14
18.4 kDa


B.
1 2 3 4 5 6 7 8 9 10
kDa
66
45
36
29
24
20
17
14
18.4 kDa

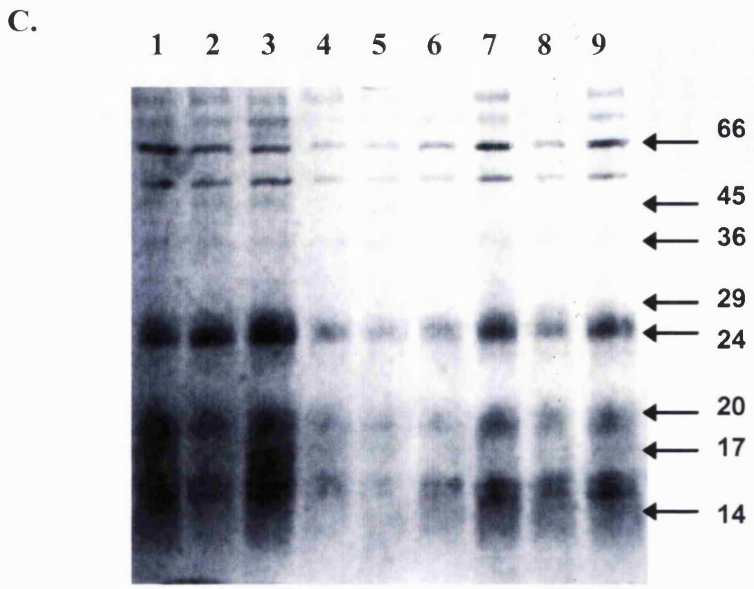


Figure 3.15 SDS PAGE of urine samples collected 12, 24, 36, 48, 60, 72 hours and on day 4, 7 and 10 post-dosing. Rats were dosed with 0.8 ml/kg CCl_4 and urine collected at the following time points: 0, 12, 24, 36, 48, 60, 72 hours and at days 4, 7, 10. In gel A, lane 1 = control; lanes 2, 3, 4, 5 and 6 = urine at 12 hour time point; lane 7 = urine at 24 hours; lanes 8, 9 and 10 = urine at 36 hour time point. In gel B, lane 1 = control, lanes 2, 3, 4 and 5 = urine at 48 hours; lanes 6, 7, 8, 9 and 10 = urine at 60 hours. In gel C, lane 1 = control; lanes 2 and 3 = urine at 72 hours; lanes 4 and 5 = urine at day 4; lanes 6 and 7 = urine at day 7; lanes 8 and 9 = urine at day 10. Gels were run as described in Section 2.11 and stained with Coomassie blue.

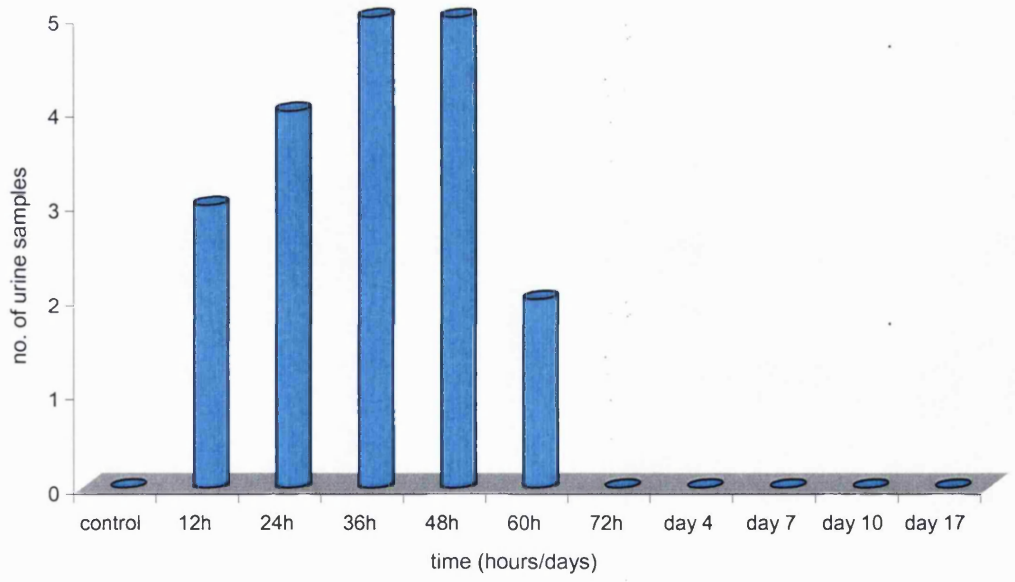


Figure 3.16 The number of urine samples out of a total of five at each time point in which the 18.4 kDa band and the 15.7 kDa peak were observed following analysis of the urine using SDS gel electrophoresis and SELDI respectively.

3.7 Nano-electrospray tandem mass spectrometry (Nano-ES-MS/MS)

The 18.4 kDa protein band identified by one-dimensional SDS-PAGE in urine from CCl_4-treated rat urine samples was digested in-gel with trypsin as described in Section 2.16. The digest was analysed by nano-ES-MS/MS, during which sample is electrosprayed into the mass spectrometer and MS/MS analysis is performed on the most intense ions (Section 2.16.5). In order to identify peptides, MS/MS data was searched using Sequest software (ThermoElectron) against a rat protein database (Finnigan Xcalibur, Revision 1.0 P/N XCALI-64012, July 2000) as described in Section 2.16.6. The aim of the Sequest approach is to find the amino acid sequence in the database that, when fragmented, would give a spectrum that correlates most closely with the experimental MS/MS spectrum.

Four peptides were identified, each unique to the 18.4 kDa protein copper/zinc superoxide dismutase (Cu/Zn SOD); **GDGPVQGVIHFEQK (*m/z* 756.1), HVGDLGNVAAGK (*m/z* 569.5), DGVANVSIEDR (*m/z* 588.1), VISLSGEHSIIGR (*m/z* 684.5).**

Each of the four peptides identified had a cross-correlation score of greater than 2.5 and a ΔC_n score of greater than 0.1. The total protein coverage was 32.5 % by amino acid count. Figure 3.17 shows a full scan mass spectrum, with the identified peptides indicated. The MS/MS spectrum of the $[M+2H]^{2+}$ ion of the peptide DGVANVSIEDR is shown in Figure 3.18.

MAMKAVCVLKGDGPVQGVIHFEQKASGEPVVVSGQITGLTEGEHGFHVHQYG
DNTQGCTTAGPHFNPHSKKHGGPADEERHVGDLGNVAAGKDGVANVSIEDRVI
SLSGEHSIIGRTVVHEKQDDLGKGGNEESTKTGNAGSRLACGVIGIAQ

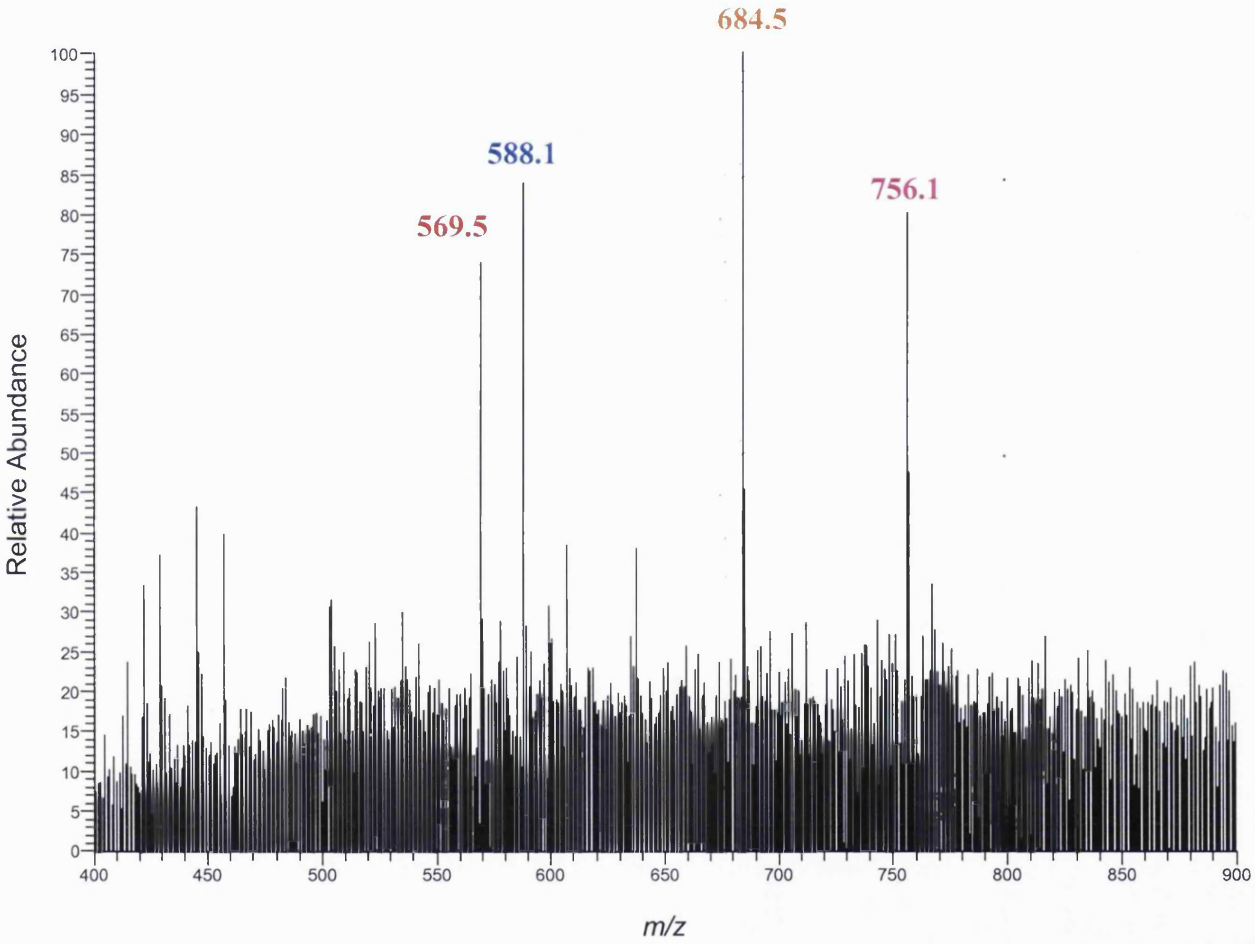


Figure 3.17 Full scan mass spectrum of an in-gel tryptic digest of SOD showing the *m/z* region between 400 and 900. The full amino acid sequence of the protein is displayed above the spectra with the four peptides that were identified indicated in colour.

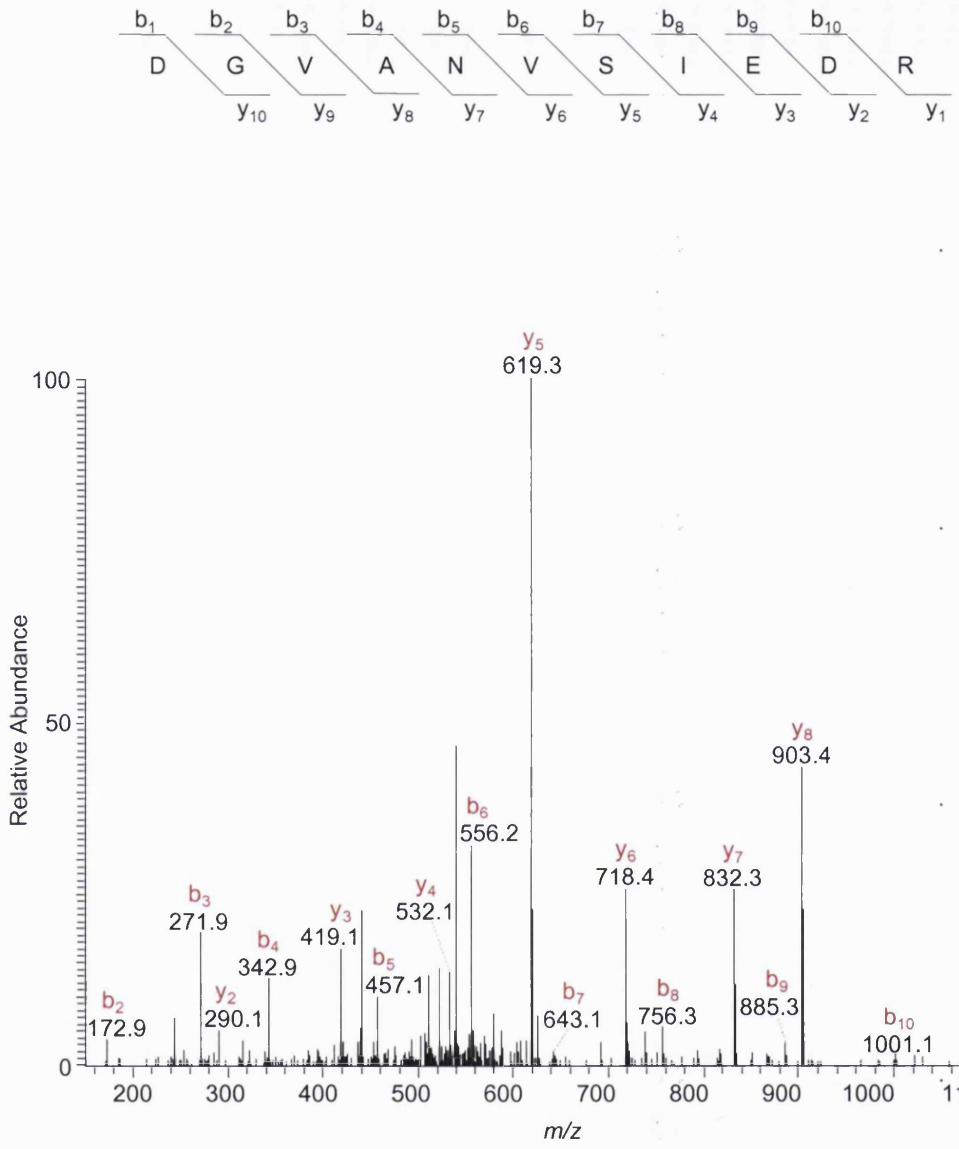


Figure 3.18 MS/MS spectrum of the tryptic peptide DGVANVSIEDR [M + 2H]$^{2+}$ ion of *m/z* 588.1, identified to originate from Cu/Zn SOD. The amino acid sequence of the peptide is shown above the spectrum. y- and b-type ions are formed by peptide bond cleavage with charge retention on the C-terminus and N-terminus, respectively. The y- and b- type ions are indicated on the spectrum.

3.6 Discussion

The objective of the dose response study and the time course study was to define a dose level of CCl_4 that induced significant hepatic injury but not renal changes and to determine the time after dosing with CCl_4 that liver injury was at a maximum level. Once these factors had been determined we wished to look for protein markers of hepatic toxicity in the urine following CCl_4-induced injury. Carbon tetrachloride was chosen as the agent to induce liver injury since it is a widely used model hepatotoxicant (de Zwart et al., 1998; Hsu, 1998). CCl_4 induces centrilobular necrosis in the liver on acute dosing but after repeated administration fibrosis occurs (Shi et al., 1998).

The dose response study enabled a dose to be determined at which hepatotoxicity could be induced but also the dose at which nephrotoxicity occurred. Hepatotoxicity was induced at the lowest dose used (0.4 ml/kg). The dose range used (0.4 to 2.8 ml/kg) was chosen since preliminary studies had been carried out to determine a maximum tolerated dose, and these had revealed that dose levels greater than 2.8 ml/kg of CCl_4 were lethal to rats of this age/weight and sex.

In the dose response study where levels of 0.4 to 2.8 ml/kg CCl_4 were used liver weights from rats treated with CCl_4 were greater than the controls at all dose levels administered (Figure 3.1). Even at the lowest dose (0.4 ml/kg) administered the liver weights were significantly greater (1.41 fold) than control ($P < 0.001$). Such increased liver weights indicate that hepatic injury has been induced. Low and colleagues (1995) carried out studies in the rat to determine if a lobar variation exists with the extent of damage induced by CCl_4. They administered CCl_4 by intraperitoneal injection and orally (1 or 5 ml/kg, and 2 ml/kg, respectively) and sampled at 24 hour post-dosing. They found no inter-lobar variation but reported significant liver weight increases in the treated rats.

Serum AST, ALT and GLDH activity measured in rats treated with CCl_4 were greater than control at all dose levels administered (Figure 3.2). At the lowest dose (0.4 ml/kg), the ALT and AST activity were significantly increased ($P < 0.01$ and $P < 0.001$, respectively). ALT activity in the CCl_4-treated rat serum was increased 50-fold over control, ALT was 30-fold greater, and GLDH activity was 200-fold greater than the activity in control serum. With increasing dose of CCl_4 the AST, ALT and GLDH

levels increased. The activity of ALT is greatest in the liver and the enzyme is therefore considered to be a good marker of necrosis and inflammation in the liver (Whitby et al., 1988). GLDH is highly liver specific and its greatest activity is in the hepatocytes of the centrilobular area, and therefore these serum enzyme results indicate that the liver injury was in the centrilobular region. Studies carried out by Campo et al. (2004) revealed that a single dose of CCl_4 at 1.0 ml/kg injected intraperitoneally produced approximately 10-fold increases in both AST and ALT activity in the CCl_4-treated rats at 24 hours post-dosing. In our studies however, dosing rats with CCl_4 at 1.2 ml/kg produced increases in serum ALT and AST activity of 74- and 46-fold, respectively over control activity. Thus the higher enzyme activities obtained during the present study may reflect the route of administration. In all the studies reported in this thesis CCl_4 was administered via gavage (i.e. as an oral gastric dose) whereas Campo and colleagues (2004) injected CCl_4 intraperitoneally.

Das et al. (2000) also administered CCl_4 intragastrically but these workers sampled at 3 hours post-dosing which was considerably earlier than our sampling time of 24 hours, they administered 3 ml of CCl_4 in liquid paraffin (1:1). Das et al. (2000) measured serum ALP, AST and ALT at this time point and reported increased activities of AST and ALT of approximately 2- and 3- fold, respectively. However, it is unlikely that these measurements at a 3 hour time point reflect maximal injury. Sheweita et al. (2001) investigated the role of antioxidants in CCl_4-induced liver injury. These studies revealed that a single dose of 400 mg/kg CCl_4 produced 4- and 6- fold increases in both serum ALT and AST activities, respectively, at 24 hours after dosing. Suja et al. (2004) administered 2 ml/kg CCl_4 by subcutaneous injection and 48 hours after dosing reported that serum AST and ALT activities were increased 3- and 6- fold, respectively. Similarly the administration of a single dose of 1.5 ml/kg CCl_4 induced significant changes in serum ALT, AST and ALP activities at 24 hours after dosing (Pawa and Ali, 2004). These workers found the activities of all three enzymes increased by 8-, 1.6- and 1.8- fold, respectively. Differences exist between these above reported studies due to the variation in the route of administration, the age of animal used and the strain of rat used. Studies carried out to investigate the importance of Kupffer cells in CCl_4-induced liver injury resulted in approximately 2-fold increases in both ALT and AST 24 hours after a single oral dose of 0.4 g/100 g (Muriel et al., 2001). Experimental variation, such as the strain or age of the rats used, or the nutritional status of the rat, may account for the differences obtained in these studies. Our present studies achieved similar

results at different dose levels to those of Edwards et al. (1993) and Muriel et al. (2001) and again this is likely to be due to experimental variations.

In the present studies histopathology of the liver tissue revealed that at 0.4 ml/kg CCl_4 there was centrilobular necrosis and this was more marked in the higher dose groups (1.2 to 2.8 ml/kg). This finding, in keeping with the serum enzyme and liver weight data, suggests that even at the lowest dose level used in this study (0.4 ml/kg) there was evidence of liver damage. Edwards et al. (1993) administered 4 g/kg CCl_4 intragastrically to female rats and sampled at 24 hours they found dramatic increases in serum AST values (16-fold increase) and also recorded centrilobular necrosis and infiltrating neutrophil polymorphs. In the present study, liver histology in the dose response study revealed centrilobular necrosis and infiltrating neutrophils. CCl_4 induces damage mainly in the centrilobular region of the liver lobule since this region has the highest concentration of the metabolising enzymes and the metabolites are free radicals with short half-lives. Free radicals are very reactive but are unable to diffuse far from the site of production and therefore the injury is localised to the area in which they are formed (Slater, 1990).

Sanzgiri and colleagues (1997) studied the uptake, distribution and elimination of CCl_4 in rat tissues after both inhalation and ingestion exposures. Since chemicals absorbed from the gastrointestinal tract enter the portal circulation and are subjected to first pass metabolism by the liver and lungs, they predicted that the inhalation of CCl_4 would result in greater deposition of the chemical to extrahepatic tissues. This was reflected in their findings. However the liver accumulated higher concentrations of CCl_4 following ingestion and in particular after a single bolus dose rather than by gastric infusion.

In the dose response study, the kidney weights were also increased in all CCl_4-treated rat groups in comparison with the control. This increase was very slight at 0.4 and 0.8 ml/kg, and therefore was not statistically different from control (Figure 3.1). At 1.2 ml/kg CCl_4 the kidney weights were statistically significantly increased over control ($P < 0.05$) and above 1.2 ml/kg there appeared to be a dose dependent increase in the relative kidney weight. At dose levels of 1.2 ml/kg and above, the urine samples contained blood. Also, analysis of serum samples revealed that both urea and creatinine were increased in all CCl_4-treated rats (Table 3.2) and were significantly greater than control at all dose levels apart from in the 1.6 ml/kg group, which had a

high intra-group variation. These findings indicate kidney damage. Above 0.8 ml/kg, histopathology of the kidneys showed proximal tubular swelling and degeneration with associated casts; these changes were not present in the controls and the lower dose CCl_4-treated groups. Therefore, at doses above 0.8 ml/kg nephrotoxicity had been induced. Since we had induced nephrotoxicity with a single dose of CCl_4 at 1.2 ml/kg it was decided that in future experiments the animals should be dosed at levels below this to ensure that any proteins detected in the urine were only the result of liver injury and not injury to the kidney. Nephrotoxicity has been reported in rats following administration of CCl_4 and it has been suggested that this injury is also associated with free radicals (Abraham et al., 1999). Ozturk and colleagues (2003) observed renal cortical damage and focal glomerular necrosis after dosing with 1 ml/kg CCl_4 subcutaneously daily for four days. Some cortical tubules were dilated and some epithelial cells showed vacuolisation. Intraluminal casts were also present in some tubules.

In the time course study, rats were dosed with CCl_4 and samples taken every 12 hours for 3 days and then on days 4, 7, 10 and 17 post-dosing. The dose of CCl_4 used in this experiment was 0.8 ml/kg since this was the highest dose used in the dose response study at which nephrotoxicity was not induced. Liver weights from CCl_4-treated rats increased with time to a maximum at 48 hours and then the weights returned towards control levels (Figure 3.5). Serum biochemistry results showed a significant increase in ALT, AST and GLDH levels from the first time point at 12 hour post-dosing and peaked at approximately 36 to 48 hours after dosing. By 72 hours after dosing the ALT levels were similar to controls, whereas the activities of both AST and GLDH fell to control levels by day 4 after dosing.

During the time course study, ALP activity and HAP-E levels were also measured (Figure 3.7). ALP activity tends to be increased in response to cholestatic lesions although the enzyme lacks specificity, whereas HAP-E is an acute phase protein and therefore increases in response to inflammation. In the present studies ALP activity was increased at the 12 hour time point but this was not significant. The ALP reached a maximum at 24 hours post-dosing ($P < 0.001$) and then began to fall towards control levels. Four days after dosing, ALP activity was similar to the controls. It is considered likely that the acute phase protein haptoglobin was increased as a result of the inflammatory response following necrotic injury after the administration of CCl_4. At 12

hours HAP-E was slightly increased but the level further rose to reach a maximum at 48 hours after dosing. By 60 hours this level had fallen slightly and was similar to control at day 10.

This pattern of serum enzyme changes compares with a study by Rao et al. (1997) where after a single dose of CCl_4, maximum ALT elevation occurred at 36 hours post dosing and declined to normal by 72 hours. De Zwart et al. (1998) administered different doses of CCl_4 (0.25, 0.5 and 1.0 ml/kg) to rats and found a dose-dependent increase in ALT, AST and GLDH levels, which was maximal at the final time point at 48 hours. A study by LeSage et al. (1999) showed increased serum ALP, total bilrubin, AST and ALT at 24 hours post-dosing which then returned to normal 7 days after the rats were given a single dose of 0.5 ml/kg CCl_4.

A single intragastric dose of CCl_4 of 2.5 ml/kg administered to rats produced significant differences in serum AST and ALT activities in treated animals, which reached a peak 48 hours after dosing (Arosio et al., 1997). Rao and Mehendale (1989) dosed rats with 2.5 ml/kg CCl_4 via intraperitoneal injection and obtained an approximately 2-fold increase in the activities of both serum ALT and AST 2 hours after dosing. By 24 hours after dosing the serum ALT activity had increased to 43-fold greater than control whereas, the serum AST activity was 78-fold greater than the control activity. Studies carried out by Yokogawa et al. (2004) showed significant increases in serum AST, ALT and ALP activities 24 hours after a single dose of 0.5 ml/kg but by 7 days these enzyme activities had returned to control values.

Noguchi et al. (2001) dosed rats with a single intraperitoneal injection of 0.1 ml/kg CCl_4 and measured serum ALT and AST as indicators of hepatotoxicity at various time points. They reported maximal increases in activity of both enzymes two days after a single dose and a return to control levels by 6 days post-dosing. A study by Salazar-Montes et al. (1999) revealed that serum AST activity was increased by 4.0-, 5.7- and 2.2- fold in rats treated with a single dose of CCl_4 (0.5 ml/kg) at days 1, 2 and 3, respectively. In the same study ALT activity was increased over control by 6-, 9- and 2.5- fold at days 1, 2 and 3 post-dosing. At time points greater than 3 days post-dosing the activities of both enzymes in CCl_4-treated rats was similar to those in control rats.

Sundari and colleagues (1997) administered phenobarbital to rats in the drinking water for 10 days and then on day 11 they exposed the rats to CCl_4 vapour. Serum AST and ALT activities started to increase in the CCl_4 exposed rats by 8 hours post-dosing, reaching a maximal at 14 hours post-exposure before starting to fall back to control activity levels.

In the present study liver histology results for CCl_4-treated rats in the time course study showed, at 12 hours to day 4 post-dosing, centrilobular hepatocellular swelling, nuclear degeneration and eventually, necrosis. However the liver samples from day 7 show mostly a normal histology. The results from this study therefore indicate that morphological changes occur relatively rapidly after dosing with CCl_4, but by day 7 the damage induced has been repaired and the liver appears structurally normal. These results agree with the other findings during this study, such as the liver weights and serum enzyme data. Liver weights were increased from 12 hours post-dosing, as were serum ALT, AST and GLDH activities. After day 4 post-dosing the serum enzymes levels were returning to control values. Le Sage et al. (1999) found that after a single dose of CCl_4 centrilobular necrosis and steatosis of hepatocytes was evident, but there was a return to normal histology by day 14. Rao et al. (1997) found maximum injury to hepatocytes 36 hours after a single dose of CCl_4, but there were swollen and lipid laden cells in the centrilobular region as early as 12 hours after a dose of 0.1 ml/kg CCl_4. In the present time course study the livers from CCl_4-treated rats showed evidence of centrilobular changes, and these were more severe at the later time points (i.e. 36 and 48 hours post-dosing).

In a study by Yata et al. (1999) histopathological changes were observed in the liver at various time points following the administration of a single dose of CCl_4 via a gastric tube. They reported findings such as the ballooning of hepatocytes and slight necrosis in the centrilobular region at 6 hours after dosing. Zonal necrosis was observed at 2 days after dosing, but normal histology was almost restored by day 7.

Moghaddam et al. (1998) examined sex differences in tissue repair following acute CCl_4 exposure (0.8 ml/kg). They found that in female rats, hepatic damage peaked at 24 hours whereas in males, maximum hepatic damage occurred at 3 hours after dosing and remained at this level until 36 hours post-dosing. The rats used in the present experiments were females. Moghaddam et al. (1998) suggested that hepatic injury to

females was approximately 2.5- fold greater than that occurring in males. Ohata et al. (1997) dosed rats with a single dose of CCl_4 at 1.0 ml/kg and found significant increases in AST and ALT activity in treated rat serum 2 hours after dosing. This elevated activity reached a maximum at the 24 hour time point after which the levels of both enzymes began to fall towards that of controls.

In the present time course study, the results for kidney weights, serum analysis and histopathology all suggest that a single dose of 0.8 ml/kg CCl_4 did not induce nephrotoxicity at any time post-dosing.

Therefore, the time course study revealed that liver injury occurs as early as 12 hours post-dosing with the maximum damage occurring at approximately 36-48 hours as determined by the serum enzyme data. By day 7 post-dosing the liver damage had been repaired and the liver appeared structurally normal, as determined by histopathological analysis.

The main objective of the present series of investigations was to identify urinary protein markers of hepatotoxicity. Therefore, urine samples collected from rats treated with CCl_4 were analysed by SDS gel electrophoresis and by Surface Enhanced Laser Desorption/Ionisation ProteinChip technology (SELDI) to look for differences in the protein content of urine between control and CCl_4-treated rats. If successful, SELDI has the ability to be employed as a high-throughput proteomic screen for evaluating hepatotoxicity in response to a variety of agents.

Urine samples from both the present dose response and time course studies were analysed using SELDI. The SELDI spectrum of urine from rats treated with CCl_4 revealed a protein peak at 15.7 kDa, which was not present in any of the control urine samples. In the dose response study all CCl_4-treated urine samples contained this protein. The urine samples from rats treated with CCl_4 at 1.2 ml/kg and above show additional protein peaks as well as that at 15.7 kDa but these are considered to be due to nephrotoxicity, as shown by the raised serum creatinine and urea results, and also histopathology of these groups.

The results of the time course study show that this 15.7 kDa protein began to appear in the urine at about 12 hours post-dosing. However the 15.7 kDa peak was only present in

three out of a total of five urine samples at this time point, suggesting that there may be considerable variation between animals at 12 hours post-dosing. Even at 24 hours, one of the five urine samples did not appear to have the 15.7 kDa protein peak. At 36 and 48 hours post-dosing, all the urine samples showed this peak. These are the time points at which maximum liver injury was observed as judged by serum enzyme analysis and histopathology. This suggests a strong connection between the 15.7 kDa peak and hepatotoxicity. By 72 hours post-dosing the 15.7 kDa peak could not be detected in the urine from rats treated with CCl_4, and again this was in agreement with the serum enzyme data and histopathology which showed recovery of the liver by this time point.

SDS PAGE of urine samples from the present dose response study revealed also the presence of an 18.4 kDa band that was present in CCl_4-treated urine samples and not in control (Figure 3.14). This protein band was consistently present in all the urine samples from rats treated with CCl_4 as part of the dose response study. In the time course study this 18.4 kDa protein was again present but not in all CCl_4-treated urine samples at all time points. The pattern of appearance of the 15.7 kDa SELDI protein and the 18.4 kDa SDS-PAGE protein was virtually identical in the time course study suggesting they were one and the same protein (Figure 3.16). This 15.7kDa/18.4 kDa protein was not detected in any of the urine samples from control rats, suggesting that its appearance was associated with the administration of CCl_4. The appearance of the protein in the urine from the time course study corresponded to the time points at which hepatotoxicity had been induced, as determined by serum enzyme analysis and histopathology. Nano-ES-MS/MS identified the 18.4 kDa band on SDS-PAGE as Cu/Zn SOD with 32.5 % coverage of its amino acid sequence.

Cu/Zn SOD is a cupro-zinc protein which was originally isolated from bovine erythrocytes (Mann and Keilin, 1938). McCord and Fridovich (1969) discovered that this protein had catalytic activity towards the superoxide anion and named it superoxide dismutase. Cu/Zn SOD exists as a dimer (composed of two identical subunits) of molecular weight of approximately 31-32 KDa (McCord and Fridovich, 1969; Weisiger and Fridovich, 1973; Steinman et al., 1974). Both subunits have an intra-chain disulphide bridge, one sulfhydryl group and an acetylated amino terminus (Fridovich, 1982). Each monomer has 151 amino acids and folds as an eight strand greek-key beta-barrel connected by three external loops (Kenneth et al., 1973).

In rats Cu/Zn SOD has a predicted molecular weight of 31.55 kDa, therefore each subunit would be expected to have a molecular weight of approximately 15.8 kDa. This 15.8 kDa subunit would therefore correspond to the 15.7 kDa protein detected by SELDI. Since the pattern of appearance of the 15.7 kDa SELDI protein and the 18.4 kDa SDS-PAGE protein in the rat urine was identical it is assumed that they are in fact the same protein. The 18.4 kDa SDS-PAGE protein has been identified as Cu/Zn SOD, and therefore we can assume that both proteins are the rat Cu/Zn SOD subunit with a molecular weight of approximately 15.7 kDa. It is therefore assumed then that Cu/Zn SOD runs anomalously on SDS-PAGE at 18.4 kDa, which is most likely due to post-translational modifications.

The superoxide dismutase (SOD) enzymes are a group of metalloenzymes that are responsible for catalysing the dismutation of superoxide anions ($O_2^{\bullet -}$) into oxygen and hydrogen peroxide (McCord and Fridovich, 1969):

$$2O_2^{\bullet -} + 2H^+ \Leftrightarrow H_2O_2 + O_2$$

This reaction proceeds spontaneously at pH 7.7 and with a rate constant of approximately $2 \times 10^5\ M^{-1}\ sec^{-1}$ (McCord et al., 1971). SOD provides a defence system against the superoxide free radical and therefore protects the cell from oxidative damage. All aerobic organisms are known to contain superoxide dismutase (McCord et al., 1971). The absence of superoxide dismutase in anaerobes proves the importance of the enzyme for the survival of organisms in the presence of molecular oxygen. SOD is widely distributed in both plants and animals, and occurs in many tissues including brain, liver, heart, erythrocytes, pancreas, intestine, muscle and kidney.

Four forms of SOD have been identified in nature and are classified according to the metal involved: manganese (Mn), iron (Fe), nickel (Ni) and copper/zinc (Cu/Zn) (also known as SOD-1). The dismutation reaction occurs via successive oxidation and reduction of the metal ion at the active site, with the superoxide free radical also undergoing both reduction and oxidation in this reaction. MnSOD is found in many bacteria and in the mitochondria of all eukaryotic cells, FeSOD in prokaryotes and plants and Cu/Zn SOD in eukaryotes.

The active site of the Cu/Zn enzyme consists of one copper and one zinc ion 6.3 Å apart, bridged by the imidazole ring of histidine 61 and is located at the base of a cavity formed by the two largest loops. The Cu^{2+} is required for activity (McCord and Fridovich, 1969; Forman and Fridovich, 1973), it is co-ordinated by four His residues (His-44, His-46, His-61 and His 118) and a water molecule, forming a tetrahedrally distorted square plane. The Zn^{2+} is bound to the protein by two additional His residues and an aspartic acid in a tetrahedral shape. The function of the Zn^{2+} is uncertain but its main role is thought to be concerned with structural stability of the enzyme. However, both the Cu^{2+} and Zn^{2+} are required to maintain the stability of the enzyme (Forman and Fridovich, 1973). The Zn^{2+} is buried within the active site whereas the Cu^{2+} is solvent accessible. No other metal can replace the copper in the active site and maintain activity whereas the replacement of Zn^{2+} with other metal ions such as cobalt do not have any effect on the stability of the enzyme.

Loop IV is an important part of the active site since residue cys 55 forms a disulphide bond with cys 144 on strand eight, and this formation enables hydrogen bonding between the carboxyl O_2 of cys 55 and an amide group on arg 141 which is essential for catalysis. It is thought that His-61 may function as a proton carrier to facilitate the protonation of the $O_2^{\bullet -}$ for the dismutation reaction.

The active site forms 10 % of the surface of the enzyme and is highly conserved with charged residues producing an electric field, thought to be responsible for directing the superoxide anion towards the active site. The presence of four histidine residues at the active site confers a positive charge to this part of the protein whereas the rest has a net negative charge (Cocco et al., 1983). Also the positively charged Arg-141 located on one side of the active site cavity is thought to play a key role in catalysis by providing an electric gradient for attracting the negatively charged superoxide radical. Chemical modification of these positively charged residues decreases enzymatic activity.

Superoxide dismutase is an unusually stable enzyme, with the ability to withstand rigorous purification processes (McCord and Fridovich, 1969; Forman and Fridovich, 1972). The enzyme retained 75 % of its activity in 8 M urea and 65 % of its activity in 10 M urea. Full activity was maintained after one hour in 4 % SDS at 25 °C (Forman and Fridovich, 1973). Cu/Zn SOD has a high percentage of glycine residues per subunit, allowing many bends in the polypeptide backbone and therefore many points of

contact between nonadjacent regions in the primary structure. These interactions are thought to increase the stability of the enzyme (Steinman et al., 1974). The side-chains and main chains of the enzyme are stabilized in their orientation by a complex network of hydrogen bonds, which may further enhance the stability of the enzyme (Tainer et al., 1982).

Many cellular proteins undergo proteolysis following cell necrosis, however due to its high stability SOD appears to be resistant. The detection of SOD-1 in the rat urine means that the protein has remained stable in the plasma and has crossed the highly effective filtration barriers in the kidney. Other less stable proteins would not survive and therefore would not appear in the rat urine. Therefore the structure and high stability of the enzyme contributes to the finding of SOD-1 in the rat urine following CCl_4-induced hepatotoxicity.

Superoxide dismutase is an important enzyme involved in counteracting the potential effects of the superoxide free radical. As a result it would be expected that SOD would be present in all cells which use oxygen, thereby having the potential to produce $O_2^{-\bullet}$ (Fridovich, 1982). Superoxide is relatively stable with a half-life of 5 seconds (Okado-Matsumoto and Fridovich, 2001; Kira et al., 2002) therefore SOD must be widely distributed in order to scavenge the superoxide close to its site of production in order to avoid oxidative damage. It has been shown that Cu/Zn SOD is found in the lysosomes, cytosol, nucleus and the intermembrane space of the mitochondria while the other major form of superoxide dismutase, Mn-SOD, is located within the mitochondrial matrix (Geller and Winge, 1982; Okado-Matsumoto and Fridovich, 2001). Therefore SOD is located both inside and outside of the mitochondria (Kira et al., 2002).

Studies have been carried out to localize the SOD activity in rat tissue and it has been shown that Cu/Zn SOD is present in liver parenchymal cells, epithelial cells of the proximal and distal tubule and collecting ducts of the kidney, striated muscle cells, epithelial cells of large bowel, pancreatic ducts, myocardial cells (Frederiks and Bosch, 1997). In rat liver Cu/Zn SOD is mainly a cytoplasmic protein and selectively accumulates in lysosomes (Liou et al., 1993).

Therefore since SOD is widely distributed, it may be possible that the appearance of SOD-1 in the rat urine is due to injury in another rat organ. In the studies carried out we

have shown that SOD-1 was detected at dose levels of CCl_4 below the threshold for kidney injury (1.2 ml/kg). However, CCl_4 can also induce injury in other organs besides the liver and kidney, such as the lungs. Therefore it is necessary to carry out studies investigating the possibility of the induction of other organ injury by CCl_4. It is important to determine if the SOD-1 in the rat urine is due to liver necrosis and therefore establish if SOD-1 can be a good urinary marker of liver toxicity.

The superoxide free radical ($O_2^{\bullet-}$) is generated by a number of biological systems by both enzymatic and non-enzymatic oxidations (Fridovich, 1982). The $O_2^{\bullet-}$ radical is able to react with hydrogen peroxide to generate the hydroxyl radical (OH^-). This reaction is named the Haber-Weiss reaction and occurs readily in buffered aqueous systems. Any aqueous system that produces $O_2^{\bullet-}$ will also generate H_2O_2 and if H_2O_2 accumulates the Haber-Weiss reaction will occur generating OH^-, which is much more potent than $O_2^{\bullet-}$ (Fridovich, 1986). It is possible therefore that $O_2^{\bullet-}$ is toxic to living cells via this non-direct method of generating a more powerful oxidant, since any aqueous system producing $O_2^{\bullet-}$ will also generate H_2O_2.

However it well known that $O_2^{\bullet-}$ can exert direct damaging effects in biological systems without the need for OH^-. It may cause the depolymerisation of acid polysaccharides and may be able to initiate the peroxidation of unsaturated lipids. Pederson and colleagues (1973) showed that in reaction mixtures containing Fe^{3+} and EDTA, lipid peroxidation was initiated by $O_2^{\bullet-}$. The superoxide free radical reacts quickly with some other radicals such as $NO^{\bullet}$ but is less reactive with non-radical species than OH^- (Halliwell and Gutteridge, 1998).

$O_2^{\bullet-}$ is removed from cells by a dismutation reaction, this is where the same species is both oxidised and reduced during the same reaction. One molecule of $O_2^{\bullet-}$ is oxidised to O_2 and the other is reduced to H_2O_2, this reaction occurs more rapidly at acidic pH (Halliwell and Gutteridge, 1998). Therefore by removing the $O_2^{\bullet-}$ from biological systems the superoxide dismutase enzymes act as a first line of defence against oxygen toxicity. SOD enzymes can react directly with certain reactive oxygen species; they react with OH• and peroxyl/alkoxyl radical since they contain histidine and other side chains which are able to react with these species (Halliwell and Gutteridge, 1998).

As described previously, carbon tetrachloride is metabolised by the cytochrome P450 oxidase system to produce the trichloromethyl free radical. This radical is able to abstract a hydrogen atom from a methylene group belonging to a membrane lipid and therefore initiate lipid peroxidation. The process of lipid peroxidation generates a number of reactive oxygen species and free radicals therefore one might expect some kind of defence system to increase in activity following CCl_4-induced toxicity. The superoxide radical is not reactive enough to abstract a hydrogen atom from a lipid and is unable to enter the hydrophobic interior of the membranes due to its charge (Halliwell and Gutteridge, 1998). However the protonated form of $O_2^{\bullet-}$ and the OH• radical are much more reactive and can initiate lipid peroxidation readily. The removal of the hydrogen leaves behind a carbon radical which can either react with another carbon radical or react with oxygen forming a peroxy radical, depending on the oxygen concentration in the system.

Therefore, understandably antioxidant defence mechanisms exist to limit the damage induced by highly reactive oxygen free radicals; such mechanisms include enzymes to inactivate peroxides, proteins to sequester transition metals and free radical scavengers (de Zwart et al., 1999). The enzyme SOD forms part of this defence system and therefore we would expect to find SOD activity to be increased following free radical injury to the liver induced by CCl_4. Studies have been carried out using antioxidants to reduce the toxic effects of CCl_4 (Shewita et al., 2001). Antioxidants such as vitamin E, garlic and ascorbic acid were administered prior to a single dose of CCl_4 and it was found that the hepatotoxicity of CCl_4 was reduced. It was thought this protection was due to an inhibition of the metabolising enzyme P450.

In the present studies, SOD-1 was identified in the rat urine following carbon tetrachloride administration and was not detected by SDS-PAGE in any of the control urine samples. This would perhaps indicate an increase in SOD-1 activity resulting from the induced oxidative stress and the further elimination of this enzyme into the rat urine. According to the SDS gels the appearance of SOD-1 does not appear to increase with increasing dose, but it was detected in all CCl_4-treated urine samples in the dose response study. In the time course study the SOD-1 band was present in all urine samples from rats treated with CCl_4 at the time points showing maximal injury, further suggesting that the presence of SOD-1 is related to CCl_4-induced hepatic injury.

Therefore the dose response and the time course study have revealed that liver injury occurs at dose levels of 0.4 ml/kg and above of CCl_4, and that maximum injury occurs at approximately 36 hours post-dosing. At these time points SOD-1 appears in all the urine samples from rats treated with CCl_4, which would indicate a connection with the CCl_4-induced liver injury. However, this needs to be confirmed and the studies described in Chapter 4 attempt to do so. It is necessary to investigate the response of SOD-1 in the rat liver following the administration of CCl_4 and to check for changes in other rat organs also. In the present studies the pattern of the appearance of SOD-1 in the urine samples corresponds with the pattern of the serum enzyme data. However, it is also important to determine if SOD is a sensitive urinary marker by looking at changes at earlier time points and at lower CCl_4 dose levels. These points will be further discussed in Chapter 4.

Chapter four

Verification of superoxide dismutase (SOD) as a potential marker of hepatotoxicity

4.1 Introduction

The primary aim of the studies reported in this Chapter was to further confirm the identification of SOD-1 in urine samples from rats dosed with CCl_4. SOD can be specifically identified using a SOD-1 antibody or by measuring its activity using a SOD activity assay. Another important objective was to determine if SOD-1 was a sensitive and specific marker for liver injury. SOD is highly expressed in many rat tissues, for example the liver, kidney, small intestine and muscle (Frederiks and Bosch, 1997). It is therefore very important to identify the source of the increased urinary SOD-1 if the enzyme is to be a useful biomarker of hepatotoxicity.

Chapter 3 reported that SOD-1 appears in rat urine following carbon tetrachloride-induced hepatotoxicity and although SOD-1 is not a liver specific protein it may have potential as a useful urinary marker of liver injury in the same way as AST and ALT are used as serum markers. CCl_4 can cause liver damage even when administered at very low dose levels, Recknagel and Ghoshal (1966) reported hepatotoxic effects after dosing rats with as little as 0.01 ml/kg CCl_4. Increased serum activity of ALT, AST and GLDH are indicators of hepatotoxicity and injury to the plasma membrane of hepatocytes results in the leakage of the enzymes into the blood stream (Amacher, 1998).

Serum ALT levels have been measured in rats treated with CCl_4 at 0.01 ml/kg and have shown increased levels over control. A study by Soni and Mehendale (1993) reported significant increases in serum ALT and AST in rats treated with CCl_4 at 100 µl/kg; raised levels occurred as early as six hours post-dosing. Rao et al. (1997) dosed rats at a range of CCl_4 dose levels including at 0.01 ml/kg and measured the response with time. In the 0.01 ml/kg group, histological analysis revealed swollen and lipid laden cells in the centrilobular region. These workers (Rao et al. 1997) reported increased serum ALT at 36 hours following a single dose of 0.01 ml/kg but this increase was not statistically significant. Murali et al. (2004) noted very limited liver injury as determined by histological examination after dosing rats with CCl_4 at 100 µl/kg but there were significant increases in serum AST and ALT.

Therefore it would appear that serum levels of ALT and AST are sensitive to low doses of CCl_4; however these enzymes are serum markers and are consequently measured after invasive sample collection. The enzymes are also non-specific to the liver and as a consequence a combination of at least two enzymes are normally measured in toxicological studies. Urinary SOD-1 may therefore turn out to be a good non-invasive, specific marker of hepatotoxicity. However, urinary SOD-1 would also need to be sensitive and respond to these lower dose levels of CCl_4. To determine if SOD-1 was a sensitive urinary marker, a low dose CCl_4 dose response study was carried out.

The results of the time course study described in Chapter 3 showed that the maximum serum enzyme response to CCl_4-induced hepatic injury occurred at 36 hours after dosing. The 18.4 KDa protein band was also detected by SDS gel electrophoresis in all the urine samples collected at this time point, as was the 15.7 KDa protein peak detected by SELDI. At 36 hours the relative liver weights were significantly greater in the CCl_4-treated groups but there was no significant effect on the relative kidney weights. At 36 hours post-dosing the activity of the serum enzymes, AST, ALT and GLDH were significantly increased in the CCl_4-treated groups. Increased activity of these enzymes indicates hepatotoxicity. Also histopathological assessment showed that significant liver damage had occurred at this time, but there was no evidence of nephrotoxicity. As a result it was assumed that the increased amount of SOD-1 in the urine was due to a toxic response in the liver.

Exposure to a toxic compound may result in changes to both the gene and protein expression profile of the liver. Fountoulakis and Suter (2002) constructed a 2D protein database of the rat liver proteins and followed this with a study on changes in gene and protein expression in the rat liver following the administration of CCl_4. These workers reported an upregulation of three proteins, including two stress proteins known as catalase and uricase, and 18 proteins showed a significant reduction in levels at 24 hours after treatment (Fountoulakis et al., 2000). Newsholme et al. (2000) reported significant changes in 17 liver proteins following drug-induced hepatomegaly; 12 of the proteins had increased expression and 5 had decreased. A study in which hepatic stellate cells were activated in vivo by administering CCl_4 to rats for 8 weeks revealed both up-regulation and down-regulation of secreted and cellular proteins (Kritensen et al., 2000).

Since we know the administration of CCl_4 to rats induces hepatocellular necrosis, it is therefore likely that the SOD-1 found in the rat urine is a result of leakage of the enzyme from the liver. However CCl_4 can induce damage in other organs and SOD is found in many other tissues besides the liver such as heart, brain and skeletal muscle (Tian et al., 1998). Therefore it is important to determine if the SOD-1 in the urine is being released specifically from the liver. It is vital that changes in SOD-1 levels in tissues such as heart and skeletal muscle are investigated following CCl_4 administration.

An early time course study was therefore carried out. This experiment would give some indication of how soon SOD-1 appears in the urine after dosing with CCl_4. It would also be possible to observe changes in SOD-1 levels in serum/plasma since the enzyme would be released into the plasma and then eliminated into the urine. Many studies have involved the measurement of SOD activity in blood following the administration of compounds to rats (Skzydlewska and Farbisszewski, 1997; Breinholt et al., 2000; Tandon et al., 2003). Liver injury due to ischaemia-reperfusion resulted in an increase in plasma Cu/Zn SOD activity after 5 minutes of reperfusion, reaching a peak at 20 minutes (Iwamoto et al., 2002). Therefore we would expect to find an increase in SOD levels in rat serum before the enzyme appeared in the urine samples.

4.2 Experimental design

4.2.1 Low dose study

45 female rats were dosed with CCl_4 in vegetable oil at 0, 0.1, 0.15, 0.2, 0.25, 0.3, 0.35, 0.4 and 0.8 ml/kg (n = 5). Control rats received vegetable oil. After dosing, rats were returned to their communal cages for 12 hours. At 12 hours the rats were placed in metabolism cages for 24 hours to collect urine from 12 to 36 hours post-dosing. At post mortem, blood was taken for serum preparation; livers were removed, weighed and liver sections placed in fixative. From the control, 0.4 and 0.8 CCl_4-treated groups the lungs (inflation fixed) and kidneys were removed and placed in fixative.

4.2.2 Early time course study

15 female rats were dosed with CCl_4 in vegetable oil at 0.8 ml/kg and sampled at 4, 12, 24 and 36 hours post-dosing (n=3), and 3 rats received just vegetable oil (controls). Urine samples were collected from 3 rats at each time point (0 to 4 hours for the first time point, then between 4 to 12 hours, 12 to 24 hours and 24 to 36 hours). Rats were housed in communal cages before being placed in metabolism cages for urine collection. At each post mortem, at the end of the urine collection period the following tissues were removed; liver, kidney, heart, lung, spleen, small intestine and skeletal muscle and freeze-clamped in liquid nitrogen, then stored at –80 ° C until analysis.

Urine samples were collected from rats at each of the following time points, as described above: 4, 12, 24 and 36 hours. The urine samples were prepared for SDS PAGE, and run on 15 % gels and then Western blotted with the SOD-1 antibody. Equal volumes of each urine sample (20 µl) were run on a preliminary SDS gel to determine the volume of each which would be necessary to normalise for the amount of albumin present in each sample. Each sample volume of urine was made up to 20 µl with water and then 4 µl of SDS sample buffer and 1µl of 2-mercaptoethanol was added. The tissues were homogenised as described in section 2.5 and the total protein concentration for each homogenate determined using the Bradford method. Samples were prepared for SDS PAGE so that the final sample contained 20 µg protein.

4.3 Results

4.3.1 Western blotting of urine

Western blotting with the SOD-1 antibody was carried out using the urine samples obtained in the dose response study described in Chapter 3. In order to test the sensitivity of the SOD-1 antibody, a commercial source of SOD-1 (Sigma) was Western blotted with the antibody. Figure 4.1 shows a Western blot of 0.05 μg (lane 1) and 0.25 μg (lane 2) of the commercial SOD-1, the intensity of the SOD-1 band is greatly increased in lane 2 proving that the antibody is very sensitive to SOD-1 protein.

When the urine samples obtained from the dose response study described in Chapter 3 were analysed using SDS-PAGE and then Coomassie blue stained the SOD-1 band was not visible in the control urine samples. However, as shown in Figure 4.1 the SOD-1 antibody is sensitive and therefore Western blotting of control urine samples showed that SOD-1 was detectable in trace amounts in control urine (Figure 4.2). Figure 4.2 shows that the amount of SOD-1 detected in CCl_4-treated urine samples (lanes 3, 4, 5 and 6) was much greater than the trace amounts found in control urine (lanes 1 and 2). Increasing the dose of CCl_4 caused an increase in the amount of SOD detected in the urine following Western blotting. Figure 4.1 shows that the SOD-1 antibody detected only one band; and this protein has an approximate molecular weight of 18.4 kDa. In Figure 4.2 the same 18.4 kDa band was detected by the SOD-1 antibody thus confirming the identification of SOD in the CCl_4-treated rat urine.

Therefore Figure 4.1 shows us that the SOD-1 antibody is sensitive to changes in SOD concentration and Figure 4.2 confirms the increase in SOD found in CCl_4-treated urine samples as identified by mass spectrometry.

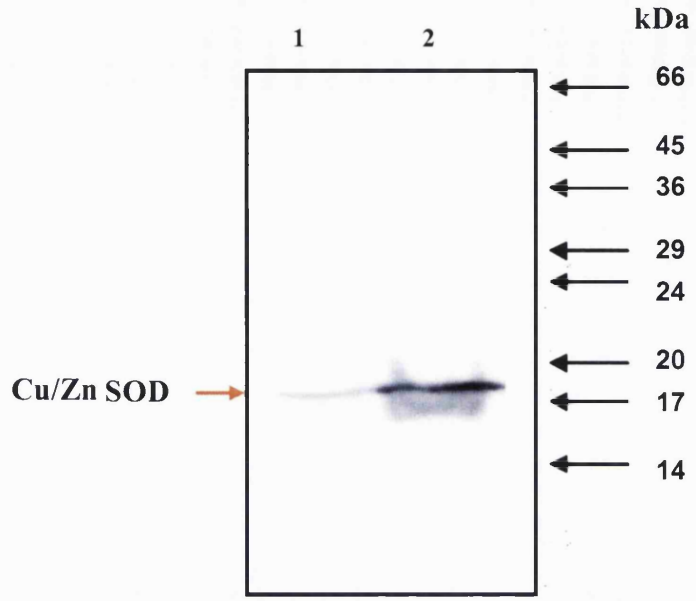


Figure 4.1 Western blot of commercial SOD standard protein using the SOD-1 antibody as described in Section 2.14. Lane 1 = 0.05 μg SOD and lane 2 = 0.25 μg SOD.

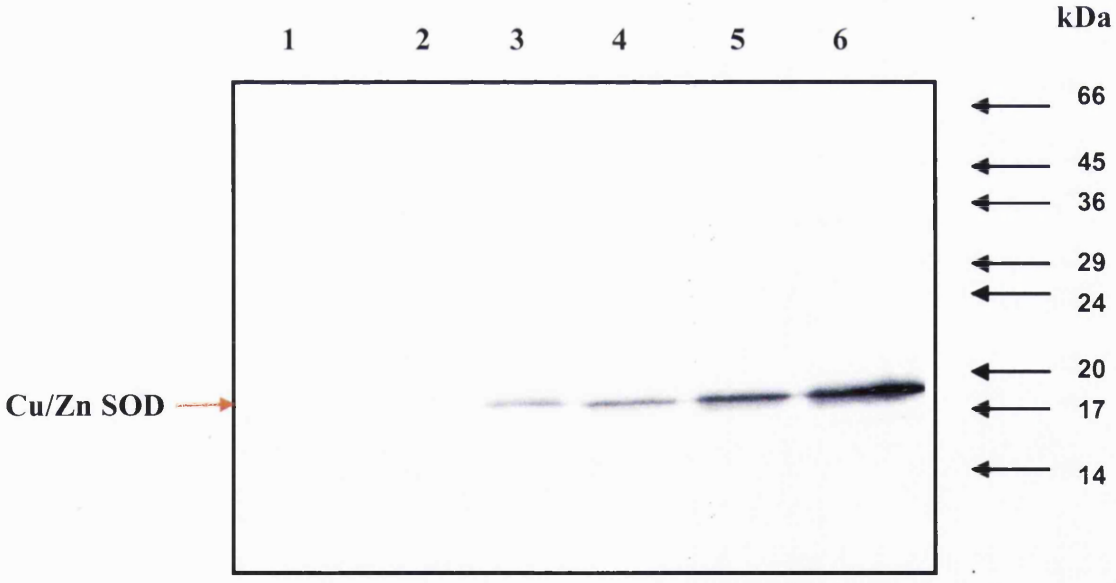


Figure 4.2 Western blot of urine samples from rats treated with carbon tetrachloride with a SOD-1 antibody as described in Section 2.14. Each sample was prepared by mixing 23 μl of urine sample, 5 μl SDS sample buffer and 2 μl 2-mercaptoethanol. Lanes 1 and 2 = control, lanes 3 and 4 = urine from rats treated with CCl_4 at 0.4 ml/kg and lanes 5 and 6 = urine from rats treated with CCl_4 at 0.8 ml/kg. Rats were treated with CCl_4 and urine samples collected from 0-24 hours post-dosing.

4.4 Low dose carbon tetrachloride study

For experimental details see section 4.2.1, page 166.

4.4.1 Liver weights

All livers from CCl_4-treated rats at all dose levels were significantly heavier than control rat livers. This was true when expressed either as absolute or relative weight. The mean control relative liver weight was 27.41 (± 2.18) g/kg, and at the lowest CCl_4 dose level used (0.1 ml/kg) the mean relative liver weight was increased to 31.56 (± 1.78) g/kg ($P < 0.05$). Relative liver weights of rats dosed with CCl_4 then increased steadily with CCl_4 dose levels up to 37.47 (± 3.33) g/kg ($P < 0.001$) in the highest dose group (0.8 ml/kg CCl_4

4.4.2 Serum analysis

The serum levels of ALT, AST and GLDH activity were increased in the CCl_4-treated rats. At the lowest CCl_4 dose administered (0.1 ml/kg), AST and ALT levels were approximately 2-fold greater than control, whereas GLDH levels were 16.5-fold greater than control levels (Figure 4.3). The activity of all three enzymes increased with increasing dose of CCl_4. At the highest dose level used (0.8 ml/kg), AST levels were 5.5-fold greater than control, ALT activity was 14-fold greater, and GLDH levels were 105-fold greater than control levels. An increase in activity of these three enzymes indicates hepatotoxicity and Figure 4.3 suggests that the injury increases with increasing dose of CCl_4.

Mean urea and creatinine levels in the serum from CCl_4-treated rats were similar to control values; there were no significant differences at any CCl_4 dose level administered (Table 4.1). An increased level of urea and creatinine in the serum is usually an indication of nephrotoxicity.

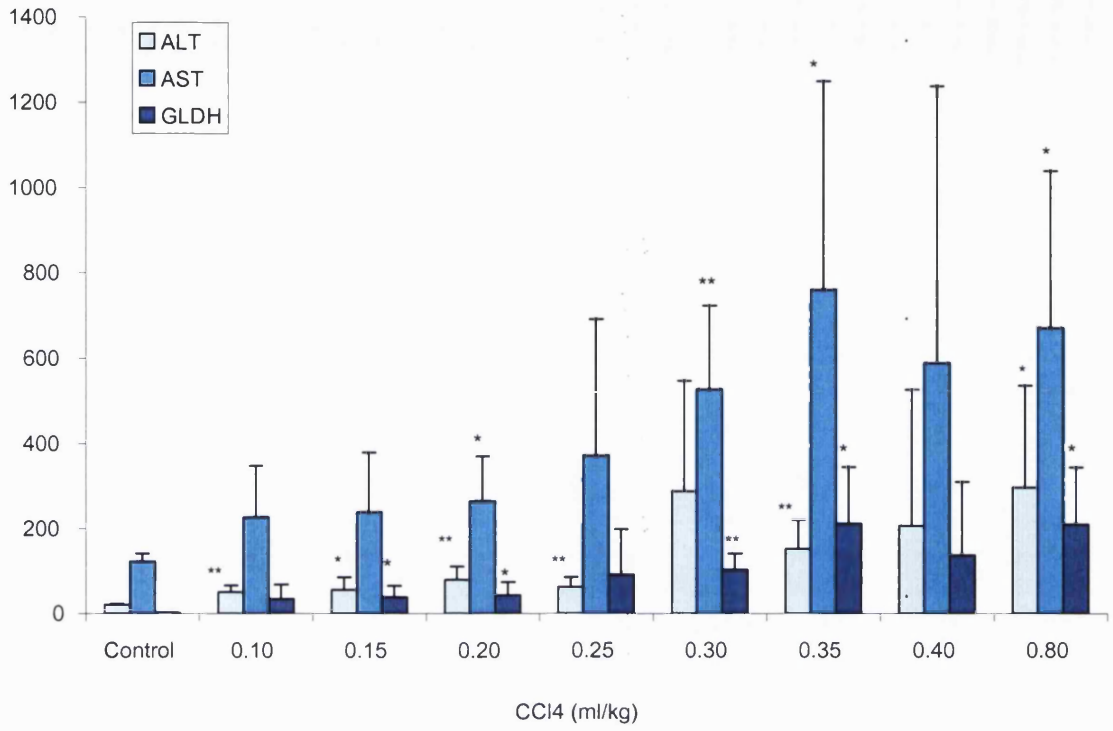


Figure 4.3 Mean ALT, AST and GLDH activity levels in the serum of rats treated with increasing dose levels of CCl_4 and sampled at 36 hours as described in Section 2.5. Values are means (error bars represent S.D.) of 5 animals. Values that differ from control by Student's t-test are shown, * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

Table 4.1 Mean urea and creatinine levels in the serum of rats treated with low increasing dose levels of CCl_4 and sampled at 36 hours post-dosing as described in Section 2.5.

CCl4	Urea (mmol/L)		Creatinine (umol/L)	
(mg/ml)	Mean	S.D.	Mean	S.D.
Control	7.01	2.07	60.00	3.98
0.10	8.00	0.82	58.00	6.48
0.15	7.23	0.60	57.00	3.67
0.20	7.68	0.99	59.00	2.45
0.25	6.33	0.49	57.00	5.59
0.30	8.01	0.89	59.00	3.67
0.35	7.88	1.22	57.00	4.09
0.40	7.20	0.34	56.00	3.11
0.80	7.47	2.42	57.00	5.77

[a]Values are means (S.D.) of 5 animals.

4.4.3 Histopathology analysis

Livers were removed from all rats during the post mortem examination for histopathological analysis. Rats treated with CCl_4 at all dose levels showed centrilobular vacuolation, with the presence of necrotic cells and infiltration of inflammatory cells (Figure 4.4). The degree of pathological change increased as the dose of CCl_4 rose. Kidneys and lungs were also examined, but no treatment related changes were observed in either organ.

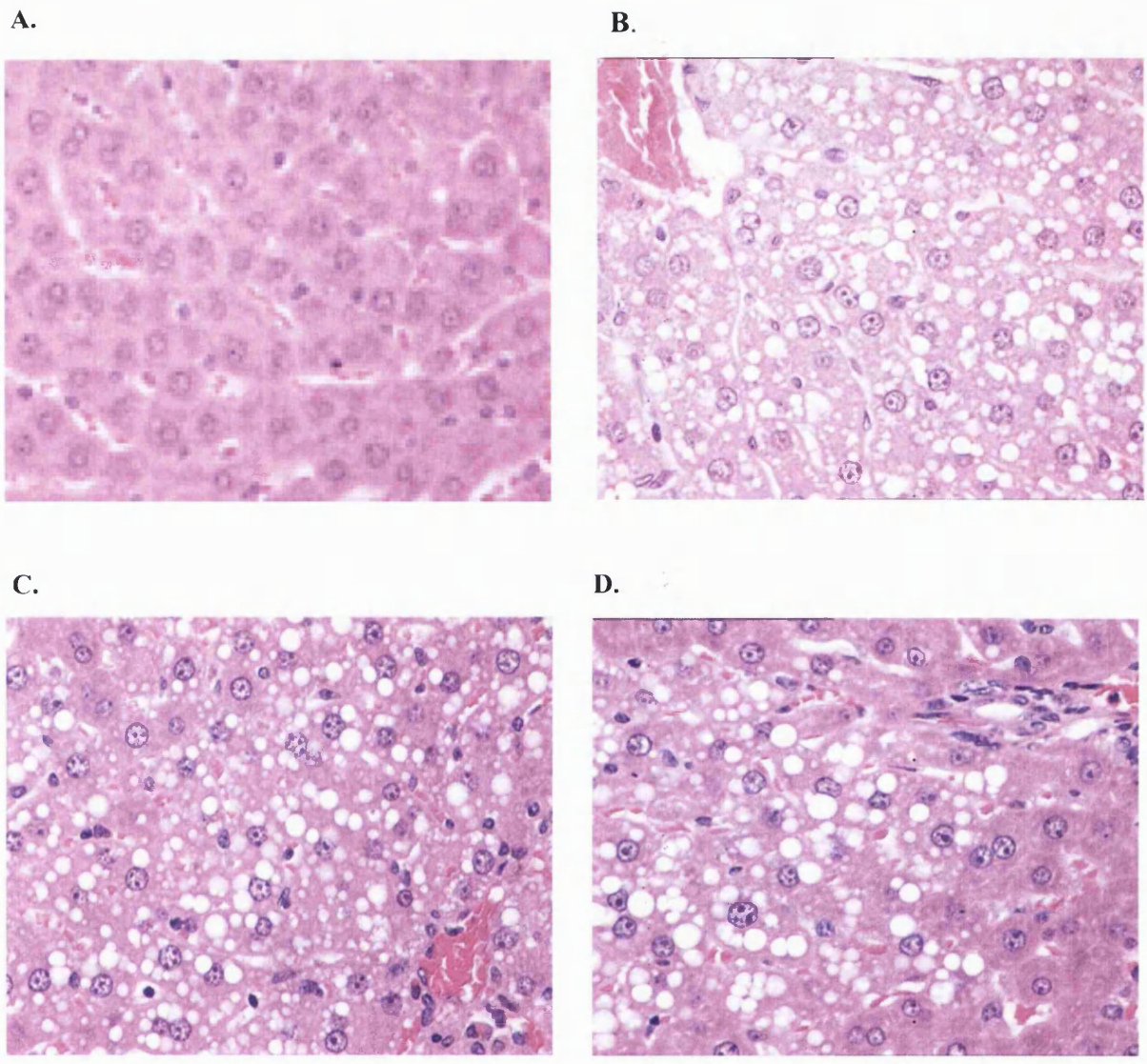


Figure 4.4 Histopathology of the liver in rats dosed with low dose levels of CCl_4 and sampled at 36 hours post-dosing. Original magnification of all images, x 400; H&E.

A. Liver from a control rat to show the normal appearance of centrilobular hepatocytes and their arrangement into anastomsing plates with sinusoids on either side.

B. Liver from a rat treated with CCl_4 at 0.1 ml/kg with diffuse centrilobular hepatocyte vacuolation.

C. Liver from a rat treated with 0.3 ml/kg at 36 hours post-dosing; hepatocellular vacuolation with necrotic cells and some infiltrating inflammatory cells.

D. Liver from an animal treated at 0.8 ml/kg CCl_4 and sampled at 36 hours after dosing; hepatocyte vacuolation with inflammatory cells.

4.4.4 Western blots of urine samples with the SOD-1 antibody

The administration of low doses of CCl_4 resulted in significant increases in the activity of serum AST, ALT and GLDH, which indicates hepatotoxicity (Figure 4.3). Therefore, since these serum markers are sensitive to low dose CCl_4 it would be interesting to determine if SOD-1 could be detected in the rat urine following the administration of low dose CCl_4. To determine the sensitivity of SOD-1 as a urinary marker, the urine samples were analysed by Western blotting with the SOD-1 antibody and also by carrying out a SOD activity assay.

Urine samples collected during the low dose study were Western blotted with the SOD-1 antibody. In contrast to Figure 4.1, no SOD-1 was detectable in control urine. There was an increase in SOD-1 with increasing CCl_4 dose, but at lower CCl_4 dose there was considerable intra-group variation e.g. at 0.1 ml/kg only 1 of 5 rats exhibited SOD-1 in urine (Figure 4.5).

4.4.5 SOD activity assay for low dose study

Urine samples collected from rats during the low dose study were analysed for SOD activity using the Ransod assay described in section 2.16. Control rats had a mean SOD activity of 424.5 (±126.7) U/L (Figure 4.6) whereas in the 0.1 ml/kg CCl_4 group the mean SOD activity was 1361.95 U/L, this was 3.2 times greater than control urine. In the highest dose group (0.8 ml/kg) SOD activity (26053.99 U/L) was 61-fold greater than in the control group.

The increased SOD activity in urine from CCl_4-treated rats was not statistically different from control at any dose level administered but this was due to considerable intra-group variation. However, the results show a general increase in SOD activity with increasing dose of CCl_4. The SOD activity measurements are similar to the Western blot results for these samples, with both showing considerable intra-group variation and increasing levels of SOD with increasing CCl_4 dose. Also, Western blotting detected SOD in one of the urine samples from the 0.1 ml/kg group, and this particular sample had a much greater SOD activity compared with the rest of the group (results not presented).

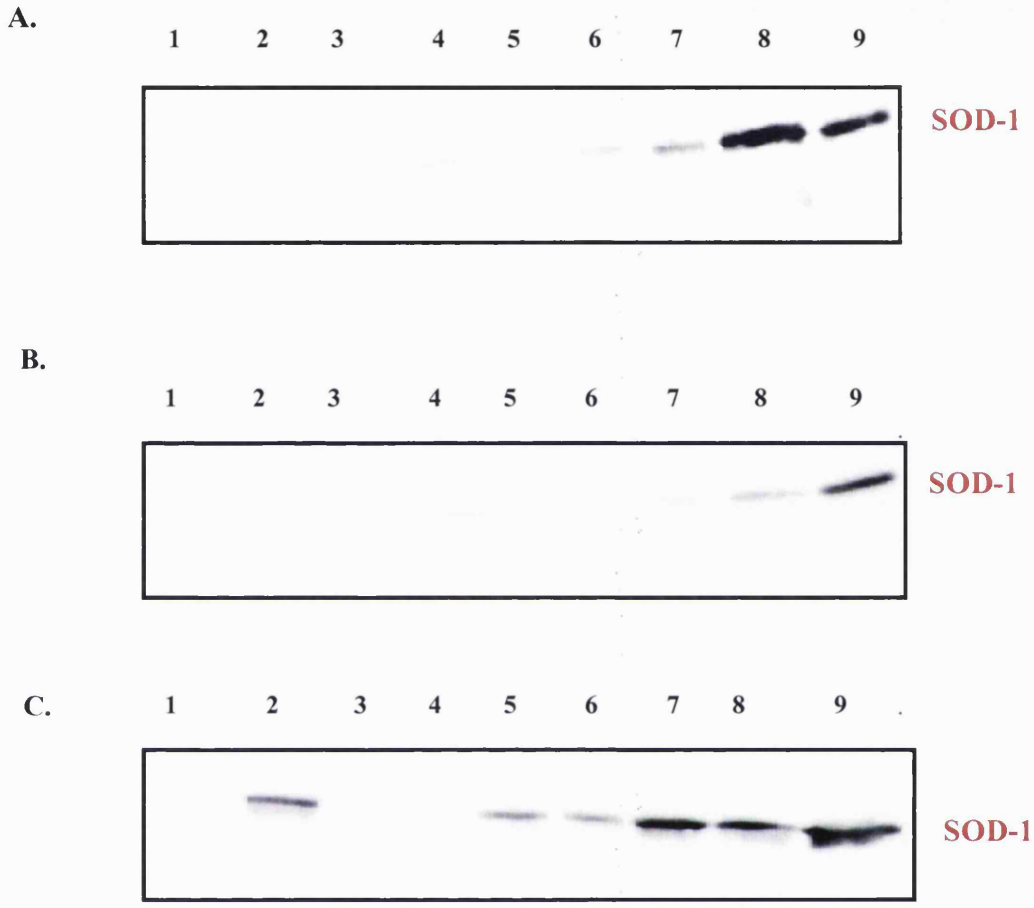


Figure 4.5 Western blots of urine samples from rats treated with CCl_4 at 0, 0.1, 0.2, 0.25, 0.3, 0.35, 0.4 and 0.8 ml/kg. Samples were prepared for SDS-PAGE by mixing 14 µl of urine, 4 µl SDS sample buffer and 2µl 2-mercaptoethanol and were Western blotted as described in Section 2.14. In all three blots: lane 1 = control, lane 2 = urine from rat treated with CCl_4 at 0.1 ml/kg, lane 3 = 0.15 ml/kg, lane 4 = 0.2 ml/kg, lane 5 = 0.25 ml/kg, lane 6 = 0.3 ml/kg, lane 7 = 0.35 ml/kg, lane 8 = 0.4 ml/kg, lane 9 = 0.8 ml/kg.

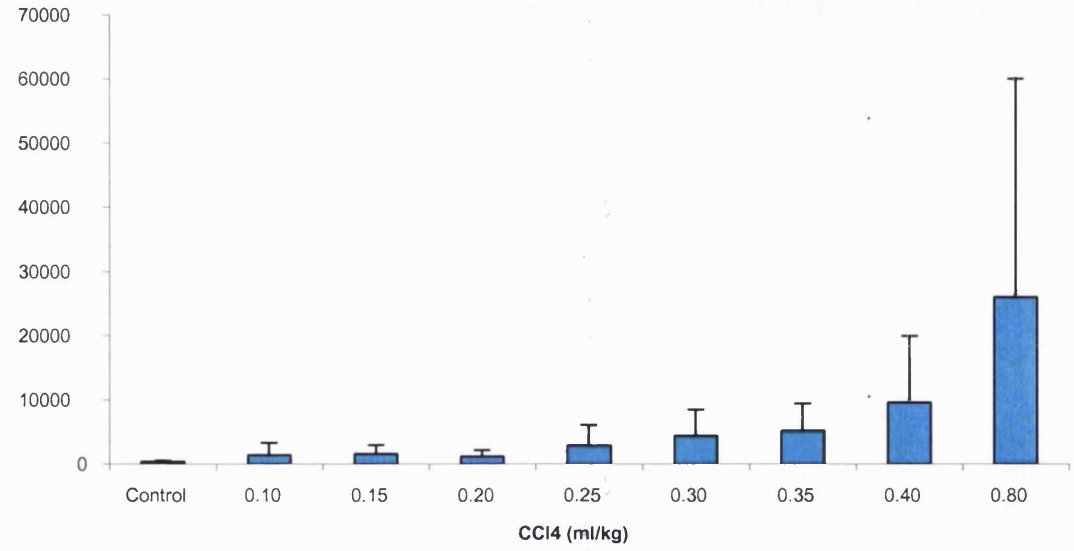


Figure 4.6 SOD activity in the urine of rats treated with low dose CCl_4. The SOD activity was measured as SOD units per litre of urine as described in Section 2.18. Values are means (error bars represent S.D.) of 5 animals.

4.5 Early time course study

For experimental details see section 4.2.2, page 166.

4.5.1 Western blotting of urine samples from rats sampled at 4, 12, 24 and 36 hours after dosing with CCl_4

Western blots of the urine samples from the different time points revealed that SOD-1 was not evident in the control samples or in any of the urine samples at the 4 hour time point. Figure 4.7 shows that SOD-1 could be detected in two out of three urine samples 12 hours after dosing rats with a single dose of CCl_4 (0.8 ml/kg) and in all the urine samples at later time points. The amount of SOD-1 observed by Western blotting was greatest in the urine samples collected at the 36 hour time point.

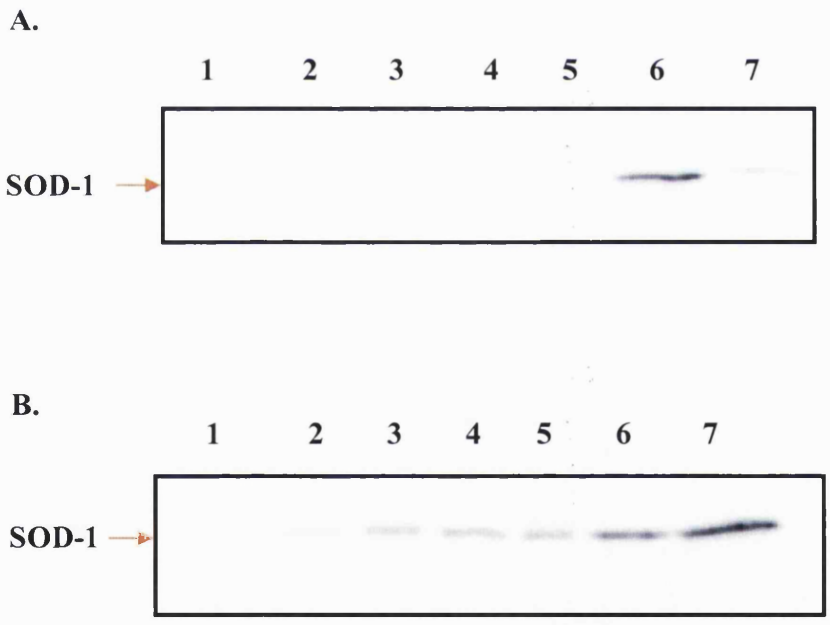


Figure 4.7 Western blots with the SOD-1 antibody of urine samples from rats dosed with CCl_4 and sampled at 4, 12, 24 and 36 hours post-dosing as described in Section 2.14. The volume of urine loaded per lane was normalised for albumin amount in each sample. A. lane 1 = control; lanes 2, 3 and 4 = urine at 4 hours; lanes 5, 6 and 7 = urine at 12 hours. B. lane 1 = control; lanes 2, 3 and 4 = urine at 24 hours; lanes 5, 6 and 7 = urine at 36 hours.

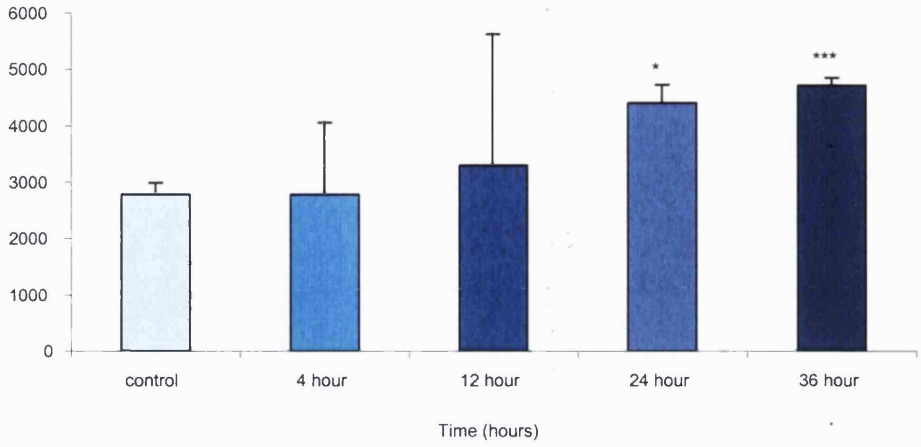
4.5.2 SOD activity in urine from early time course study

Urine samples were collected from rats at 4, 12, 24 and 36 hours after dosing with CCl_4 at 0.8 ml/kg and urine assayed for SOD activity. Control urine had a mean SOD activity of 2787.3 U/L. At four hours post-dosing the SOD activity in urine from rats treated with CCl_4 was similar to control. SOD activity in CCl_4-treated rat urine samples was greater than control 12 hours after dosing, and was further increased at both 24 and 36 hours (Figure 4.8). The SOD activity was significantly greater than control in CCl_4-treated urine samples at both 24 and 36 hours post-dosing ($P < 0.05$ and $P < 0.001$). Figure 4.8B shows the individual SOD activity for each of the urine samples collected during this study. The SOD activity of the urine samples collected at 12 hours is increased above control activity. This increase is not significant due to the much smaller activity that was measured for sample number 5 compared with the other two samples collected at the 12 hour time point. The numbers on the x-axis indicate the lane on the Western blots shown in Figure 4.7 that each sample corresponds to. The much lower SOD activity for sample 5 explains why the Western blot in Figure 4.7 shows that SOD was not detected in this sample (lane 5). The SOD activity for this sample was much lower than that of both the control urine samples.

The SOD activity for the control samples in this early time course study had a mean of 2787.3 U/L whereas the control urine obtained during the low dose response study had a mean SOD activity of 424.5 U/L. This difference may be due to the different methods used to carry out the activity assay. The assay used to measure the SOD activity in the dose response urine samples was automated whereas that for the early time course study was a manual assay. This is discussed further in section 4.8.

However, both the Western blot results and the SOD activity assay showed that the amount of SOD in the rat urine is increased in CCl_4-treated rats by 12 hours after dosing. They also showed that the SOD activity increased with time post-dosing up to 36 hours.

A.



B.

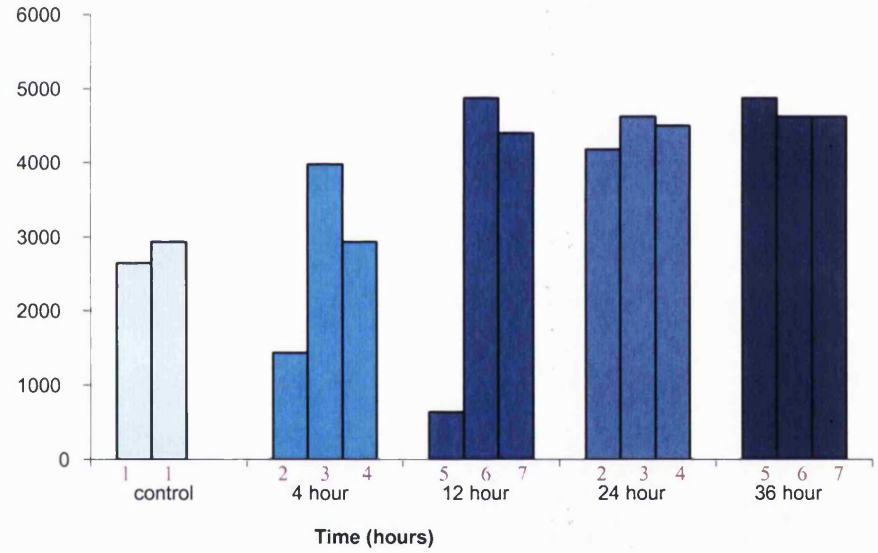


Figure 4.8A Mean SOD activity in urine from rats dosed with CCl_4 at 0.8 ml/kg and urine collected 4, 12, 24 and 36 hours after dosing. Values are means (error bars represent S.D.) of three animals except in the control group where values are means of 2 animals. Values that differ from control by Student's t-test are shown, * $P < 0.05$, *** $P < 0.001$.
Figure 4.8B shows the SOD activity of the individual urine samples at each time point. The coloured numbers along the x-axis indicate the corresponding lane on the Western blots shown in Figure 4.7, purple colour for blot A and red for blot B. The SOD activity was measured as SOD units per litre of urine as described in Section 2.18.

4.5.3 Superoxide dismutase in tissue homogenates from rats treated with carbon tetrachloride

Rats were dosed with CCl_4 at 0.8 ml/kg and sampled at 4, 12, 24 and 36 hours post-dosing. At each post mortem organs were removed and tissue homogenates were prepared as described in section 2.7, tissue homogenates were then Western blotted with the SOD-1 antibody as described in section 2.14. Tissue homogenates were prepared from kidney, heart, lung, small intestine, skeletal muscle and spleen.

Figure 4.9 shows the Western blots of liver homogenates, the SOD-1 antibody detected only one band at 18.4 kDa as in the urine samples. The amount of SOD detected in the CCl_4-treated rat livers appears to be decreased compared to control at the 12 hour time point. However there was a definite decrease in the amount of SOD detected at both 24 and 36 hours in all samples analysed. These results therefore show that SOD expression is not increased following CCl_4-induced hepatotoxicity. This proves that following CCl_4-induced liver necrosis SOD leaks from the cell into the plasma and is then eliminated via the urine.

The SOD activity assay and the Western blots of the urine samples from this study revealed that SOD was not increased in the urine at 4 hours post-dosing and this is backed up by the fact that the amount of SOD-1 in the liver homogenate at this time point was not decreased. At 12 hours the urine samples showed an increase in SOD-1 in two of three samples. Figure 4.9 shows that there appears to be a decrease in SOD-1 in two of the liver samples (lanes 6 and 7). These liver homogenate samples are from the same animals in which an increase in SOD was detected in the urine samples. Therefore the changes in SOD-1 found in the liver homogenates correspond to those detected in the rat urine. The 36 hour time point has been shown to be the time point at which maximum injury has been induced following CCl_4 treatment in previous studies described in Chapter 3. Therefore, the tissue homogenates prepared from all the other rat tissues removed at the 36 hour time point were Western blotted with the SOD-1 antibody and compared with control. Figure 4.10 shows the Western blots of each individual tissue homogenate. These Western blots revealed no differences in the amount of SOD-1 observed in the CCl_4-treated tissue homogenates when compared with control homogenates at the 36-hour time point.

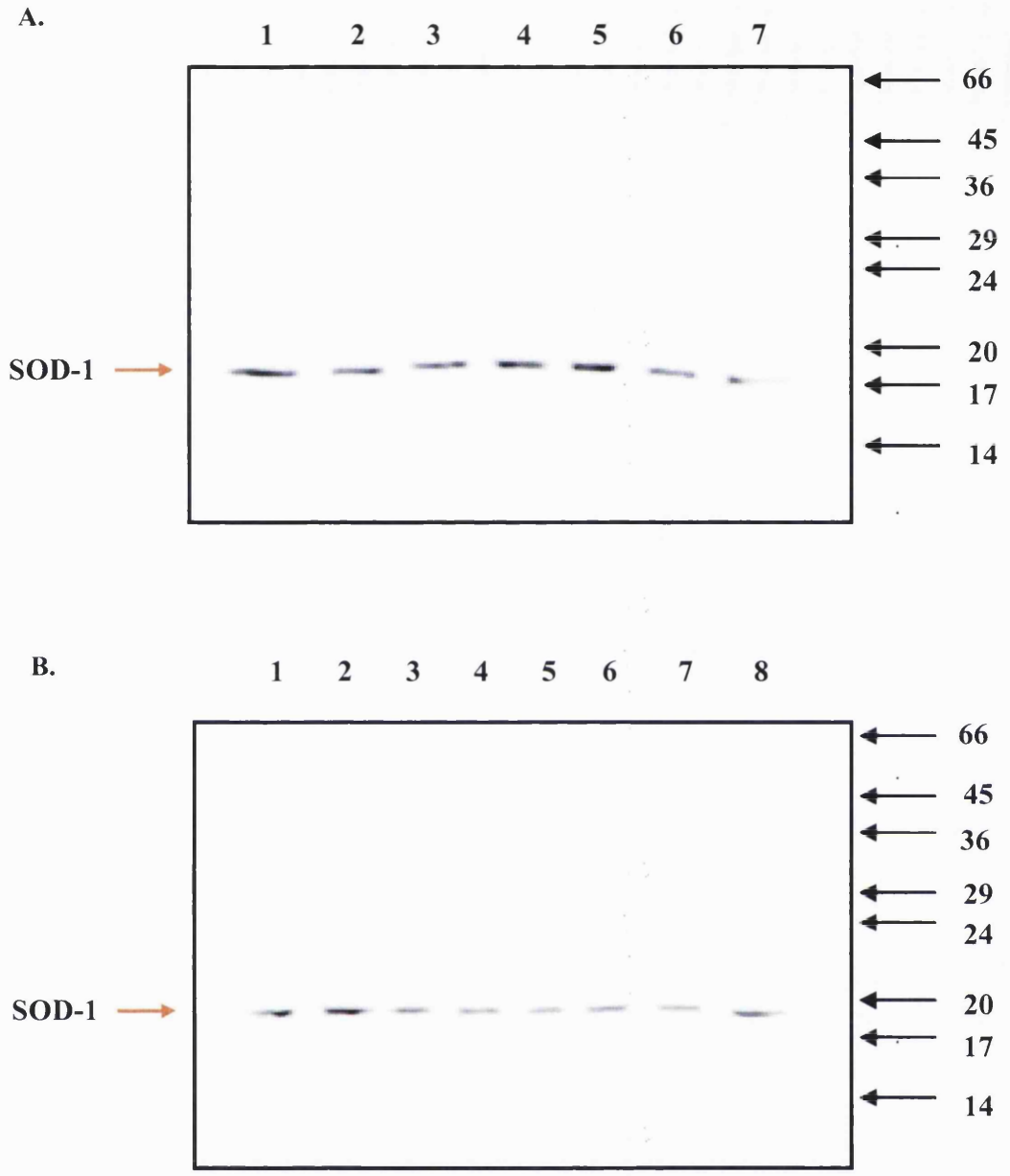


Figure 4.9 Western blots of liver homogenates prepared from liver taken from rats at different time points after dosing with CCl_4 at 0.8 ml/kg as described in Section 2.14. Each sample lane contains 20 μg of total protein. A. lanes 1 = control; lanes 2, 3 and 4 = liver homogenates at the 4 hour time point; lanes 5, 6 and 7 = 12 hours. B. lanes 1 and 2 = control; lanes 3, 4 and 5 = 24 hours; lanes 6, 7 and 8 = 36 hours.

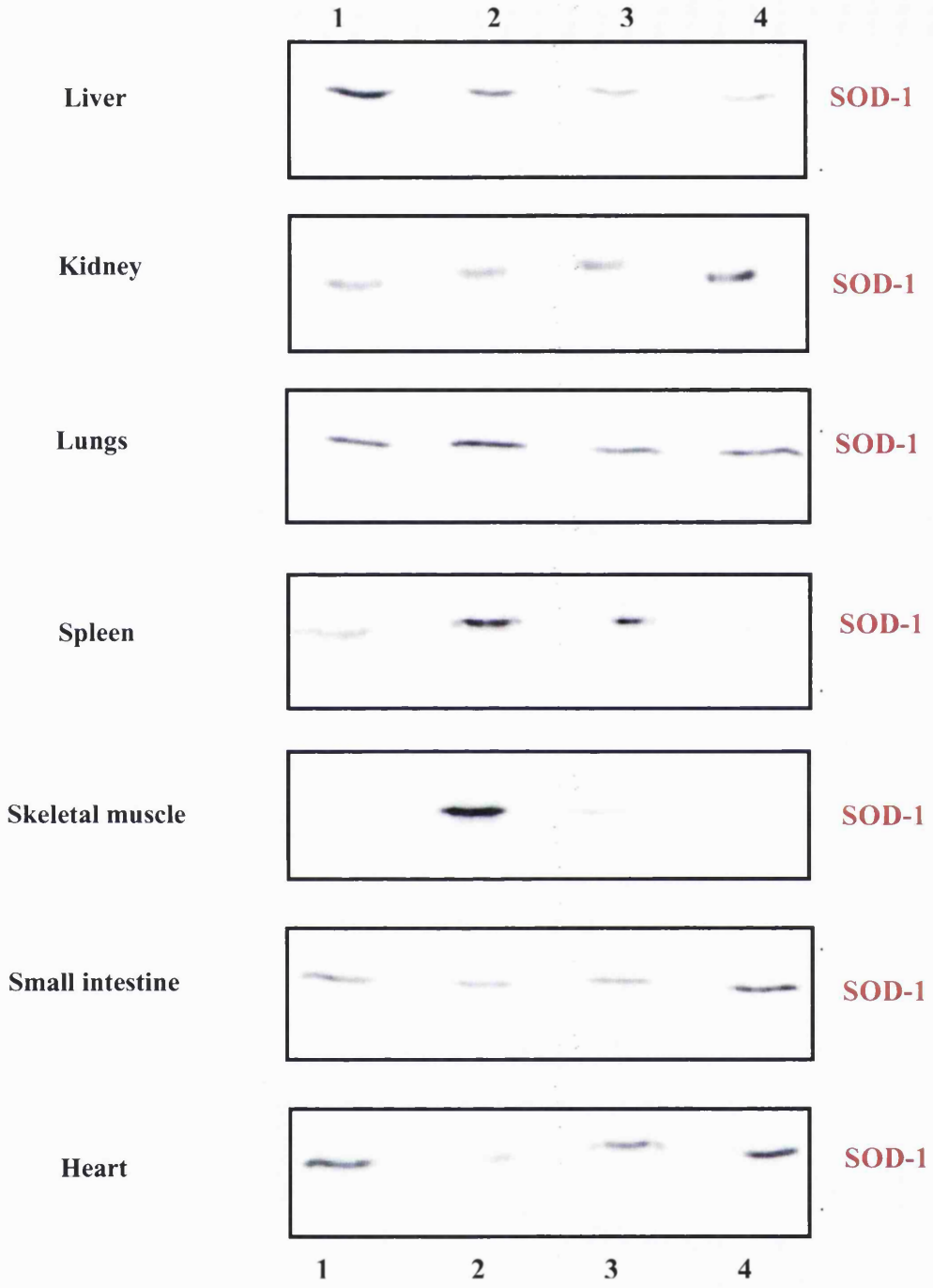


Figure 4.10 Western blots of tissue homogenates prepared from tissues taken from rats (as described in Section 2.7) dosed with CCl_4 at 0.8 ml/kg and sampled at 36 hours post-dosing. In all Western blots lanes 1 and 2 = control, lanes 3 and 4 = homogenates from the 36 hour time point. All lanes contain 20 μg total protein.

4.6 Identification of SOD protein in serum samples

The studies described above show that SOD levels decrease in the rat liver and not in any other rat organ in response to CCl_4 treatment. Analysis of the urine samples from rats showed that SOD-1 appeared in the urine following the administration of CCl_4. The administration of CCl_4 to rats eventually induces liver necrosis and this leads to the breakdown of the hepatocellular membranes. Following the breakdown of the plasma membranes the enzymes are free to leak out of the cell and into the blood, before being eliminated from the body. Therefore we would expect SOD to be released into the blood from the hepatocytes and then eliminated via the urine. Consequently, studies were carried out to detect the appearance of SOD in the blood. Blood was removed from rats dosed with CCl_4 at 0, 0.4 and 0.8 ml/kg at 36 hours post-dosing and serum prepared as described in section 2.3. Serum samples were then run on SDS PAGE to identify the SOD-1 band.

Figure 4.11 shows that when serum proteins were separated by SDS PAGE and the gel stained with Coomassie blue, the large amounts of albumin present obscured most of the other protein bands. It was not possible to see any of the lower abundance proteins present in the serum sample, this was the case with both control and CCl_4-treated serum. Therefore, we could not visualise the SOD band in serum from rats treated with CCl_4. Serum samples were Western blotted with the SOD-1 antibody with the hope that the antibody would be able to detect small quantities of SOD-1 not visible by SDS PAGE (Figure 4.12). The Western blot shows that the large quantity of albumin present non-specifically bound either the SOD-1 antibody or the secondary antibody as determined by the signal at 66, 000 Da. This made identification of the actual SOD-1 band difficult.

Therefore, the next step towards the identification of SOD-1 in serum was to remove as much albumin as possible from the serum samples. To do this, the serum samples were gel filtrated using a Superdex 75 gel filtration column on FPLC as described in section 2.20. But first we needed to estimate the elution volume at which we would expect to find SOD. The Superdex 75 gel filtration column was calibrated by running protein standards: bovine serum albumin (66,000 Da), carbonic anhydrase (29,000 Da) and cytochrome C (12,300 Da). Figure 4.13 shows the gel filtration trace for the protein standards and the calibration curve.

Figure 4.13A shows that BSA eluted from the Superdex 75 column between 8 and 12 ml, therefore we would expect the albumin in the serum samples to elute at this volume also. Carbonic anhydrase has a molecular weight of 29, 000 Da and elutes between 11 and 14 ml. From the calibration curve (Figure 4.13B) we can estimate that the native form of SOD (a dimer with molecular weight of 32 kDa) would elute from the Superdex 75 column at approximately 12.1 ml. Should single subunits of SOD exist, they would be expected to elute at approximately 14.2 ml. Therefore when running the control and CCl_4-treated serum we collected fractions eluting from the Superdex 75 column between 10 and 20 ml.

Therefore 200 µl of each serum sample was filtered and loaded on to the column and 0.3 ml fractions were collected between the elution volume of 10 and 20 ml. Fractions were pooled in groups of two and concentrated down to 100 µl samples as described in section 2.20. Figure 4.13 C and D show gel filtration traces for control and CCl_4-treated serum samples. The concentrated fractions were then prepared and run on SDS PAGE. Figure 4.13 C and D shows that a very large protein peak elutes from the column between 6–12 ml; this protein is most likely albumin. We expected to find SOD in the fractions collected between 10 and 20 ml.

When the concentrated fractions were run on SDS-PAGE the protein concentration in the FPLC fractions was very low and therefore the gels needed to be silver stained to visualise the protein bands (Figure 4.14). However, even after silver staining the gels it was not possible to determine whether the 18.4 kDa band was present or not. When these fractions were Western blotted with the SOD-1 antibody no SOD-1 could be detected (Results not shown). A mixture of commercially available SOD-1 and BSA was prepared, run on SDS-PAGE and Western blotted with the SOD-1 antibody and this blot showed two bands; one at 66 kDa and one at 18.4 kDa (Results not shown). Therefore it was assumed that BSA and therefore presumably rat serum albumin could non-specifically bind either the SOD-1 antibody or the secondary antibody; further Western blots using secondary antibody alone revealed the albumin was non-specifically binding the SOD-1 antibody

In our serum samples the total amount of albumin present in the serum samples could not be removed by gel filtration since the molecular weight of albumin (66, 000 Da) is

not considerably greater than that of SOD (32,000 Da). Consequently, the remaining albumin was probably non-specifically binding the SOD-1 antibody.

Therefore a SDS gel was run with the fractions from CCl_4-treated rat serum collected from the gel filtration column and blotted on to PVDF membrane as usual. But, before placing the blot in primary antibody the albumin was removed by cutting across the membrane at the 45 kDa molecular weight protein marker. The result of this can be observed in Figure 4.15 where, although faint, the SOD-1 protein has been detected by Western blotting in the fractions that had eluted from the Superdex 75 column between 10 and 13 ml. The most intense bands are in lanes 2 and 3 showing that the peak of SOD-1 eluted between 11.3 and 12.4 ml. This is in close agreement with the predicted elution volume of 12.1 ml as determined by the calibration curve.

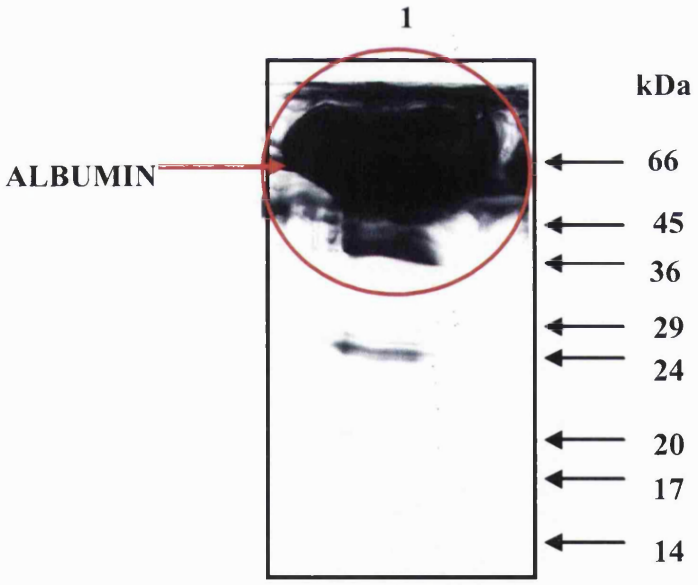


Figure 4.11 SDS gel of a serum sample froma rat treated with CCl_4 at 0.8 ml/kg (one sample only). For SDS PAGE 5 µl of serum sample was mixed with 4 µl SDS sample buffer and 1 µl 2-mercaptoethanol and gel run as described in Section 2.11.

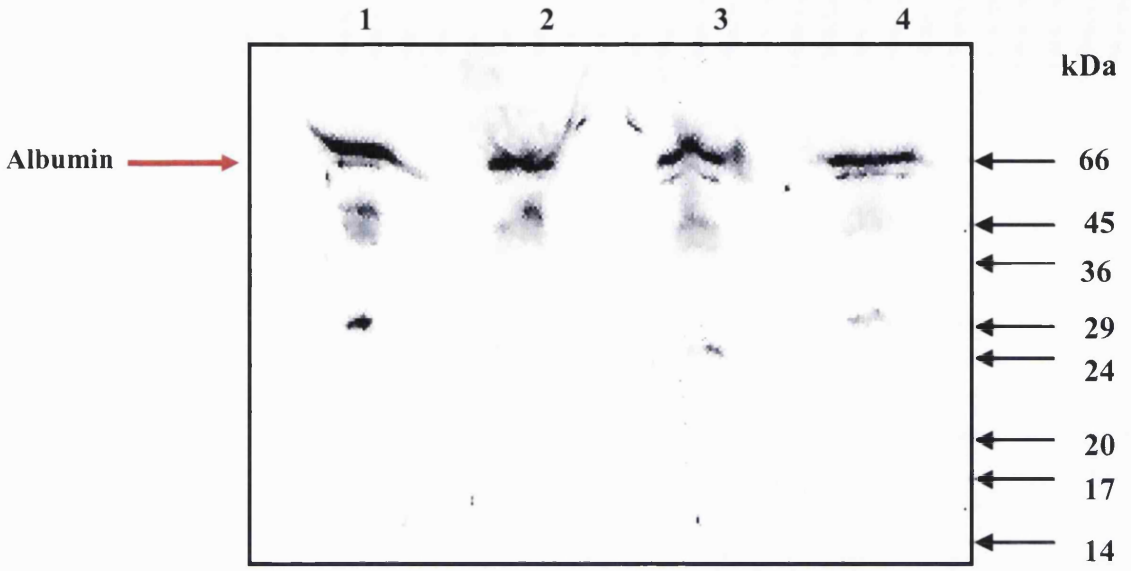


Figure 4.12 Western blot of serum samples from rats treated with 0, 0.35, 0.4 and 0.8 ml/kg and sampled at 36 hours post-dosing. Each lane contained 5 µl of serum sample that was mixed with 4 µl SDS sample buffer and 1 µl 2-mercaptoethanol. Western blotting was carried out as described in Section 2.14. Lane 1 = control, lane 2 = serum from rat treated with CCl_4 at 0.35 ml/kg, lane 3 = serum from rat treated with CCl_4 at 0.4 ml/kg and lane 4 = serum from rat treated with CCl_4 at 0.8 ml/kg

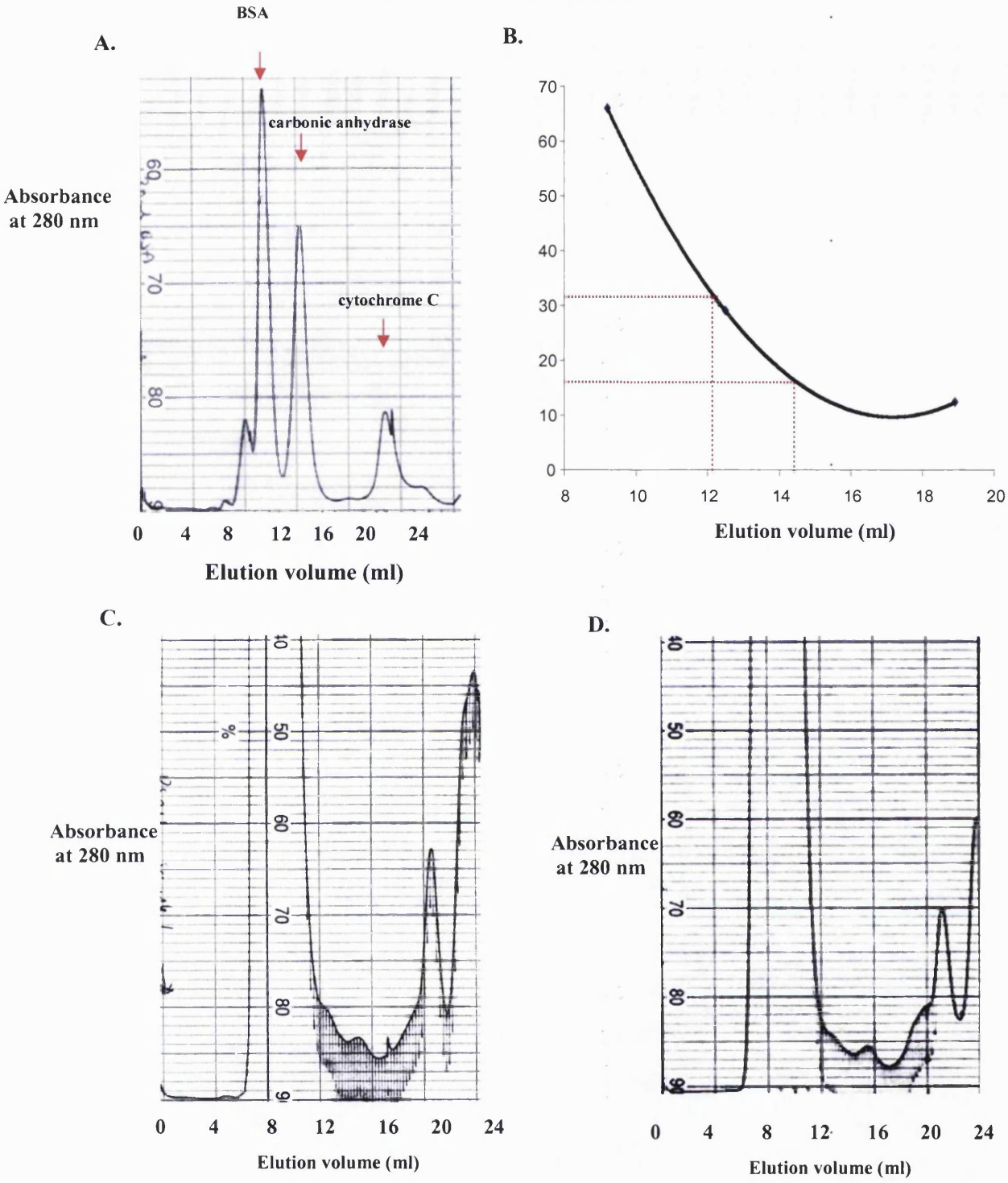


Figure 4.13 Gel filtration traces. A. Protein standard: BSA, carbonic anhydrase and cytochrome C, B. Calibration curve, C. Serum from control rat, D. Serum from rat dosed with CCl_4 at 0.8 ml/kg. For each standard 20 µl of a 10 mg/ml stock solution was mixed with 200 µl gel filtration buffer (200 mM NaCl, 10 mM Tris HCl, pH 7.2 @ 25 °C, 1 mM EDTA). 200 µl of each serum sample was filtered and loaded on to the superdex 75 column as described in Section 2.20.

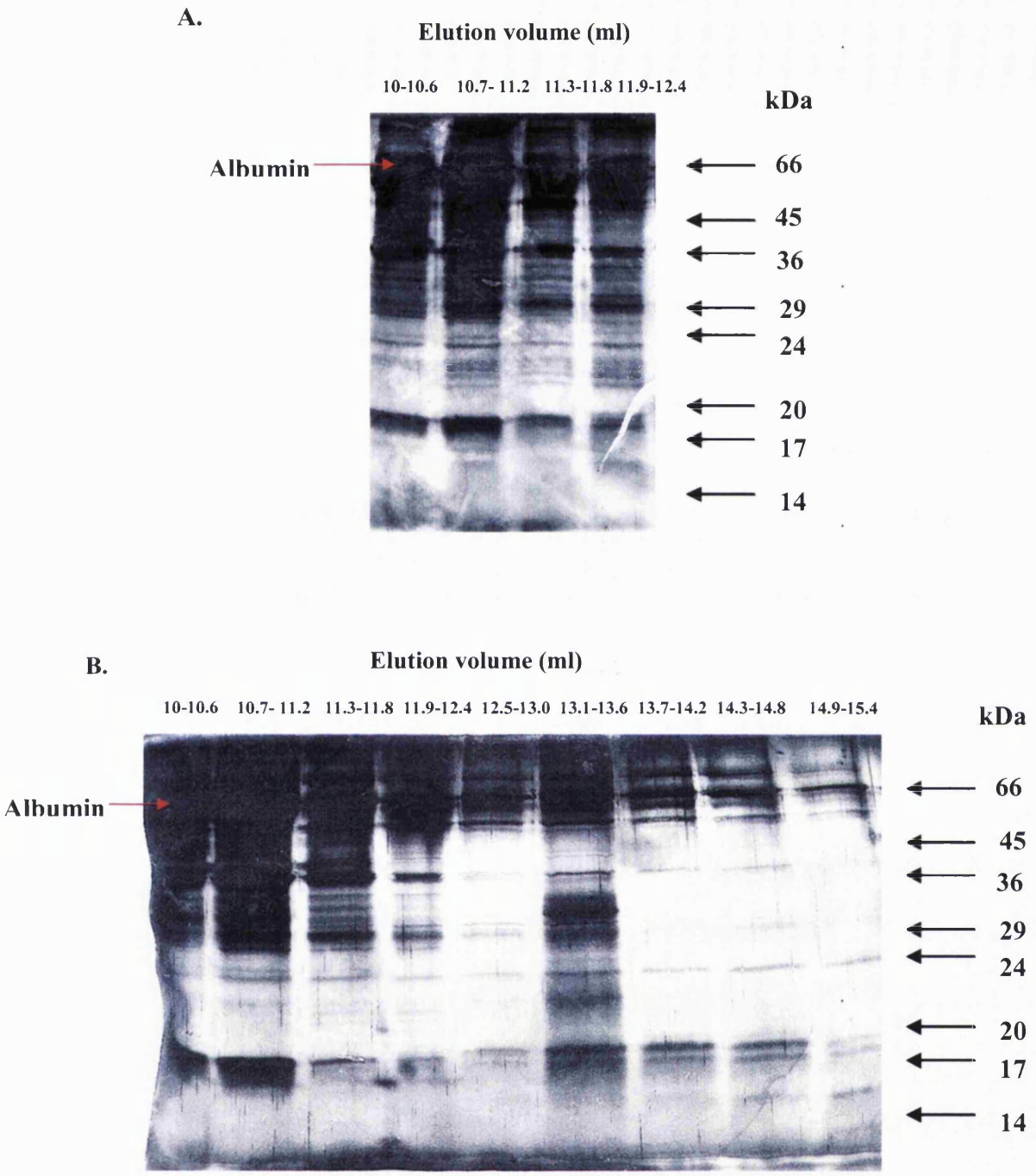


Figure 4.14 Silver stained SDS gels of serum fractions after gel filtration. Each lane was loaded with 23 µl of sample was mixed with 5 µl SDS sample buffer and 2 µl 2-mercaptoethanol as described in Section 2.11. A. Serum fractions from a control rat. B. Serum fractions from a rat treated with CCl_4 at 0.8 ml/kg. Each lane is numbered according to elution volume.

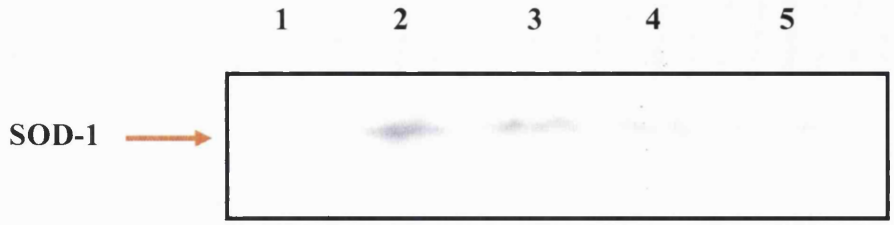


Figure 4.15 Western blot of fractions from Superdex 75 gel filtration of serum from a rat treated with CCl_4 at 0.8 ml/kg as described in Section 2.14. Before placing the blot into the primary antibody the PVDF membrane was cut below the 45kDa molecular weight marker to remove the albumin from the blot. Lanes 1 = elution volume 10.6-11.2 ml, lane 2 = 11.3-11.8 ml, lane 3 = 11.9- 12.4 ml, lane 4 = 12.5- 13.0 ml, lane 5 = 13.1-13.6 ml.

4.7 SOD activity in serum

In the low dose study, as described in section 4.3, SOD activity was measured in the serum samples prepared from blood collected at the post mortem 36 hours after dosing rats with increasing dose levels of CCl_4. Control rats had a mean serum SOD activity of 954 U/L. The mean SOD activity in serum from rats treated with CCl_4 was greater than control at all dose levels administered, although in the 0.1 and 0.4 ml/kg groups this difference is not statistically significant. In general the SOD activity in serum appears to increase with increasing dose of CCl_4 however this trend does not fit all the treatment groups including the 0.4 and 0.8 ml/kg groups. However in urine the SOD activity measured from these rats is greater than control and there is a clear dose response relationship in the urine samples.

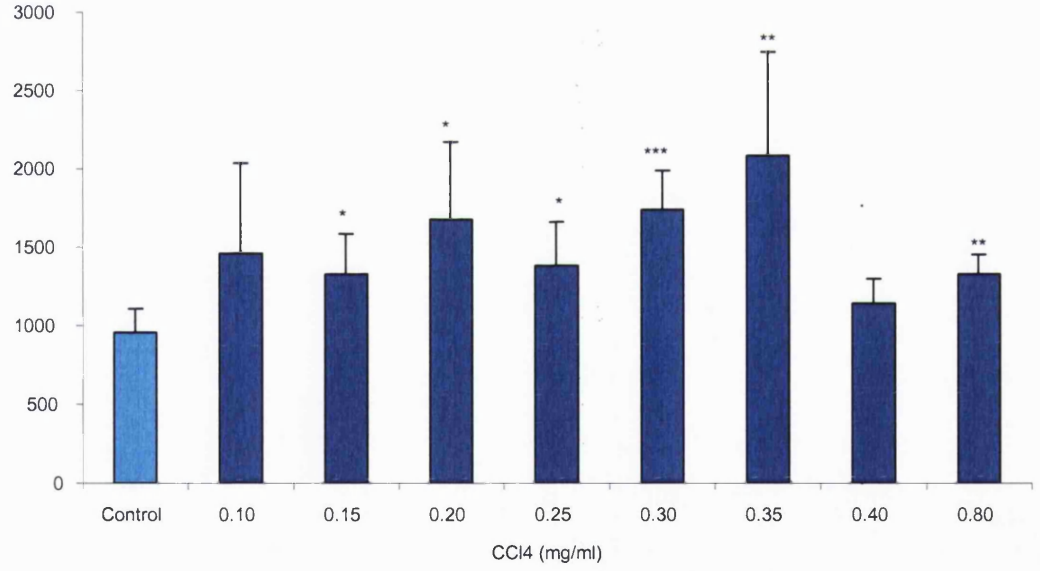


Figure 4.16 Mean SOD activity in serum from rats treated with increasing low dose levels of CCl_4 and sampled at 36 hours post-dosing as measured using an automated assay kit as described in Section 2.18. Values are means (S.D.) of five animals. Values that differ from control by Student's t-test are shown, * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

4.8 Discussion

Cu/Zn SOD was identified in the urine of rats treated with CCl_4 by one dimensional gel electrophoresis and mass spectrometry (Chapter 3). This was confirmed by Western blotting the same urine samples with an antibody specific for Cu/Zn SOD (Figure 4.2). The Western blot revealed that, although in much smaller amounts SOD-1 is present in control urine. SOD-1 is a small protein; the dimer has a molecular weight of approximately 32 kDa with each of the sub-units having a molecular weight of 15.8 kDa (Okado-Matsumoto and Fridovich, 2001). As a result, it may be possible for some of it to be filtered into the urine. The urine from rats treated with 0.4 ml/kg CCl_4 has much greater SOD-1 than control rats; and the amount of SOD-1 detected is increased after increasing the dose of CCl_4 to 0.8 ml/kg. Therefore Western blotting confirmed that the appearance of the 18.4 kDa protein in rat urine after dosing with carbon tetrachloride was SOD-1, as previously identified by mass spectrometry. SOD is an unusually stable enzyme and is resistant to heating and denaturation by sodium dodecyl sulphate (SDS) and urea (Fridovich, 1982, Halliwell and Gutteridge, 1998). This explains how SOD-1 can be detected in the urine after being released from the liver, by withstanding proteolytic cleavage in the blood and then undergoing filtration in the kidneys to the urine.

By Western blotting the commercial SOD-1 standard we obtained some idea as to how sensitive the SOD antibody is and also an estimate of the amount of SOD-1 present in the urine samples. Figure 4.1 shows that as low as 0.05 μg of protein can be clearly detected by the SOD-1 antibody. By comparing Figures 4.1 and 4.2 we can estimate that our control urine samples may contain as much as 0.05 μg SOD-1 per 23 μl, whereas sample 6 (urine from a rat treated with CCl_4 at 0.8 ml/kg) has on estimate greater than 1 μg of SOD-1 in the 23 μl sample.

The appearance of SOD-1 in the rat urine may or may not be due to liver injury but SOD-1 has the potential to be a urinary marker. An ideal biomarker is highly specific for the injury induced by the toxicant; shows an early response after exposure; can be measured by non-invasive techniques and portrays a relationship between response and the damage induced (de Zwart et al., 1998). Therefore, a good urinary marker should be sensitive to small increases in the dose of the toxin administered. A low dose study was

carried out with the objective of investigating urinary SOD-1 even at low dose CCl_4 and determining the change in urinary SOD-1 with small increases in the dose of CCl_4 administered. We know that CCl_4 is toxic to the liver when administered at low dose levels (Harris et al., 1982; Rao et al., 1997), therefore we would expect SOD-1 to appear in the urine following low dose CCl_4.

Figure 4.3 shows that the serum enzymes AST, ALT and GLDH are greater than control at the lowest dose of CCl_4 (0.1 ml/kg); and at this dose level the activity of ALT is statistically different from control ($P < 0.01$). Increasing the dose of CCl_4 caused a dose response rise in the activity of all three enzymes. These serum enzyme results confirmed the presence of liver injury. The serum urea and creatinine results showed no significant differences between control and treated rats (Table 4.1), thus suggesting that no kidney damage was inflicted at these low doses of CCl_4. Histopathology confirmed the presence of liver damage; CCl_4-treated rat livers had centrilobular vacuolation with necrotic and inflammatory cells present (Figure 4.4). There were no CCl_4 treatment related histopathological changes in either the kidneys or lungs.

Figure 4.5 shows the Western blots of urine samples from three out of a total of five rats in the low dose study. In one of the urine samples an increase was detected at 0.1 ml/kg CCl_4 (Figure 4.5C) and two of the urine samples had increased SOD-1 at 0.20 ml/kg (results not presented). In the 0.25 ml/kg and 0.3 ml/kg groups, SOD-1 was detected in four out of the five urine samples. In the higher dose groups (0.35 ml/kg and greater) SOD was detected in all five urine samples. These results suggest some degree of variation as to the extent of injury induced. However, detection of SOD-1 in the urine at these lower doses suggests that SOD-1 may be a relatively sensitive urinary marker.

To further prove the finding of SOD in the urine the activity of the enzyme was measured. The SOD activity assay was carried out on urine samples collected from rats during the low dose study. Although there were no statistically significant differences between control and treated, all the CCl_4-treated groups had a greater mean SOD activity in urine than control, and the SOD activity increased with increasing CCl_4 dose (Figure 4.6). This supports the Western blot showing that SOD-1 appears in urine in response to CCl_4 treatment 12-36 hours post-dosing. The fact that one can detect a change in SOD activity even at the lowest dose administered (0.1 ml/kg) indicates that SOD could be described as a sensitive urinary marker. At the highest dose (0.8 ml/kg)

of CCl_4 administered to rats the mean SOD activity is increased 61-fold over control activity levels. At this dose histopathology had revealed no treatment-related changes in either the lungs or kidney so the likely source of the SOD is the liver.

It is clear that SOD-1 is increased in urine at low dose levels of CCl_4 but it is also important to investigate the SOD activity in urine at early time points after the administration of CCl_4 because an ideal biomarker also shows an early response (de Zwart et al., 1998). The early time course study enabled urine to be collected from rats at 4, 12, 24 and 36 hours post-dosing. The 36 hour time point was included as urine had been collected from rats during the low dose study at 36 hours post-dosing and therefore this time point would act as a positive control.

CCl_4 is known to induce early changes in the liver morphology, within one hour protein synthesis is reduced and the endoplasmic reticulum loses ribosomes (Reynolds and Molsen, 1979). One to three hours after dosing, triglycerides accumulate as fat droplets and the rough endoplasmic reticulum shows signs of membrane damage.

Western blotting of urine samples from this study revealed that SOD-1 could not be detected in the control urine samples and also no SOD-1 was visible in the urine samples collected at 4 hours post-dosing. At the 12 hour time point SOD-1 was detected in the urine of two of the three rats treated with CCl_4, showing variation within the group at this time point. This intra-group variation is similar to the appearance of the 18.4 KDa band (now identified as SOD-1) and the 15.7 kDa peak detected by SELDI in the time course study described in Chapter 3. Both the 18.4 kDa band and the 15.7 kDa peak were present in only some of the urine samples collected 12 hours after dosing. This indicates that even 12 hours after dosing not all the rats in the group have been equally affected by the CCl_4 and some degree of variation exists concerning the extent of liver damage induced. However, SOD-1 was present in all the urine samples 24 hours after dosing and the amount of SOD-1 detected was increased in the urine samples collected at the 36 hour time point. These results reveal that SOD-1 was detected in urine from rats treated with CCl_4 from 12 hours after dosing, and the amount detected increased with time.

Figure 4.8 shows that SOD activity in urine was similar to control at 4 hours after dosing with CCl_4, which was in agreement with the Western blot results for these urine

samples (Figure 4.7) and that SOD activity increases with time to a maximum at 36 hours post-dosing.

We assumed that the increased SOD activity in the urine from rats treated with CCl_4 is a result of the enzyme leaking from damaged hepatocytes into the bloodstream before ending up in the urine. SOD-1 did not increase in urine as determined by Western blot and activity assay measurements at a time point as early as 4 hours post-dosing. Therefore it is not a very early indicator of liver damage. However, at 12 hours when liver injury is apparent then SOD-1 is a marker of injury.

The mean SOD activity for the control urine samples in Figure 4.8 was considerably greater than the control SOD activity in the low dose study (Figure 4.6). It is possible that the control urine samples from the rats in the time course study had higher than normal SOD activity. The high control activity in Figure 4.8 does not correlate with 'no signal' on Western blot, and the difference between control activity and the activity at 36 hours in Figure 4.8 does not correlate with the difference in Western blot signal in Figure 4.7. The sample number was lower than in the low dose study, (2 controls in the early time course as opposed to 5 in the low dose study).

SOD activity was measured in the urine samples from the time course study but unlike the previous SOD activity results for the low dose the assay was carried out manually. These results were likely then to be less comparable to the previous set due to the method the assay was carried out.

These differences may be a result of a number of things including the fact that the assay requires the substrate to be mixed with the enzyme and then an absorbance difference to be calculated. The automated method can therefore perform these actions much more accurately and will process all the samples equally. Another problem, which may have been encountered in the assay, is that a false SOD activity can be measured as a result of any agent which inhibits superoxide generation, for example by inhibiting the xanthine oxidase enzyme (Halliwell and Gutteridge, 1998).

However, although the activity assay was carried out differently for the low dose and early time course studies the results concurred. Both sets of results agree that SOD activity was increased in the CCl_4-treated rat urine samples. The elevated SOD activity

increased both with the CCl_4 dose administered to the rat and with time after dosing that urine samples are collected.

The enzyme SOD is found in many rat organs; liver, kidney, pancreas, brain, muscle, small intestine and heart (Fredericks and Bosch, 1997). We assume that the SOD-1 found in the rat urine was a result of CCl_4-induced injury to the liver. If this was the case then we ought to see a change in SOD-1 detected in the liver sample after CCl_4 administration. The Western blot of liver homogenates from rats treated with CCl_4 during the early time course study (Figure 4.9) showed no difference in the amount of SOD-1 detected at the four hour time point. If the administration of CCl_4 to rats induced an increase in SOD expression then we would expect to observe an increase in the amount of SOD-1 detected in the liver homogenates in this study. However, the only difference in the amount of SOD-1 detected is a decrease. Although the liver homogenates obtained at the 12 hour time point show some variation within the samples, the amount of SOD-1 detected in two of the CCl_4-treated rat livers is less than control. By 24 hours after dosing all the liver homogenates have less SOD-1 than control livers as do the liver homogenates from the 36 hour time point.

Therefore, these results correspond with the amount of SOD-1 found in the urine samples from the same rats. SOD activity was increased in two of the CCl_4-treated rat urine samples at the 12 hour time point and then in all the samples at the later time points. The two rats with a decrease in the amount of SOD-1 detected in the liver homogenates are the two that had increased SOD activity in the urine samples following CCl_4-induced hepatotoxicity.

Jeon et al. (2003) found that on administration of CCl_4 to rats SOD activity in liver was slightly decreased but not to a statistically significant level, but when they also administered an antioxidant (chitosan) along with CCl_4 the SOD activity was 2.5 times higher than in the CCl_4 group. Dosing rats with a single 0.5 ml/kg dose of CCl_4 caused a decrease in SOD activity in liver which was reversed by the administration of the antioxidant protein turmeric (Subramanian et al., 1999). Ramkumar et al. (2003) measured the SOD activity in liver of rats treated with CCl_4 and found that it was reduced when compared to control. They discovered that this reduction was less in food-restricted animals. SOD activity was decreased in liver in both spontaneously

hypertensive rats and Wistar-kyoto rats following the subcutaneous administration of CCl_4 at 0.4 ml/100 g twice a week for four weeks (Hsu, 1995).

A number of other groups report a decrease in SOD activity in the liver following CCl_4 administration. Ilavarasan et al. (2003) investigated the effects of an antioxidant, *Thespesia populanea*, against the effects of CCl_4. They discovered that SOD activity was decreased in liver following a single dose of CCl_4 at 5 ml/kg but this decrease was reversed by the antioxidant. These results reflect a number of studies carried out looking at the effects of antioxidants on CCl_4 treatment (Bhattacharyya et al., 2003; Hsiao et al.,2001 and 2003; Chidambara et al., 2002 and Venukumar and Latha, 2002). In all these studies CCl_4 treatment reduced the amount of SOD in the liver but the use of an antioxidant reversed this decrease so that the SOD activity was similar to control values.

If the increased SOD detected in the urine was a result of CCl_4-induced liver injury then we would not expect the SOD activity in other rat tissues to change with treatment. This was the case when we examined the SOD-1 content in rat tissues by Western blotting. The Western blots of kidney, heart, spleen, lungs, small intestine and skeletal muscle homogenates (Figure 4.10) showed no obvious change in SOD-1 between control and treated tissue homogenates at the 36 hour time point. The 36 hour time point was chosen since the liver homogenates from rats 36 hours after dosing showed the maximum decrease in the amount of SOD-1 detected by Western blotting.

Many other studies have been carried out in which SOD activity in the liver has been measured to determine the state of the antioxidant defence system in the rat following administration of a toxic compound. Thioacetamide (TAA) is a potent hepatoxicant, a single dose of 6.6 mmol/kg causes severe liver necrosis (Sanz et al., 2002). When administered to rats, TAA caused a decrease in Cu/Zn SOD activity in the liver at both 24 and 48 hours after dosing in both weanling and adult rats (Sanz et al., 1998 and 2002). Abul et al. (2002) induced cirrhosis in rats using TAA and found a significant decrease ($P < 0.0001$) in SOD activity in the liver of cirrhotic rats.

Cadmium is another potential pro-fibrogenic compound, which may induce oxidative damage by inducing lipid peroxidation (del Carmen et al., 2002). The administration of a single dose of cadmium at 2.5 mg/kg caused significant decreases in Cu/Zn SOD

activity in rat liver at 6, 24 and 48 hours post-dosing (Casalino et al., 2002). Jurczuk et al. (2004) investigated the effects of co-exposure to both ethanol and cadmium to the oxidative system of rats. SOD activity was decreased in the liver of rats after 12 weeks of exposure to cadmium, ethanol or both cadmium and ethanol when compared to control.

Ohta et al. (1999) administered a single dose of ∝-naphthylisothiocyanate (ANIT) to rats and found a decrease in SOD activity in the liver of treated rats at 24 hours post-dosing, but by 72 hours after dosing the SOD activity had recovered to control levels. The SOD activity in liver has been studied after treatment with acetone (Orellana et al., 2000) and it was found that acetone caused a decrease in SOD activity after 7 days of exposure.

A single dose of lindane (60 mg/kg) administered to rats caused a decrease in liver SOD activity at days 1 and 2 post-dosing (Junqueira et al., 1997). Kim and Lee (1997) dosed rats with a single dose of 75 mg/ml benzo(a)pyrene and this caused the SOD activity in liver and kidney to decrease compared with control. Ochratoxin (OTA) causes a number of pathological changes in the liver and kidney of rats. Four weeks exposure to OTA induced a decrease in SOD activity in the liver and kidneys (Meki and Hussein, 2001). Brown et al. (1998) overloaded rats with carbonyl iron for 10 weeks and found a decrease in Cu/Zn SOD activity in the liver, but there did not appear to be any change to the Cu/Zn SOD mRNA levels. Lu et al. (2004) investigated the effects of the antioxidants idoxifene and estradiol on hepatic fibrosis in rats. They found that liver Cu/Zn SOD activity was reduced in the DMN model of fibrosis, but the administration of idoxifene and estradiol reversed this decreased activity.

However, other studies have found an increase in the SOD activity in rat livers following the administration of toxic compounds. Skrzydlewska and Farbiszewski (1997) investigated the effects of methanol on the antioxidant status of both the brain and liver in rats. A single dose of 6g/kg body weight produced an increase in liver SOD activities at 6 hours post-dosing, with the levels returning to control values by 5 days. Acute treatment of rats with chloroquine resulted in no real effects to the antioxidant enzyme in the liver, whereas treatment with chloroquine for 4 weeks caused an increase in the SOD activity in the liver (Magwere et al., 1997).

Rathore et al. (1998) found that a single dose of isoproterenol to rats caused an increase in SOD activity in the liver but after a second dose of isoproterenol the SOD activity decreased in the liver. A single dose of 3-Nitropropionic acid to rats induced an increase in the liver SOD activity (Fu et al., 1995). Dwivedi et al. (1998) found an increase in SOD in liver when they dosed rats with quinalphos at both 0.52 and 1.04 ml/kg.

Other groups report no changes to SOD activity in the liver following exposure to a hepatotoxin. Kern et al. (2002) administered 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) to rats and found no change in the SOD activity in the liver when compared to control. Studies using TCDD suggest that it damages organs through the generation of free radicals and oxidative stress. A study carried out by Videla et al. (2000) reports no change in SOD activity in liver following administration of lindane, phenobarbital or joint phenobarbital and lindane to rats.

The results of the low dose and early time course studies suggest that the presence of SOD-1 in the urine is a result of a loss of SOD-1 from the liver. This is likely to be caused by the enzyme leaking from necrotic hepatic cells into the plasma and then being filtered into the urine since it exists as a small molecular weight protein and therefore can easily pass through the filtration barriers. If this were the case then it would be interesting to determine if we could detect any change in SOD-1 in the plasma following CCl_4-induced hepatotoxicity.

The identification of proteins in blood/plasma/serum poses a problem because of the high concentration of albumin present which masks the appearance of other proteins. After gel filtration of the serum samples to separate the albumin from the rest of the sample and by cutting the PVDF membrane before incubation in primary antibody it was possible to see SOD-1 in the serum fractions from rats treated with CCl_4 at 0.8 ml/kg by Western blotting (Figure 4.15). When the SOD activity was measured in the serum samples from rats treated with low doses of CCl_4 (Figure 4.16), the SOD activity was increased in the CCl_4-treated samples over control. But the increase was not as great as that in the urine samples. There appeared in general to be an increase with increasing dose of CCl_4 but this trend did not fit all groups including the 0.4 and 0.8 ml/kg groups. The SOD activity in the serum was measured 36 hours after dosing. Since SOD-1 appeared in urine from approximately 12 hours it is possible that more

obvious changes in SOD activity in serum would have been detected at earlier time points before the SOD is filtered into the urine. Other serum enzymes currently measured as indicators of hepatotoxicity include ALT, AST and GLDH; these enzymes leak into the blood stream following hepatocellular necrosis (Amacher, 1997). Increased ALT and AST activities have been found after dosing rats with CCl_4 at 2 - 3 hours post-dosing (Ohta et al., 1997; Wang et al., 1997; Das et al., 2000; Janakat and Al-Merie, 2002).

Figure 4.3 shows the mean serum ALT, AST and GLDH activities for the low dose study; in the 0.1 ml/kg group the only significant difference was for ALT activity. Serum GLDH activity was significantly increased in the 0.15 ml/kg group and the lowest dose at which AST was significantly increased was in the 0.2 ml/kg group. The serum SOD activity was significantly increased at 0.15 ml/kg CCl_4 (Figure 4.16). There were no significant differences between control and treated rat serum for all the enzymes measured including SOD in the 0.4 ml/kg group (Figures 4.3 and 4.16). This shows that SOD appears to follow the same trend in activity as ALT, AST and GLDH.

Breinholt et al. (2000) administered lycopene to rats for 2 weeks and found that SOD activity increased in rat plasma. A study carried out by Tandon et al. (2003) administered cadmium to rats for five days and discovered an increase in SOD activity in blood, liver and brain. Orellana et al. (2000) investigated the effect of bile duct ligation (BDL) on the oxidative response in rat. They found that BDL caused a decrease in SOD activity in the liver but there was no change in SOD in the red blood cells. Iwamoto et al (2002) measured the change in Cu/Zn SOD in plasma following ischaemia-reperfusion in rat liver. The SOD activity was increased in plasma from five minutes to two hours after ischaemia-reperfusion. MacDonald-Wicks and Garg (2003) carried out studies investigating changes in oxidative stress induced by CCl_4 after modulating dietary fat and found that the SOD activity in rat plasma was increased following CCl_4 administration. Kumaravelu et al. (1995) measured the SOD activity in erythrocytes in rats treated with CCl_4 at 1.195 g/kg three times a week for 2 weeks and found that the activity was increased over control. The administration of eugenol caused the SOD activity to decrease to control levels.

Therefore we can conclude that SOD activity is increased in rat urine following CCl_4 induced hepatotoxicity. This increase is a result of the loss of SOD-1 from the hepatic

cells as determined by the decrease in SOD-1 levels in the liver from 12 hours post-dosing. No changes in SOD-1 levels have been detected in other rat tissues following CCl_4 administration. The increased SOD activity in rat urine can be detected following doses of CCl_4 as low as 0.1 ml/kg, indicating that it is a sensitive urinary protein to liver toxicity. SOD-1 is present in serum samples and there is a slight increase in activity in serum from treated rats even at 36 hours post-dosing. The serum enzyme studies suggest that SOD-1 may be described as similar in sensitivity to GLDH and perhaps more sensitive than AST in response to CCl_4-induced hepatotoxicity.

Chapter five

Development of a rat model of hepatic fibrosis and the identification of novel urinary markers

5.1 Introduction

The studies carried out in Chapters 3 and 4 investigated the acute response of the rat liver to a single oral dose of CCl_4. The enzyme SOD-1 was identified in the rat urine following hepatic injury. However, the aim of the investigation described in this Chapter was to firstly develop a model for liver fibrosis by the chronic administration of CCl_4 and then use this model to investigate urinary protein markers in chronic hepatic injury.

The current methods for assessing liver fibrosis are liver biopsy and histopathology. However, in humans these can be associated with patient morbidity and mortality as a result of surgical intervention (Friedman, 2003). Many serum markers of liver fibrosis are also currently measured in a clinical setting in the evaluation of fibrosis including hyaluronic acid, procollagen III propeptide, collagen-IV, MMP's, TIMP's and many others. Hyaluronic acid is a glycosaminoglycan synthesized as a component of the extracellular matrix by stellate cells in the liver, and the measurement of hyaluronic acid in serum correlates with the degree of fibrosis (Patel et al., 2003; Olga and Nikolai, 2003). Also, persistently high serum concentrations of procollagen III propeptide are associated with hepatic fibrosis (Savolainen et al., 1988) and the serum procollagen III propeptide level is directly correlated with the expression of TGF-β1 (Guéchot et al., 1994). Laminin is a glycoprotein that is associated with the distribution of collagen IV, and during the development of fibrosis the synthesis of both increases as they form a basement membrane-like structure within the space of Disse (Korner et al., 1996; Walsh et al., 2000). Recent studies have suggested that plasma prolidase may also be a useful marker of liver fibrosis (Abraham et al., 2000). This enzyme is important for the degradation of collagen. Other serum markers presently used in the clinical situation include TGF-β1, HGF, α_2-macroglobulin and haptoglobin.

However, the ideal serum marker of liver fibrosis should be liver specific and sensitive and unfortunately many of the current markers fail to meet both requirements, with many failing to detect fibrosis until the late stages (Friedman, 1999). As a result, liver fibrosis is measured in the serum using a combination of serum markers. Imbert-Bismut and colleagues (2001) reported that in man a combination of five or six biochemical markers can be highly predictive in the diagnosis of liver fibrosis. Consequently, it

would be useful to identify a single liver specific marker of fibrosis, which is highly sensitive and could act as a sole measurement in the diagnosis of liver fibrosis. The measurement of serum markers requires the removal of blood hence making it an invasive method. The assessment of liver fibrosis in humans by liver biopsy does pose a risk to the patient. There is therefore a need for a good non-invasive marker that could be identified at an early stage in the process of fibrosis. At present, research is being carried out in an attempt to develop anti-fibrotic drugs and therefore a non-invasive marker would provide a rapid and easy method for measuring any response to such compounds (Friedman, 2003).

CCl_4 induces liver fibrosis following repeated administration and if dosing continues cirrhosis eventually develops (Luckey and Petersen, 2001). Repeated administration of CCl_4 causes focal necrosis and during these processes cell membranes are disrupted and broken down. As a result the liver will undergo regeneration to replace the damaged hepatocytes and this will result in the formation of nodules contained within fibrotic septa (Perez-Tamayo, 1983). The CCl_4 rat model is a widely used model to induce fibrosis/cirrhosis and can induce a form of cirrhosis that closely resembles that of alcoholic cirrhosis in humans both histologically and systemically (Proctor and Chatamra, 1982).

In the present studies it was therefore necessary to carry out a preliminary investigation to determine a dose level of CCl_4, which following repeated administration would induce fibrosis without causing mortality. The CCl_4 would be administered intragastrically since this route exposes the liver to a greater concentration of CCl_4 over a longer period of time. The CCl_4 enters the liver via the portal vein before leaving for the arterial blood (Proctor and Chatamra, 1982). Although liver fibrosis and cirrhosis were thought to be irreversible, evidence exists to suggest that they may be reversible (Arthur, 2002, Benyon and Iredale, 2000). Iredale et al. (1998) investigated the reversibility of liver fibrosis following CCl_4 treatment; they reported a noticeable breakdown in collagen in the liver and a return towards normal liver structure after a 4 week recovery period when rats received no treatment. The current study will also investigate a six week recovery period at the end of dosing, during which the rats will receive no treatment.

To establish that fibrosis has been induced by chronic CCl_4 administration it was necessary to carry out a histopathological assessment of the liver tissue. The measurement of serum enzymes and liver weights (as before) was useful as indicators of hepatic injury. As discussed previously, CCl_4 does not only induce damage in the liver, and other organs may be targeted, particularly the kidney, therefore during these studies it will be necessary to carry out a histopathological examination of the kidneys as a method of determining renal injury.

Once the model of fibrosis has been developed, urine from these rats was analysed (as described in Chapters 3 and 4) for the identification of urinary protein markers. Urine will be analysed by one-dimensional SDS-PAGE after 6 weeks of CCl_4 treatment (weeks 1 to 6) and also during the 6 week recovery period (weeks 7 to 12). During the present acute dose studies with CCl_4 (Chapters 3 and 4) Cu/Zn SOD was identified in the rat urine in response to hepatic injury. Therefore it was interesting to determine if SOD-1 appeared in rat urine following repeated (chronic) administration of CCl_4 and also whether SOD-1 could still be identified in the urine at the end of the six weeks recovery period. Therefore, urine samples from this chronic dose study were Western blotted with the SOD-1 antibody and SOD activity measurements were also be made.

5.2 Experiment 1: Study to determine a dose level of carbon tetrachloride which on repeated administration would induce liver fibrosis.

In the dose response study described in Chapter 3 rats were administered a single dose of CCl_4 and sacrificed 24 hours later. In this study hepatic injury was induced after a single dose of CCl_4 at 0.4 ml/kg. At dose levels above 1.2 ml/kg renal injury also occurred as determined by blood in the urine samples and histopathological examination of the kidneys. Therefore, it was thought that the repeated administration of CCl_4 at dose levels below 0.4 ml/kg would be sufficient to induce fibrosis, and would be below 1.2 ml/kg to avoid renal injury. A single dose of CCl_4 at 0.1 ml/kg induced hepatic injury in the form of centrilobular vacuolation as described in Chapter 4, therefore it was thought that dose levels in this range may induce fibrosis following twice weekly administration of CCl_4 for 6 weeks.

84 rats (mean body weight 213.7 g) were divided into 7 groups of 12 animals each. Rats were dosed by gavage, with vegetable oil (control) or CCl_4 dissolved in vegetable oil at 0.06, 0.12, 0.18, 0.24, 0.30 and 0.36 ml/kg. Rats were treated twice a week (on a Monday and Thursday) for 6 weeks with amounts of CCl_4 administered corrected for changes in body weight once a week. Throughout the experiment, animals were housed in communal cages and had free access to diet and water *ad libitum*; they were weighed weekly. At the end of the six-week period of dosing (weeks 1 to 6) 6 rats from each treatment group were killed and samples taken, while the remaining 6 rats per group were left untreated in communal cages for a further six week recovery period (weeks 7 to 12). This experimental design allowed the reversibility of liver changes to be studied. At the post mortem examination at week 6 and 12, kidneys and livers were weighed and sections placed in fixative; blood was taken for serum preparation. No urine samples were collected during this experiment.

5.3 Results for experiment 1

5.3.1 Observations during the study

Throughout the 6 week period of CCl_4 dosing all the animals appeared healthy and there were no clinical signs of toxicity. Body weights were recorded each week throughout the dosing period (weeks 1 to 6) and also during the recovery period (weeks 7 to 12). During the six weeks of dosing, the animals in CCl_4-treated groups did not gain as much weight as the control rats (Figure 5.1). At the end of six weeks dosing period, control rats had increased in body weight by 11.24 %, whereas animals in the 0.18 ml/kg CCl_4 group increased in weight by 2.35 %, and at 0.36 ml/kg the rats increased in body weight by 1.33 %.

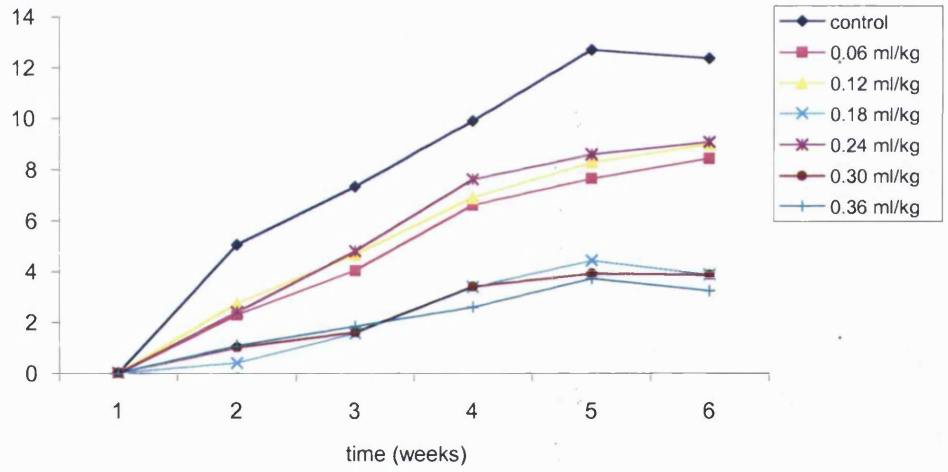


Figure 5.1 Percentage body weight gain for rats treated with a range of CCl_4 dose levels twice a week for six weeks and sampled at six weeks. Body weights were recorded each week of the study. Values are means of six rats per dose group.

5.3.2 Liver weights

At the six-week post mortem, the livers from the CCl_4-treated rats appeared to be paler than those of controls and slightly enlarged, and this observation was more apparent in the higher dose groups (i.e. 0.24, 0.30 and 0.36 ml/kg). The livers taken from CCl_4-treated rats were significantly heavier than the control liver weights, when expressed

both as absolute and relative liver weight. Figure 5.2A shows the mean relative liver weight for rats killed at the end of the six-week dosing period. A dose response relationship exists, with the highest dose group (0.36 ml/kg) having the heaviest liver weight. This increase in liver weight indicates that liver injury has occurred and a dose response relationship exists.

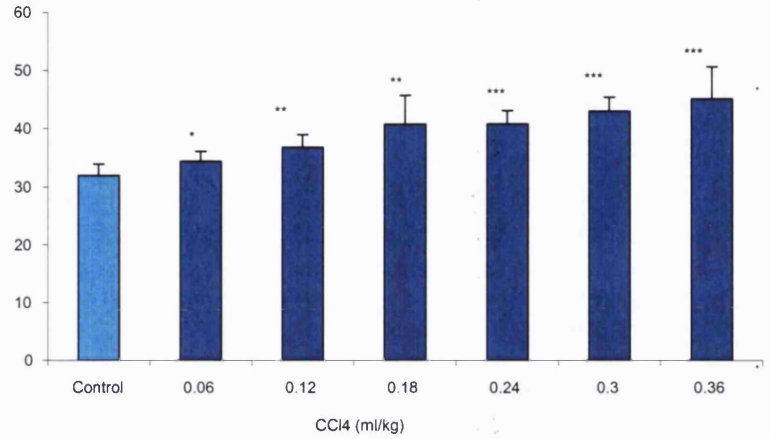
In the group of rats that were dosed for six weeks and then left until week 12 to recover, the livers were removed at the 12-week post mortem. In this group, most of the liver weights from CCl_4-treated animals were slightly heavier than the controls (Figure 5.2B) however, the only statistically significant differences were in the 0.18 and 0.24 ml/kg groups ($P < 0.05$).

5.3.3 Kidney weights

At the post mortem at week 6, the relative kidney weights of the CCl_4-treated rats were no different to control kidney weights (Figure 5.3A). These results indicate that nephrotoxicity has not occurred.

In the group of rats treated with CCl_4 for six weeks and then allowed to recover for six weeks, the relative kidneys weights are lighter than control kidney weights (Figure 5.3B). However, the only statistical difference between control and CCl_4-treated kidney weights is in the 0.18 ml/kg group ($P < 0.05$).

A.



B

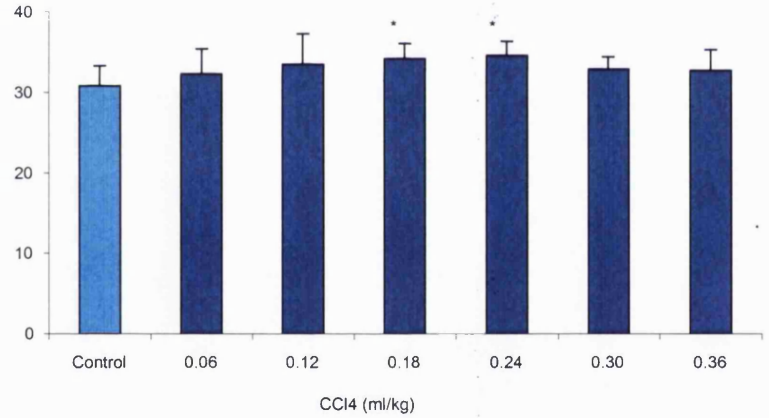
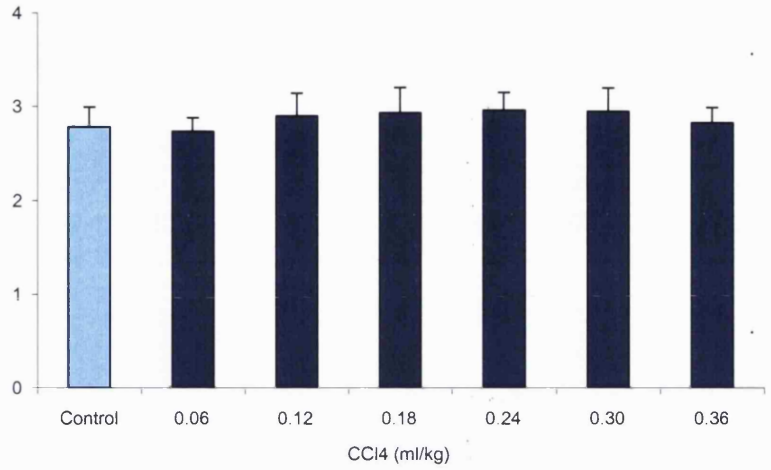


Figure 5.2 Relative liver weights for rats treated with a range of CCl_4 dose levels twice a week for six weeks and sampled at six weeks (A), and relative liver weights for rats treated with CCl_4 twice a week for six weeks followed by a six week recovery period (B). Values are means of six animals per dose group (error bars represent S.D.). Values that differ from control by Student's t-test are shown, * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

A.



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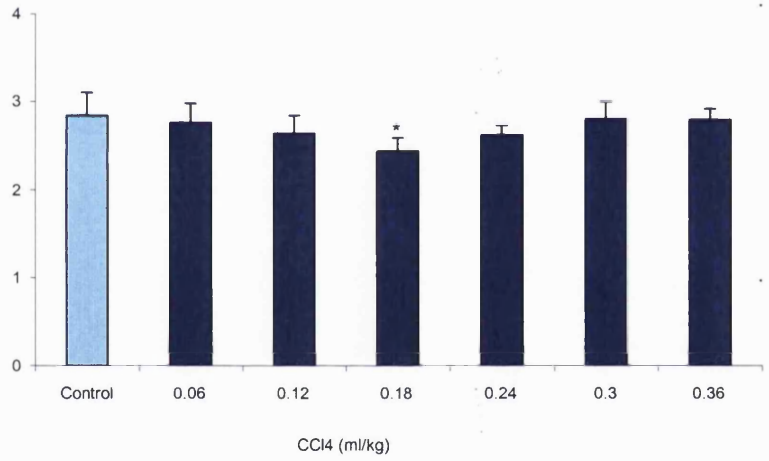


Figure 5.3 Relative kidney weights for rats treated with a range of CCl_4 dose levels twice a week for six weeks and sampled at six weeks (A), and relative kidney weights for rats treated with CCl_4 twice a week for six weeks followed by a six week recovery period (B). The kidney weight was expressed as a mean of both the right and left kidneys from each rat and the relative weight calculated. Values are means of six animals per dose group (error bars represent S.D.). Values that differ from control by Student's t-test are shown, * $P < 0.05$.

5.3.4 Serum clinical biochemistry

At the six week post mortem, serum ALT, AST and GLDH were increased in all the CCl_4-treated rats and this increase was greater in the higher dose groups (Figure 5.4). At 0.06 ml/kg the only significant increase was for ALT ($P < 0.001$) but all three enzymes were greater than control. At 0.12 ml/kg both ALT and GLDH were significantly greater than control. There is a very large increase in the mean activity of all three enzymes in the 0.18 ml/kg group, but this level slightly falls in both 0.24 and 0.30 ml/kg groups. The lower mean activity of ALT, AST and GLDH in the 0.24 and 0.30 ml/kg groups is a result of one or two animals at these dose levels having much lower activities of the enzymes that the rest of the group. Therefore, these results show that, even at the lowest dose of CCl_4 used (0.06 ml/kg) significant hepatic injury was induced by the repeated administration of the compound.

Other serum parameters that were increased in the serum as a result of treating rats with CCl_4 twice a week for six weeks were alkaline phosphatase (ALP), bile acids and total bilirubin. ALP activity was increased at the lowest dose level of CCl_4 administered ($P < 0.05$) and the activity increased with increasing dose. At the highest dose, ALP activity was approximately five times greater in the CCl_4-treated group than in the controls (Figure 5.5A). Figure 5.5B shows that serum albumin was slightly decreased in the rats treated with CCl_4 at all dose levels when compared to control levels. However, the decrease was only statistically significant for animals treated at 0.30 and 0.36 ml/kg ($P < 0.05$). The total bilirubin (Figure 5.5C) was increased in all CCl_4-treated rats but at the lowest dose (0.06 ml/kg) this increase was not statistically significant. The mean bilirubin level in rats dosed at 0.36 ml/kg was 2.4 times greater than control (Figure 5.5C).

Blood was also taken for serum preparation at the twelve week post mortem, at the end of the 6 week recovery period. Serum enzyme analysis revealed that in general the levels of ALT, AST and GLDH in rats, which had been treated with CCl_4, were similar to control levels (Figure 5.6). But AST levels were significantly lower than control in the 0.24, 0.30 and 0.36 ml/kg groups.

Following the six-week recovery period, ALP activity in serum was similar in CCl_4-treated rats as in control for most groups (Figure 5.7A). The activity was slightly decreased in the 0.18, 0.30 and 0.36 ml/kg treated groups but these differences are not statistically significant (Figure 5.7A). Figure 5.7B shows that at 12 weeks, albumin levels are still slightly lower in the CCl_4-treated rats than in control but again, there are no statistically significant differences between control and CCl_4-treated groups. The amount of bilirubin in the serum from rats treated with CCl_4 is similar to control following six weeks of no treatment (Figure 5.7C).

Urea and creatinine levels in CCl_4-treated rats were not statistically significantly different from the controls at the six-week post mortem (Table 5.1). After 12 weeks the urea and creatinine levels in the serum were similar to control at all dose levels apart from a decrease in creatinine levels in the 0.36 ml/kg group (Table 5.2).

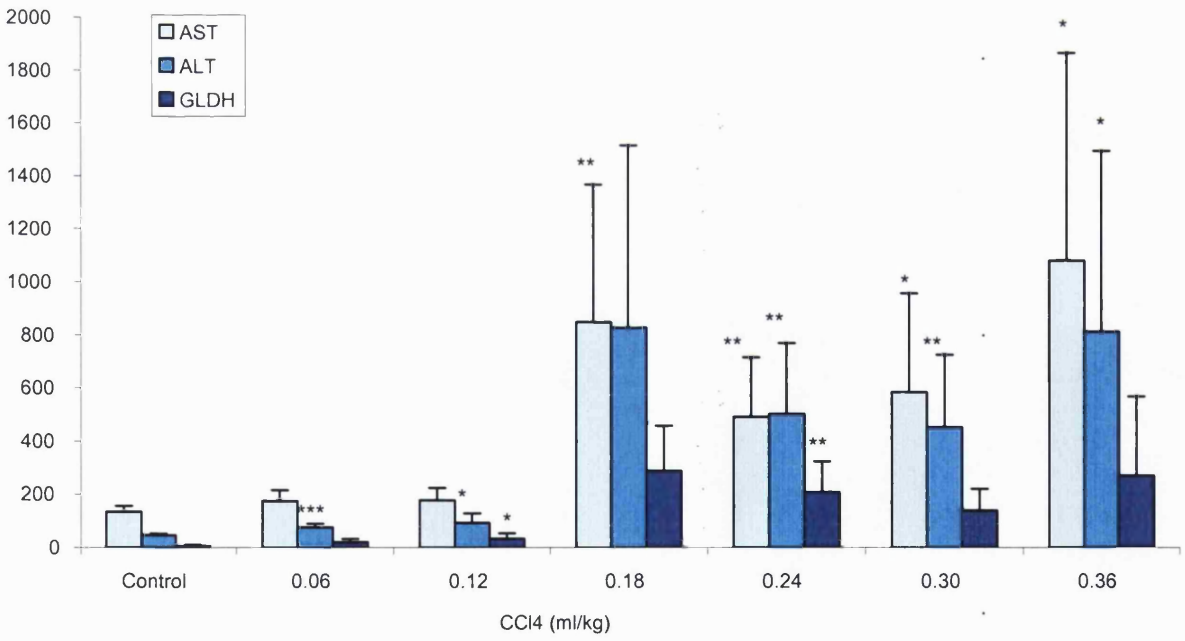


Figure 5.4 Mean ALT, AST and GLDH levels for rats treated with CCl_4 at a range of dose levels twice a week for six weeks, and sampled at six weeks. Enzyme assays were carried according to methods described in Section 2.5. Values are the means of 6 animals per dose level group (error bars represent S.D.). Values that differ from control by Student's t-test are shown, * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

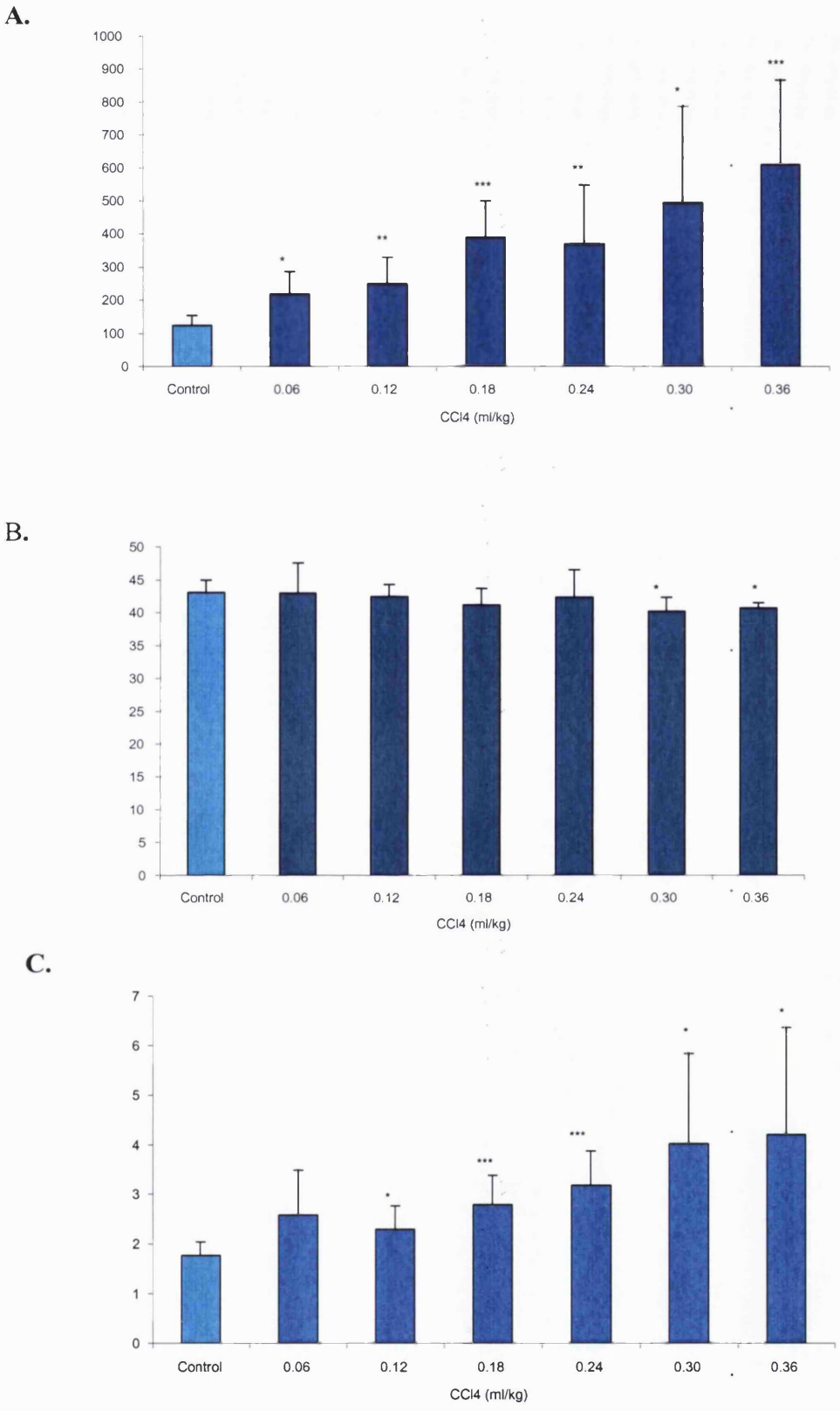


Figure 5.5 Mean ALP (A), albumin (B) and bilirubin (C) levels measured in serum from rats treated with CCl_4 at a range of dose levels twice a week for six weeks, and sampled at six weeks. Enzyme activities were measured according to the methods described in Section 2.5. Values are means of 6 animals (error bars represent S.D). Values that differ from control by Student's t-test are shown, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

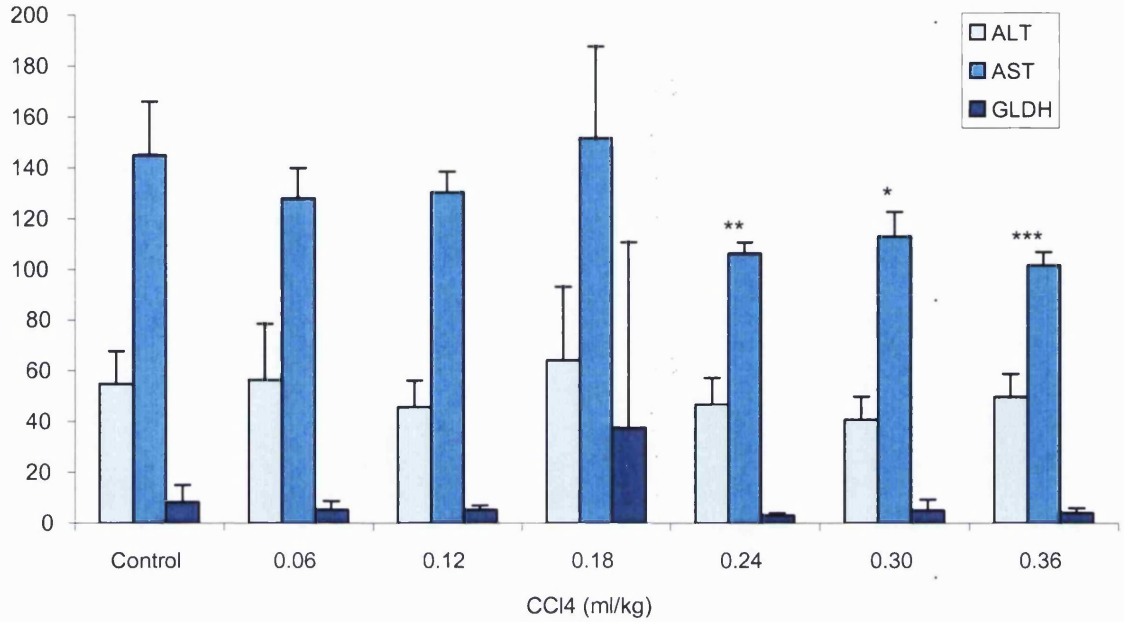
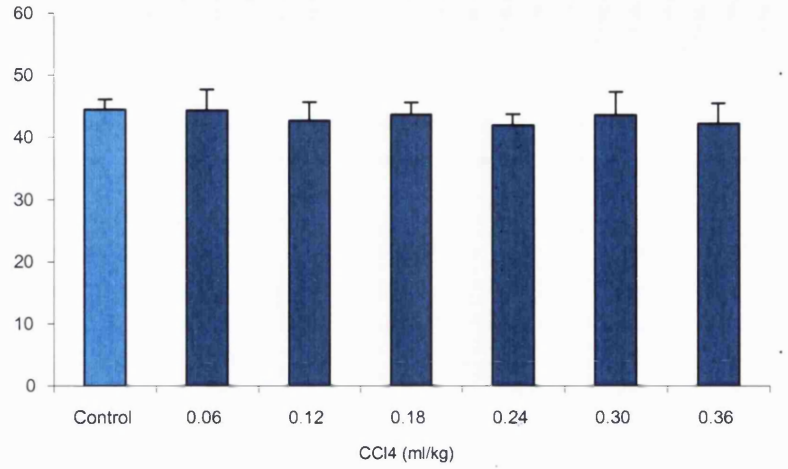


Figure 5.6 Mean ALT, AST and GLDH levels for rats treated with CCl_4 at a range of dose levels twice a week for six weeks, and left untreated for six weeks and sampled at the end of the six week recovery period. Enzyme assays were carried out according to methods described in Section 2.5. Values are the means of 6 animals per dose level group (error bars represent S.D.). Values that differ from control by Student's t-test are shown, * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

A.



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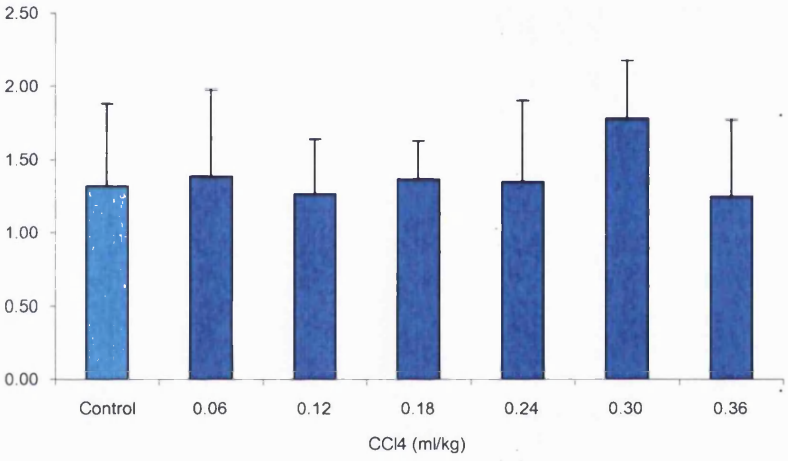

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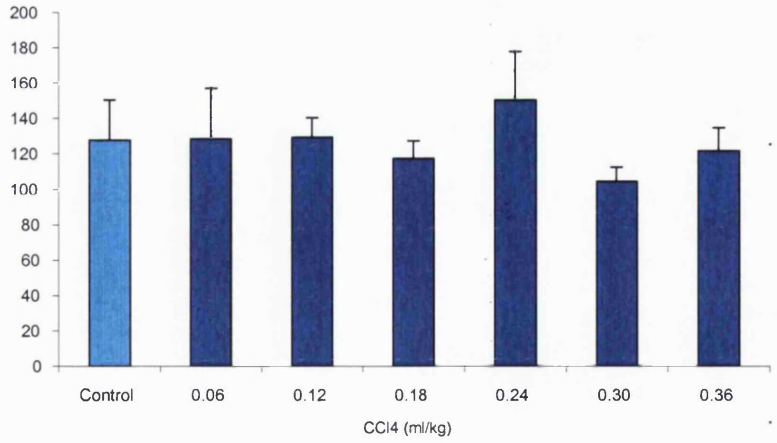


Figure 5.7 Mean ALP (A), albumin (B) and bilirubin (C) levels measured in serum from rats treated with CCl_4 at a range of dose levels twice a week for six weeks, and left untreated for six weeks and sampled at the end of the six week recovery period. Enzyme activities were measured according to the methods described in Section 2.5. Values are means of 6 animals (error bars represent S.D). Values that differ from control by Student's t-test are shown, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

Table 5.1 Mean urea and creatinine levels measured in serum from rats treated with CCl_4 at a range of dose levels twice a week for six weeks, and sampled at six weeks[a]. Assays were carried out according to the methods described in Section 2.5.

CCl4	Urea (mmol/L)		Creatinine (umol/L)	
ml/kg	Mean	S.D.	Mean	S.D.
0 (control)	7.65	0.68	58.83	4.58
0.06	9.10	1.48	57.00	9.12
0.12	8.70	0.97	56.83	4.36
0.18	8.39	0.7	58.83	5.53
0.24	8.19	0.97	57.33	5.01
0.30	7.40	1.09	55.83	3.37
0.36	8.42	0.53	60.00	6.10

[a]Values are means of five animals (and S.D.).

Table 5.2 Mean urea and creatinine levels measured in serum from rats treated with CCl_4 at a range of dose levels twice a week for six weeks, and left untreated for six weeks and sampled at the end of the six week recovery period[a]. Assays were carried out according to the methods described in Section 2.5.

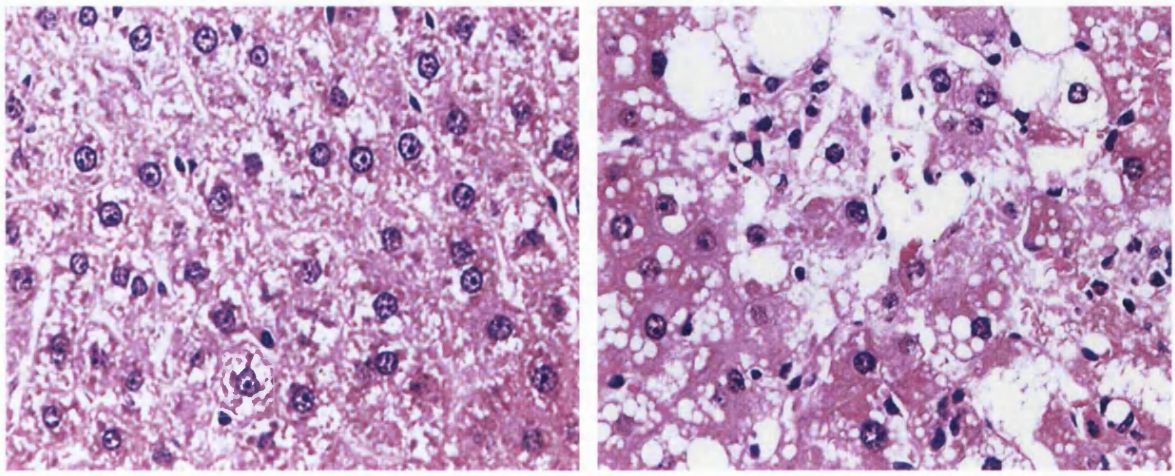
CCl4	Urea (mmol/L)		Creatinine (umol/L)	
ml/kg	Mean	S.D.	Mean	S.D.
0 (control)	7.70	0.70	60.17	2.14
0.06	7.07	0.75	57.50	6.22
0.12	7.48	0.83	56.83	4.36
0.18	7.37	0.36	60.67	8.59
0.24	8.15	1.12	53.78	10.46
0.30	7.45	1.13	59.20	4.32
0.36	7.20	0.85	54.17**	3.66

[a]Values are means of five animals (and S.D.). Values that differ from control by Student's t-test are shown, ** $P < 0.01$.

5.3.5 Histopathology

At both the 6 week and 12 week post mortem, livers and kidneys were removed and placed in formalin for histopathological examination. After six weeks of treatment with CCl_4 the livers of treated rats showed related hepatocellular degeneration and necrosis (Figure 5.8). Cells in the centrilobular region showed cytoplasmic swelling and vacuolation, cytoplasmic and nuclear degeneration, and necrosis. Inflammatory cells were sometimes present. Around the centrilobular veins and periportal regions, early and mature collagen was present, which extended into the parenchyma along sinusoidal tracks. The fibrosis present in the CCl_4-treated livers showed considerable variation between CCl_4 dose groups, and between individuals within each group. There was a trend for the degree of fibrosis to increase with increasing CCl_4 dose level. There were no CCl_4-treatment related findings in the kidneys at the 6 week autopsy or at the end of the period of recovery. Liver sections were chosen at random for Sirius Red staining. This revealed the presence of collagen fibres in some of the liver sections. The fibrosis was often perivascular in distribution and sometimes included delicate "bridging" between the centrilobular and periportal areas. In some sections, the capsular region was also involved. Following the six weeks recovery period the livers had returned to normal. No clear or consistent evidence of fibrosis was evident on Sirius Red staining.

A. **B.**



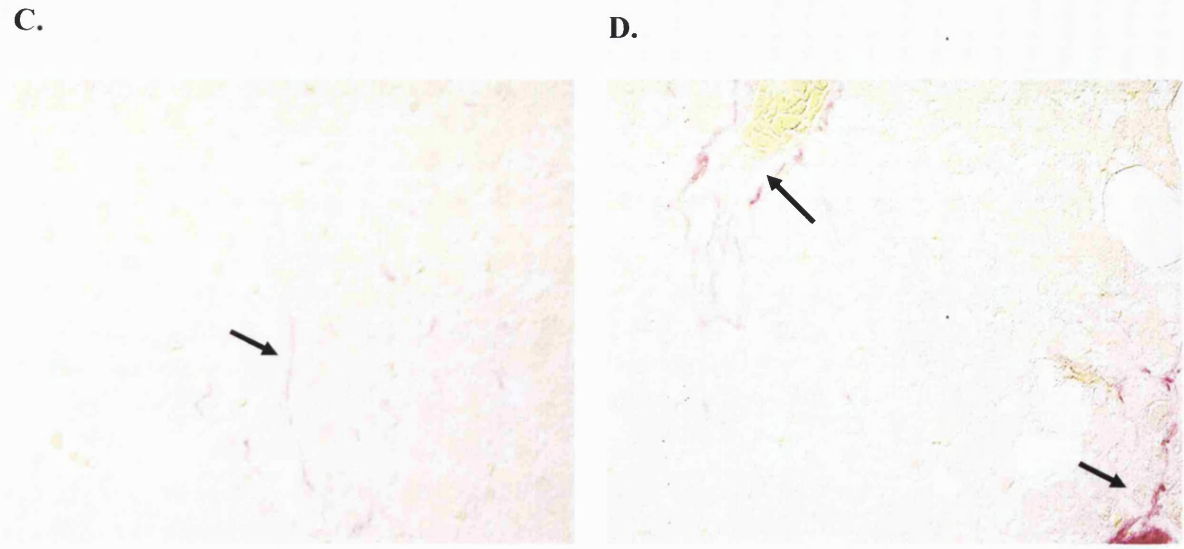


Figure 5.8 Histology of the liver from rats treated with CCl_4 at increasing dose levels twice a week for six weeks and sampled at six weeks.

A. Liver from a control rat to show the normal arrangement and appearance of hepatocytes. Original magnification of image, x 400 (H&E).

B. Centrilobular region in the liver of a rat treated with CCl_4 at 0.36 ml/kg twice weekly for 6 weeks showing diffuse marked hepatocellular vacuolation and degeneration with necrotic cells. Original magnification of image, x 400 (H&E).

C. Liver from a rat treated with CCl_4 at 0.18 ml/kg twice weekly for 6 weeks; the very slight presence of (arrow) delicate fibrillar strands of collagen is evident, scattered in the parenchymal tissue. Original magnification of image, x 100 (Sirius Red).

D. Liver from a rat treated with CCl_4 at 0.24 ml/kg twice weekly for 6 weeks; slight perivascular fibrosis is seen with some evidence of "bridging" between the centrilobular and periportal areas (arrows). Original magnification of image, x 100 (Sirius Red).

The histological assessment for Experiment 1 shows that liver fibrosis was slight and there was a lot of intra-animal variation even within the same dose group. However there was a trend for the degree of fibrosis to increase with increasing CCl_4 dose level. Therefore, a second study was carried out but CCl_4 was administered at higher dose levels than in Experiment 1.

5.4 Experiment 2: Generating a rat model of liver fibrosis

Experiment 1 showed that, using the method described in Section 5.2, fibrosis was generated but there was considerable variation in the degree of fibrosis induced at the dose levels administered. Therefore, in the second study the same method was used but at higher dose levels.

96 rats were divided into 8 groups of 12 animals each. Rats were dosed by gavage, with oil (control) or CCl_4 at 0.4, 0.5, 0.6, 0.7, 0.8, 0.9 and 1.0 ml/kg. The rats were treated twice a week (on a Monday and Thursday) for 6 weeks with the level of CCl_4 administered corrected for changes in body weight once a week. Throughout the experiment, animals were housed in communal cages and had free access to diet and water *ad libitum*; they were weighed weekly. At the end of the six-week period of dosing (weeks 1 to 6) 6 rats from each treatment group were killed, while the remaining 6 rats per group were left untreated in communal cages for a further six week recovery period (weeks 7 to 12). This experimental design allowed the reversibility of the liver changes to be studied.

After three weeks of CCl_4 dosing, six rats from the control, six from the 0.7 and six from the 1.0 ml/kg groups were placed in metabolism cages and urine collected for 24 hours as described in Section 2.2. After 24 hours the rats were returned to the communal cages. At the end of the dosing period, at 6 weeks, the same rats were placed in metabolism cages and urine collected again for 24 hours. Urine samples were also collected at the end of the six week recovery period. Urine samples were stored at –80 ° C until later analysis.

5.5 Results for experiment 2

5.5.1 Observations during the study

Throughout the 6 week period of CCl_4 dosing all the animals appeared healthy and there were no clinical signs of toxicity. Body weights were recorded each week throughout the dosing period (weeks 1 to 6) and also during the recovery period (weeks 7 to 12). During the six weeks of dosing, the animals in CCl_4 treated groups did not gain as much weight as the control rats (Figure 5.9). At the end of six weeks dosing period, control rats had increased in body weight by 27.34 %, whereas in the 1.0ml/kg CCl_4 group the rats increased in body weight by 7.21 %.

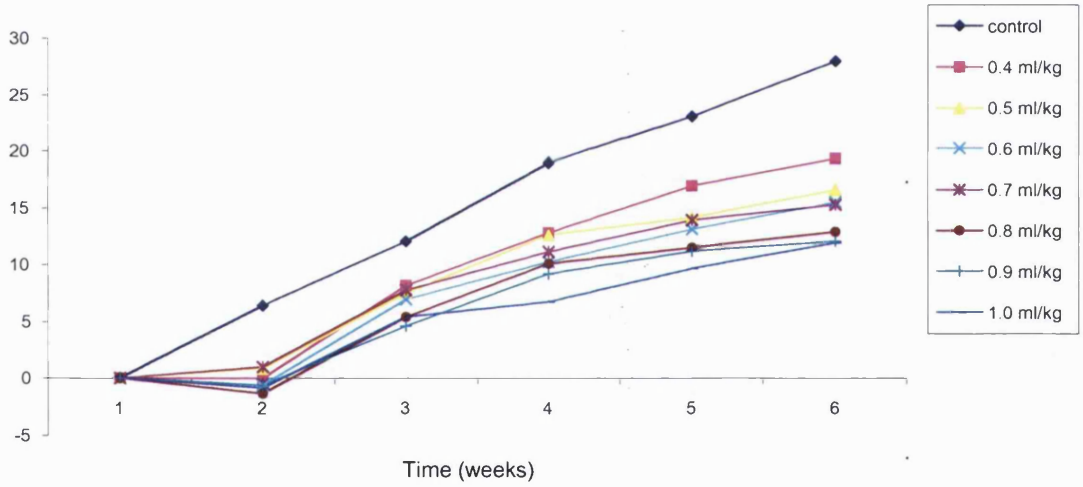


Figure 5.9 Percentage body weight gain for rats treated with a range of CCl_4 dose levels twice a week for six weeks and sampled at six weeks. Body weights were recorded each week of the study. Values are means of six rats per dose group.

5.5.2 Liver weights

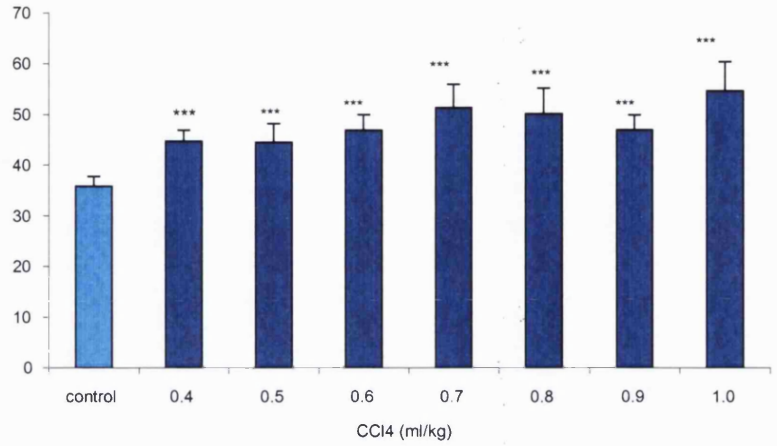
At the post mortem mean liver weights (both absolute and relative) for rats treated with CCl_4 twice a week for six weeks were significantly heavier than the control livers at all the CCl_4 dose levels used ($P < 0.001$) (Figure 5.10A). At the lowest dose used (0.4 ml/kg) the relative liver weights from treated rats were 24.3 % heavier than control. There was a 52.3 % increase in relative liver weights in the rats treated with 1.0 ml/kg CCl_4 compared to control.

After the 6 week recovery period, at 12 weeks post-dosing, the relative liver weights for CCl_4-treated rats were still a little heavier than control (Figure 5.10B) but the only statistically significant differences were in the 0.7 and 0.8 ml/kg groups. The mean relative liver weight for the 0.7 ml/kg group was 12.76 % heavier than control whereas there was a 15.31 % increase in relative liver weight for rats treated with CCl_4 at 0.8 ml/kg

5.5.3 Kidney weights

The relative kidney weights from CCl_4-treated rats at the six week post mortem were slightly but significantly heavier than control at all dose levels used (Figure 5.11A). But at the 12 week post mortem after 6 weeks of recovery there were no statistically significant differences between kidney weights of control and CCl_4-treated rats (Figure 5.11B). The increase in kidney weights in CCl_4-treated rats after 6 weeks of dosing may be an indicator of nephrotoxicity but histological examination of the kidneys is required to confirm this.

A.



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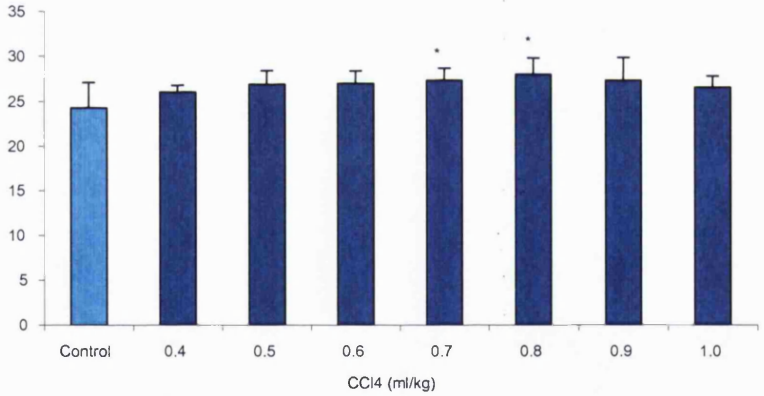
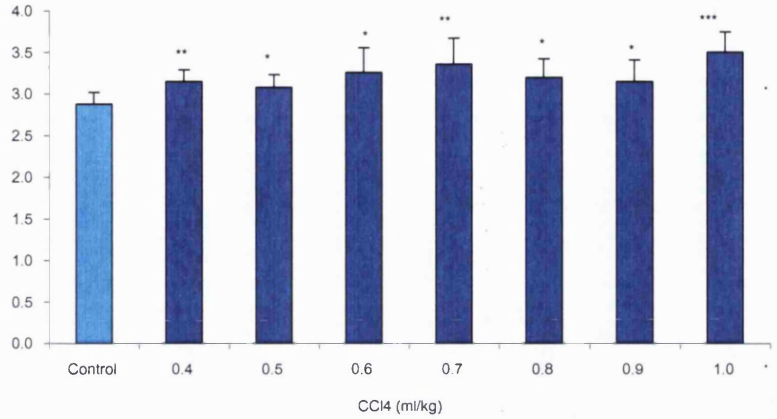


Figure 5.10 Relative liver weights for rats treated with a range of CCl_4 dose levels twice a week for six weeks and sampled at six weeks (A), and relative liver weights for rats treated with CCl_4 twice a week for six weeks followed by a six week recovery period (B). Values are means of six animals per dose group (error bars represent S.D.). Values that differ from control by Student's t-test are shown, * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

A.



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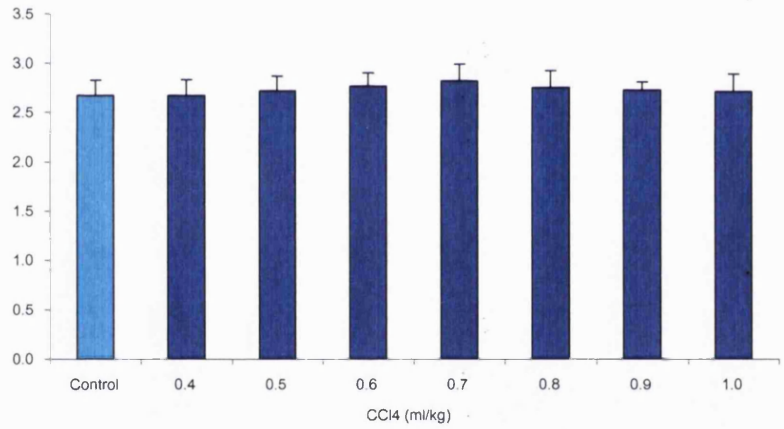


Figure 5.11 Relative kidney weights for rats treated with a range of CCl_4 dose levels twice a week for six weeks and sampled at six weeks (A), and relative kidney weights for rats treated with CCl_4 twice a week for six weeks followed by a six week recovery period (B). The kidney weight was expressed as a mean of both the right and left kidneys from each rat and the relative weight calculated. Values are means of six animals per dose group (error bars represent S.D.). Values that differ from control by Student's t-test are shown, * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

5.5.4 Serum analysis

After six weeks of dosing serum ALT, AST and GLDH activities were increased in all CCl_4-treated rats compared to the controls (Figure 5.12). The mean ALT activity in control rats was 39.5 U/L and this increased 5.7-fold in the 0.4 ml/kg group to 225.7 U/L. The activity then increased as the dose increased until at 1.0 ml/kg it had risen 39.1-fold to 1544.3 U/L. AST activity was significantly increased in all CCl_4-treated groups apart from 0.7 ml/kg. The mean AST activity in control rats was 123.2 U/L and this increased 2.6-fold in the 0.4 ml/kg group to 325.5 U/L. The activity then increased as the dose increased until at 1.0 ml/kg it had risen 15.5-fold to 1912.33 U/L. The mean control GLDH activity was 4.63 U/L and this increased 23.9-fold in the 0.4 ml/kg to 110.65 U/L. The activity then increased as the dose increased until at 1.0 ml/kg it had risen 156-fold to 722.3 U/L.

In serum samples taken at the 6 week autopsy ALP activity was increased in all CCl_4-treated groups (Figure 5.13A). At the lowest dose administered the ALP activity was 1.5 times greater than control ($P < 0.05$). ALP activity tended to increase in the serum as the CCl_4 dose increased, the value for the 0.7 ml/kg group was very high due to one animal having an abnormally high ALP activity (Figure 5.13A). At 1.0 ml/kg CCl_4 ALP activity was 2.72-fold greater than control. The albumin measured in the serum from rats treated with CCl_4 was decreased in all treatment groups compared to control values. Figure 5.13B shows that this decrease was significant at 0.5, 0.7, 0.8 and 1.0 ml/kg CCl_4. Bilirubin and bile acids were also increased in all CCl_4-treated rats when compared to control levels (Figures 5.13C and 5.13D, respectively). The increase was significant at all dose levels for bilirubin apart from the 0.7 and 0.8 ml/kg groups where animal to animal variation was much higher. The bile acid measurement in the serum revealed that at the lowest dose administered the value was significantly higher ($P < 0.001$) in the CCl_4-treated rats than control. At the highest CCl_4 dose level the mean bile acid value in the rat serum was 3.8 times greater in the CCl_4-treated rats than in controls (Figure 5.13D).

At six weeks the serum urea levels were significantly increased in all CCl_4-treated groups but the level did not appear to increase with increasing dose of CCl_4 (Table 5.3).

The creatinine levels were significantly decreased in rats treated with CCl_4 at 0.6, 0.7, 0.8 and 1.0 ml/kg.

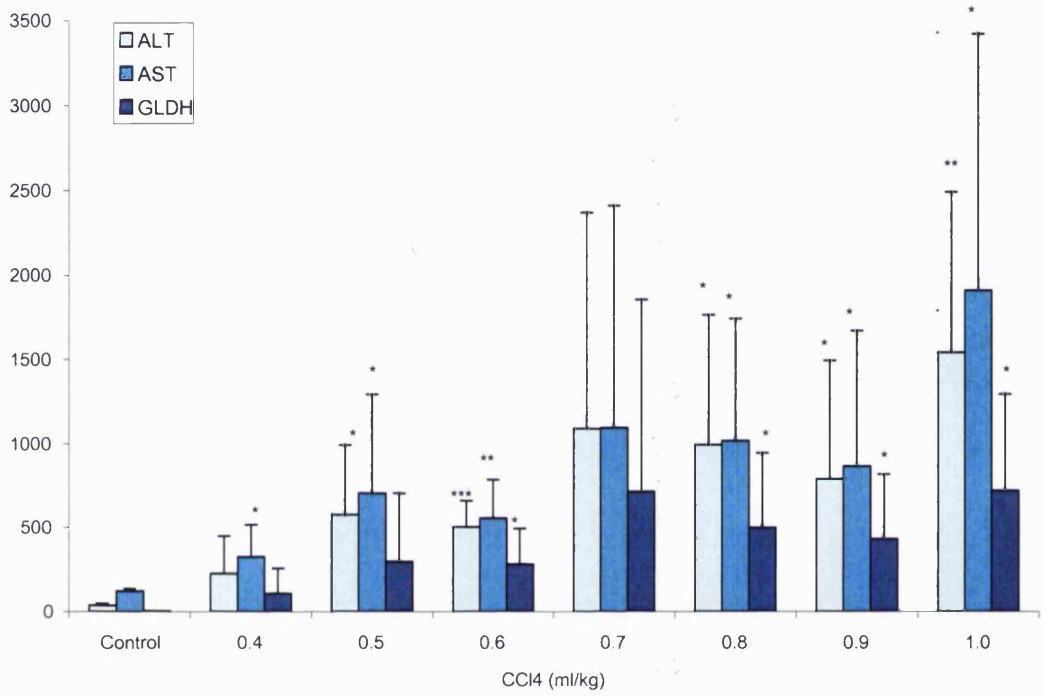


Figure 5.12 Mean ALT, AST and GLDH levels for rats treated with CCl_4 at a range of dose levels twice a week for six weeks, and sampled at six weeks. Enzyme assays were performed as described in Section 2.5. Values are the means of 6 animals per dose level group (error bars represent S.D.). Values that differ from control by Student's t-test are shown, * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

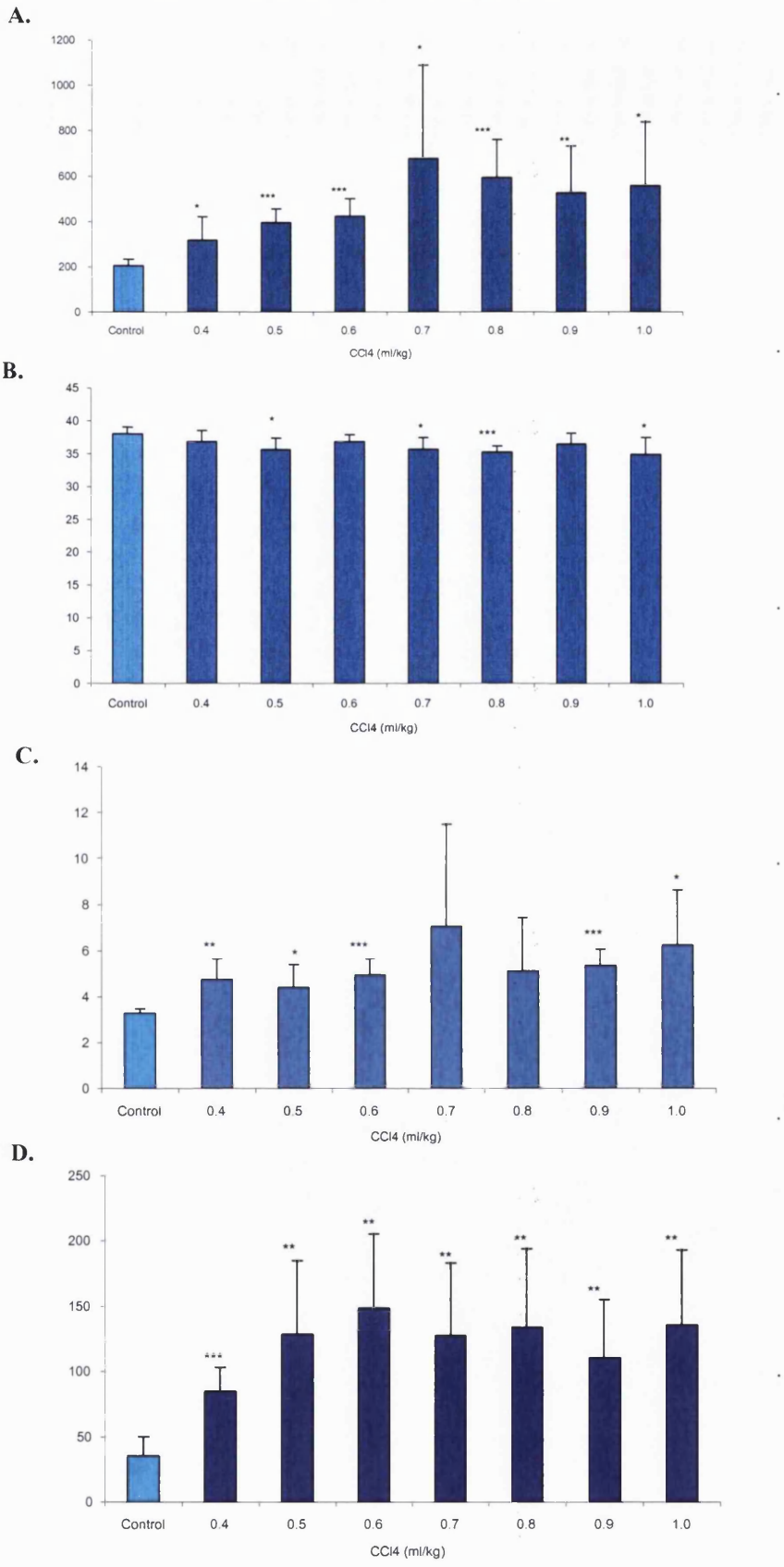


Figure 5.13 Mean ALP (A), albumin (B), bilirubin (C) and bile acid (D) levels measured in serum from rats treated with CCl_4 at a range of dose levels twice a week for six weeks, and sampled at six weeks. Enzyme activities were measured according to the methods described in Section 2.5. Values are means of 6 animals (error bars represent S.D). Values that differ by control by Student's t-test are shown, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

Table 5.3 Mean urea and creatinine levels measured in serum from rats treated with CCl_4 at a range of dose levels twice a week for six weeks, and sampled at six weeks[a]. Assays were carried out according to the methods described in Section 2.5.

CCl4	Urea (mmol/L)		Creatinine (umol/L)	
(ml/kg)	Mean	S.D.	Mean	S.D.
0 (control)	7.43	0.37	57.83	2.71
0.4	9.63**	1.17	56.67	1.97
0.5	9.79***	0.91	56.50	3.94
0.6	9.84***	0.80	54.50*	0.89
0.7	9.42**	1.08	51.17*	4.45
0.8	9.24*	1.40	52.17*	3.54
0.9	9.19**	1.17	56.17	3.43
1.0	9.15***	0.78	51.67*	5.13

[a]Values are means of five animals (and S.D.). Values that differ from control by Student's t-test are shown, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

Serum analysis at 12 weeks (i.e. after the 6 week recovery period) revealed that ALT and GLDH activities were similar to the controls at all dose levels (Figure 5.14). However, rats that received doses of 0.7 ml/kg CCl_4 or higher twice a week for 6 weeks showed a significantly lower serum AST activity after a subsequent 6 week period of recovery (Figure 5.14). Following the 6 week recovery period serum ALP, albumin and bilirubin levels returned to control values, and there were no real differences between control and CCl_4-treated animals (Figure 5.15). Serum bile acids levels after the 6 week recovery period are highly variable within the dose groups and again show no difference between control and CCl_4-treated animals (Figure 5.15D).

At 12 weeks the urea levels for rats treated with CCl_4 were greater than control levels in the lower dose groups (0.4, 0.5 and 0.6 ml/kg). However, in the higher dose groups (0.7, 0.8, 0.9 and 1.0 ml/kg) urea levels were lower than control levels. There were no statistically significant differences between control and CCl_4-treated serum creatinine levels at 12 weeks (Table 5.4).

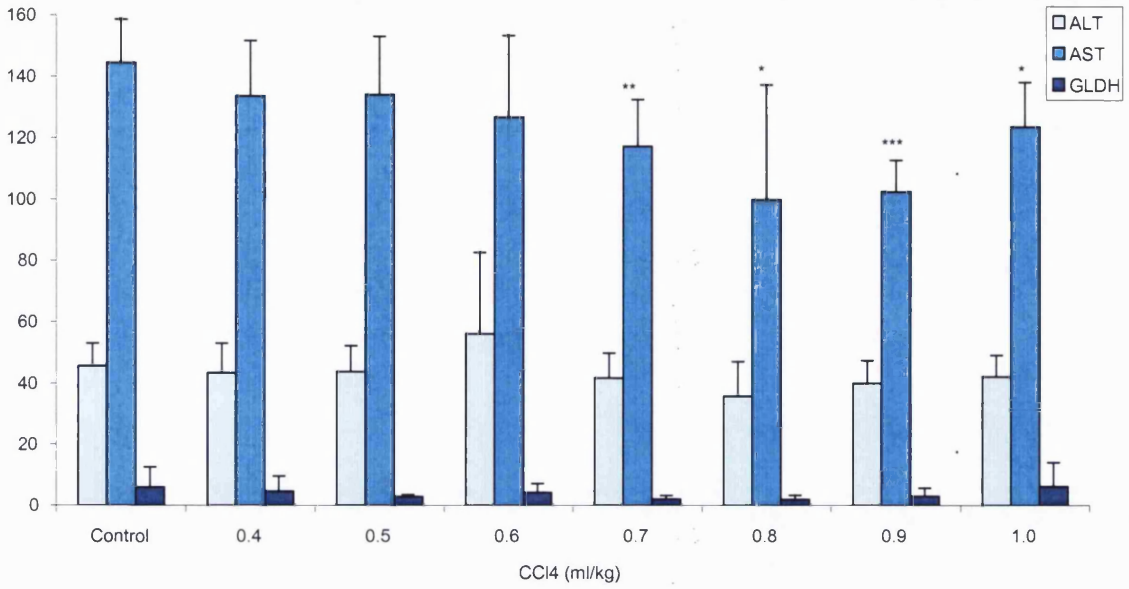


Figure 5.14 Mean ALT, AST and GLDH levels for rats treated with CCl_4 at a range of dose levels twice a week for six weeks, and left untreated for six weeks and sampled at the end of the six week recovery period. Enzyme assays were carried out according to methods described in Section 2.5. Values are the means of 6 animals per dose level group (error bars represent S.D.). Values that differ significantly from control by Student's t-test are shown, * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

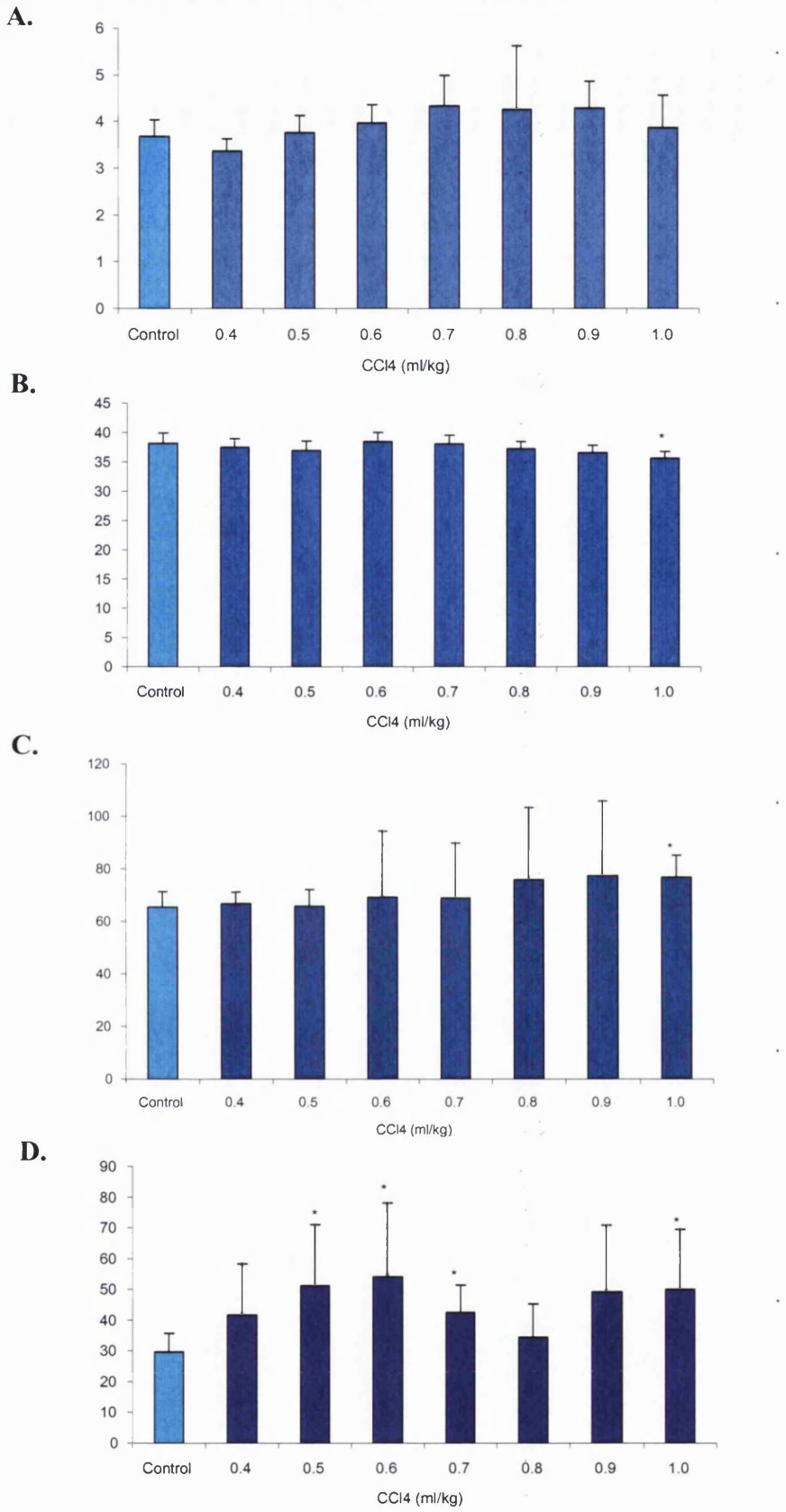


Figure 5.15 Mean ALP (A), albumin (B), bilirubin (C) and bile acid (D) levels measured in serum from rats treated with CCl_4 at a range of dose levels twice a week for six weeks, and left untreated for six weeks and sampled at the end of the six week recovery period. Enzyme activities were measured according to the methods described in Section 2.5. Values are means of 6 animals (error bars represent S.D). Values that differ from control by Student's t-test are shown, * $P < 0.05$.

Table 5.4 Mean urea and creatinine levels measured in serum from rats treated with CCl_4 at a range of dose levels twice a week for six weeks, and left untreated for six weeks and sampled at the end of the six week recovery period[a]. Assays were carried out according to the methods described in Section 2.5.

CCl4	Urea (mmol/L)		Creatinine (umol/L)	
(ml/kg)	Mean	S.D.	Mean	S.D.
0 (control)	7.48	0.36	57.50	13.17
0.4	8.17**	0.24	63.33	5.79
0.5	8.65	1.38	63.83	4.54
0.6	9.09**	1.13	64.50	3.27
0.7	7.20	1.36	60.67	5.43
0.8	6.80	1.37	55.67	11.47
0.9	6.93	0.87	56.00	3.74
1.0	6.40*	1.02	58.17	3.76

[a]Values are means of five animals (and S.D.). Values that differ significantly from control by Student's t-test are shown, * $P < 0.05$, ** $P < 0.01$.

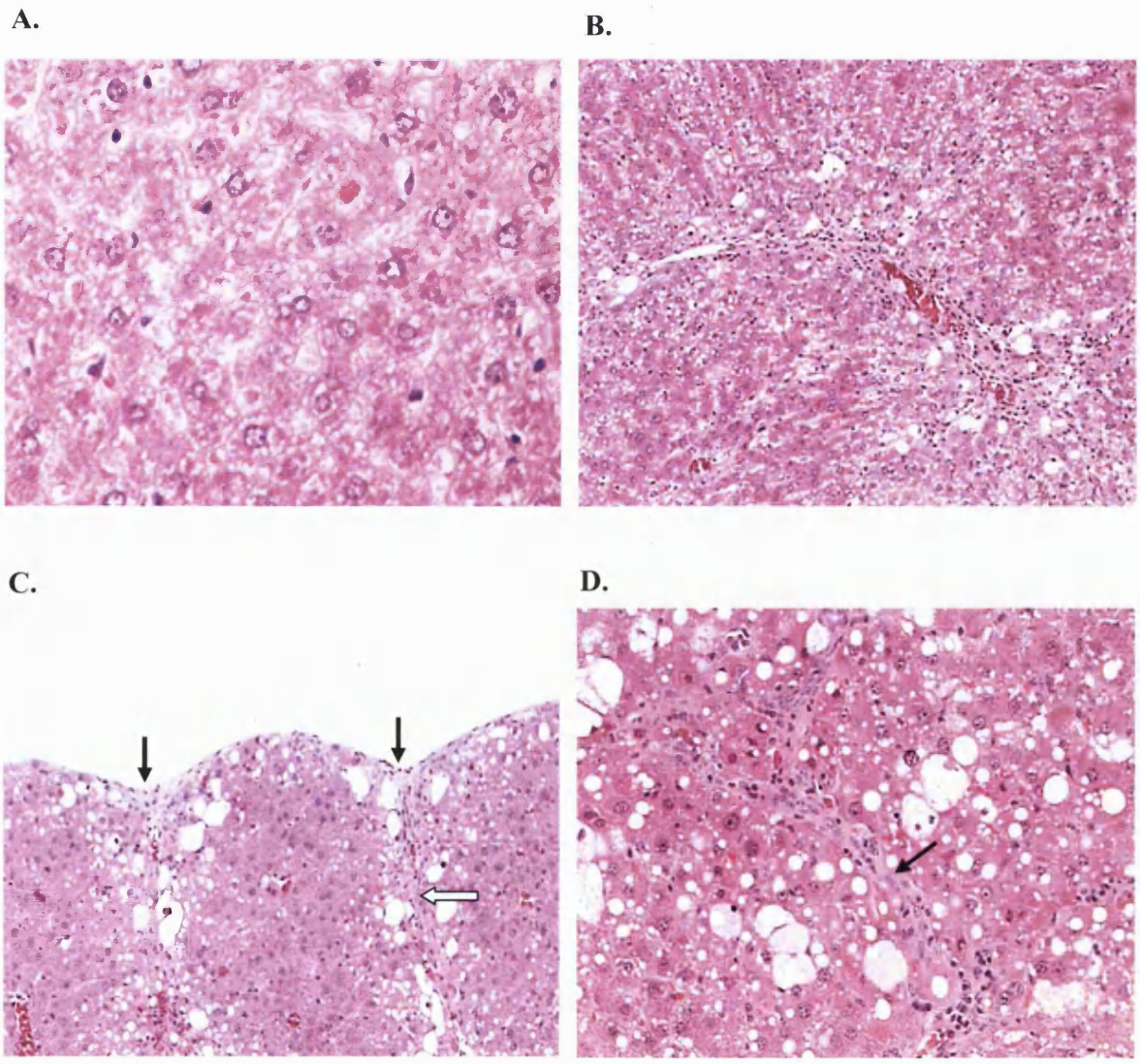
5.5.5 Histopathology of liver sections

At the 6 week and the 12 week post mortem livers were removed and placed in formalin for histopathological assessment. Livers and kidneys were stained with H&E and with Sirius Red (a special stain for collagen). H&E staining of liver tissue at 6 weeks revealed hepatocellular vacuolation in the centrilobular region and fibrosis with capsular pitting in the CCl_4-treated rat livers (Figure 5.16). Kidneys were also removed at post mortem and histopathological analysis carried out. H+E staining of kidney sections did not reveal any treatment-related changes.

In tissues taken at the end of the 6 week dosing period of randomly selected animals in all the CCl_4-treated groups Sirius Red staining of the liver sections revealed evidence of hepatic fibrosis in all randomly selected animals in all the CCl_4-treated groups (Figure 5.17). In all rats selected there was perivascular fibrosis and delicate "bridging" between centrilobular, periportal and capsular areas. There was no evidence of fibrosis in any of the kidney sections stained with Sirius Red.

At the 12 week post mortem, after the 6 week recovery period livers and kidneys were also removed for histopathological assessment. In these liver sections H&E staining detected fibrosis in some of the animals at 0.7 ml/kg CCl_4 and higher. In some animals mature collagen was present in subcapsular regions and was associated with capsular pitting or contraction. Some liver sections also had parenchymal fibrosis with fibrous tissue present around centrilobular veins, periportal regions, and extending into the parenchyma along sinusoidal tracks. There were no treatment-related changes in the kidneys with H+E staining.

Sirius Red staining of liver sections from rats treated with CCl_4 for six weeks followed by a six week recovery period showed evidence of fibrosis in all randomly selected animals, although there was intra-group variation in relation to the degree of fibrosis detected (Figure 5.18).



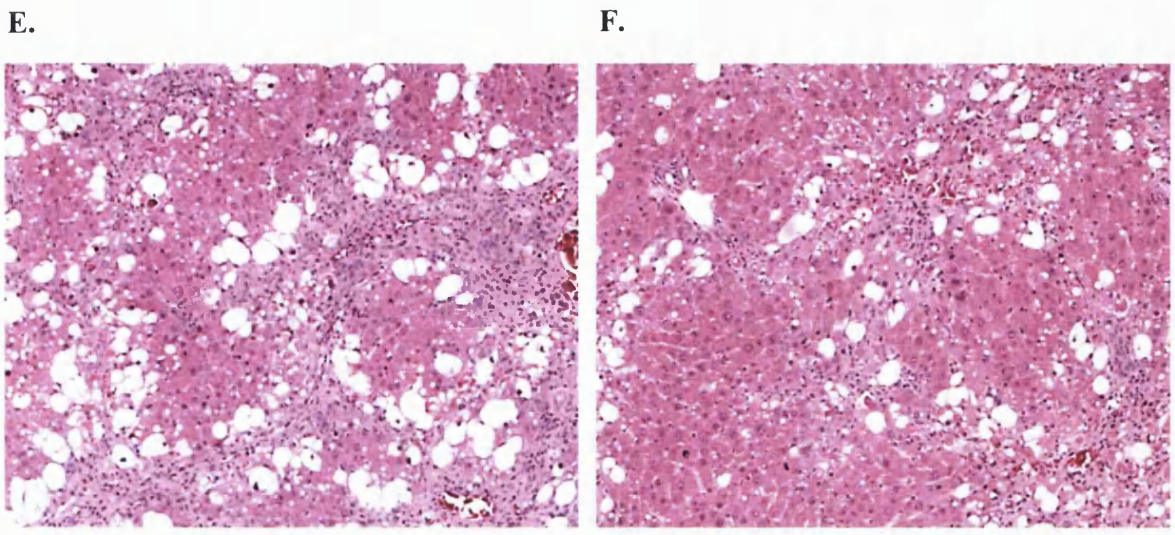


Figure 5.16 Histopathology of the liver sections from rats treated with increasing dose levels of CCl_4 twice a week for six weeks and sampled at six weeks.

A. Liver from a control rat to illustrate the normal appearance and arrangement of hepatocytes. Original magnification of image x 400; H&E.

B. Liver from a rat treated with CCl_4 at 0.4 ml/kg, twice weekly for 6 weeks; there is hepatocellular vacuolation in the centrilobular area with some necrotic and inflammatory cells. There is collagen formation around the centrilobular veins and periportal regions, and the collagen extends into the parenchyma along sinusoidal tracts. Original magnification of image x 100; H&E.

C. Liver from a rat treated with CCl_4 at 0.6 ml/kg twice weekly for 6 weeks. In the subcapsular region there is hepatocellular vacuolation, degeneration and necrosis, with capsular pitting or contraction (black arrows). There is collagen formation in the capsular area which extends downwards into the liver parenchyma (open arrow). Original magnification of image x 100; H&E.

D. Liver from a rat treated twice weekly for 6 weeks with CCl_4 at 0.8 ml/kg; there is hepatocyte vacuolation and degeneration with collagen formation extending through the parenchyma along sinusoidal tracts (arrow). Original magnification of image x 100; H&E.

E. Liver from a rat treated with CCl_4 twice weekly for 6 weeks at 0.9 ml/kg. Significant fibrosis is evident in a bridging formation linking centrilobular and periportal areas, and surrounding areas of hepatocyte vacuolation and degeneration. Original magnification of image x 100; H&E.

F. Liver from a rat treated with CCl_4 twice weekly for 6 weeks at 1.0 ml/kg. There is significant fibrosis which is tending to link centrilobular and periportal areas. Hepatocytes are vacuolated with evidence of degeneration. Original magnification of image x 100; H&E.

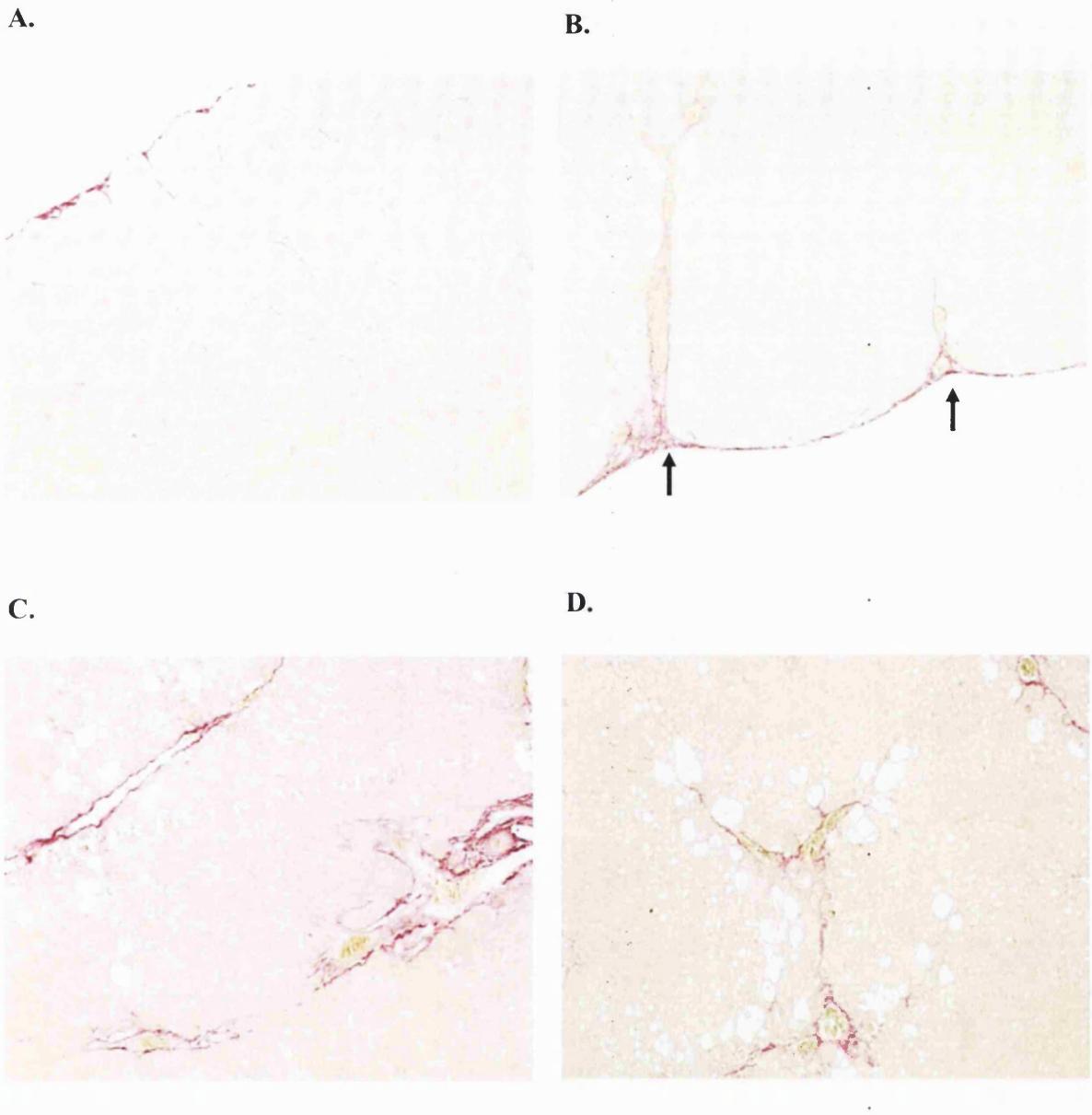


Figure 5.17 Histopathology of the liver from rats treated twice a week with CCl4 at varying dose levels and sampled at six weeks. Original magnification of all images, x 400; Sirius Red.

A. Control liver from a rat treated with oil (vehicle) twice weekly for 6 weeks to show the capsular distribution in normal animals.

B. Liver from a rat treated with CCl_4 twice weekly at 0.4 ml/kg for 6 weeks. There is capsular pitting (arrows) with fibrosis extending into the parenchymal tissue.

C. Liver from a rat treated with CCl_4 twice weekly for 6 weeks at 0.7 ml/kg; perivascular fibrosis in the centrilobular and periportal areas, with some evidence of bridging along the sinusoidal tracts.

D. Liver from a rat treated for 6 weeks, twice weekly at 1.0 ml/kg. Perivascular fibrosis with bridging along the sinusoidal tracts within the liver parenchyma is evident.

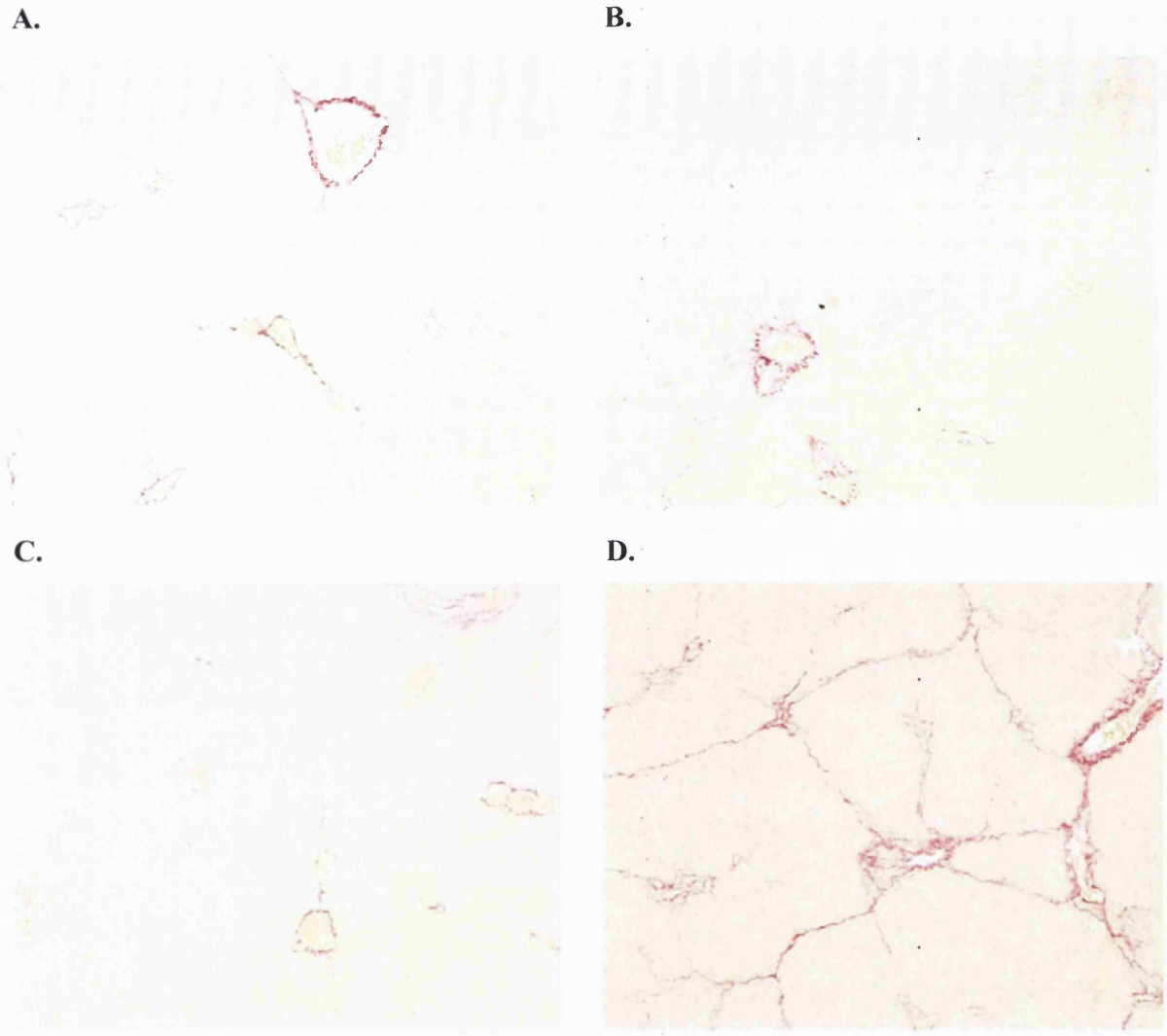


Figure 5.18 Histopathology of the liver from rats treated twice a week with CCl_4 at increasing dose levels for six weeks, then left untreated for a six week period of recovery. Original magnification of all images, x 100; Sirius Red stain.

A. Liver from a rat treated with CCl_4 for 6 week at 0.4 ml/kg and left untreated for a 6 week recovery period. There is perivascular fibrosis with some evidence of delicate partial bridging within the liver parenchyma.

B. Liver from a rat treated with CCl_4 for 6 weeks at 0.7 ml/kg and left untreated for a 6 week recovery period; there is slight perivascular fibrosis in the periportal and centrilobular areas.

C. Liver from a rat treated with CCl_4 for 6 weeks at 0.9 ml/kg and left untreated for a 6 week recovery period; there is slight perivascular fibrosis in the periportal and centrilobular areas.

D. Liver from a rat treated with CCl_4 for 6 weeks at1.0 ml/kg and left untreated for a 6 week recovery period. There is perivascular fibrosis in both the centrilobular and portal areas, with delicate bridging of parenchymal fibrosis between the areas.

5.6 Analysis of urine for novel protein markers of hepatic fibrosis

5.6.1 Reagent strips

All urine samples collected during experiment 2 were analysed using reagent strips as described in section 2.6. Urine collected from rats after 3 weeks and 6 weeks of dosing twice a week with CCl_4 at 0, 0.7 and 1.0 ml/kg (n = 6 in each group) were positive for bilirubin in all samples from rats treated with CCl_4. Control urine samples had trace protein levels whereas most of the CCl_4-treated urine samples had protein readings of at least 1 g/L. All other parameters measured in the urine samples (i.e. glucose, ketones, specific gravity, blood, pH, protein, urinobilinogen, nitrite and leucocytes) were similar in both control and CCl_4-treated urine. The reagents strips revealed that urine collected during the recovery period (at three and six weeks of the recovery period) had similar readings for all parameters including bilirubin and protein regardless of whether the rat had been treated with CCl_4 or not.

5.6.2 SDS-PAGE

In experiment 2, urine samples were collected from three rats from each of the control, 0.7 and 1.0 ml/kg CCl_4 groups after three weeks of dosing and also at the end of dosing (i.e. at six weeks). These urine samples were analysed using one-dimensional SDS PAGE. When the urine samples were analysed by SDS PAGE the only difference between control and CCl_4-treated urine was the presence of the SOD-1 band (i.e. the 18.4 kDa band) in the urine from rats treated with CCl_4 (Figure 5.19A) (Not all results presented). SOD-1 was present in all the urine samples from rats treated with CCl_4 and not in any of the control samples at both three and six weeks after the start of the study. Urine samples from the same rats were also collected after the six week recovery period and analysed by SDS-PAGE. These gels revealed that the SOD-1 band could no longer be detected in any of the rat urine samples from CCl_4-treated animals (Figure 5.19C). The only different according to 1D SDS-PAGE between urine from control rats and rats with liver fibrosis was the SOD-1 band. No other protein bands were observed in the urine samples.

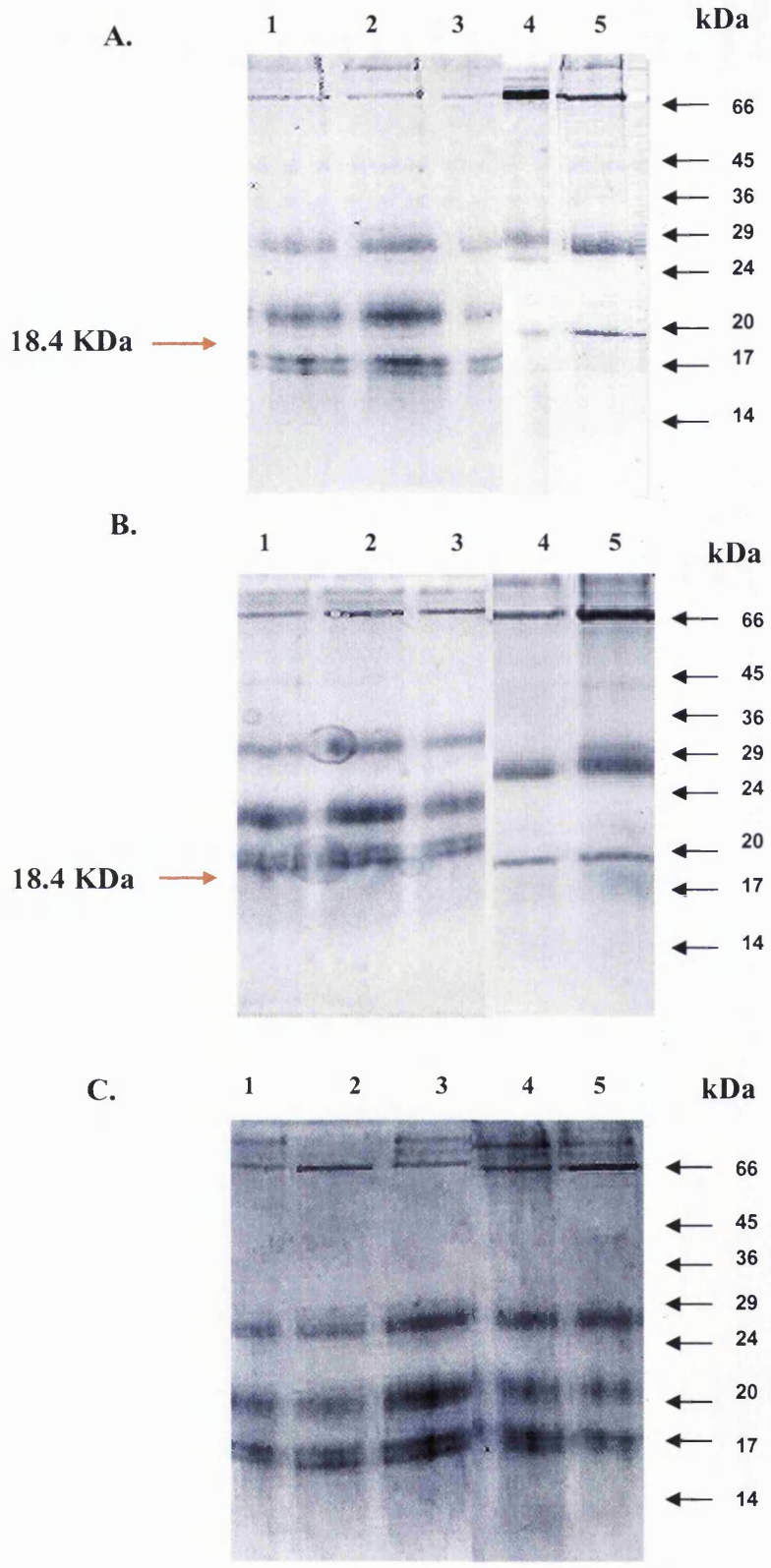


Figure 5.19 SDS-PAGE of urine samples collected during the induction of fibrosis as described in section 5.2.2. A. Urine collected after three weeks of dosing twice a week with CCl_4. B. Urine after six weeks of dosing. C. Urine at the end of the six week recovery period. In all gels: lanes 1, 2 and 3 = control urine; lanes 4 and 5 = urine from rats treated with CCl_4 at 0.7 ml/kg. Gels were run as described in Section 2.11.

5.6.3 Western blotting with the SOD-1 antibody

Urine samples collected after three weeks and six weeks of CCl_4 dosing at 0 (control) and 0.7 ml/kg dose levels were Western blotted with the SOD-1 antibody. This revealed that SOD-1 was greatly increased in the CCl_4-treated urine samples compared with control after both three and six weeks of dosing (Figure 5.20A and B). However when the urine samples collected at the end of the six week period of recovery were Western blotted with the SOD-1 antibody, SOD-1 could no longer be detected, and this indicates that there is no new injury to the liver.

5.6.4 SOD and GLDH activity assay

Urine samples collected after six weeks of dosing twice a week with CCl_4 at 0, 0.7 and 1.0 ml/kg were assayed for SOD activity as described in section 2.16. After six weeks of dosing rats with CCl_4 twice a week with either 0.7 ml/kg or 1.0 ml/kg SOD activity was increased 6-fold above control. The mean control SOD activity was 633.250 U/L; this was increased to 3911.83 and 3835.33 U/L in the 0.7 and 1.0 ml/kg groups, respectively (Figure 5.21A).

GLDH activity was also measured in the urine samples collected after six weeks of dosing. The GLDH activity in control samples was 0.43 U/L and this was increased in the 0.7 ml/kg group to 0.73 U/L. The GLDH activity was increased to 1.20 U/L (2.8 fold greater than control) in the urine samples from rats treated with CCl_4 at 1.0 ml/kg (Figure 5.21B).

In this study, the increase in SOD activity in rat urine following the induction of fibrosis is clearly more significant than the increase in GLDH activity. Therefore, the measurement of SOD activity in urine samples may be a better indicator of liver fibrosis than GLDH.

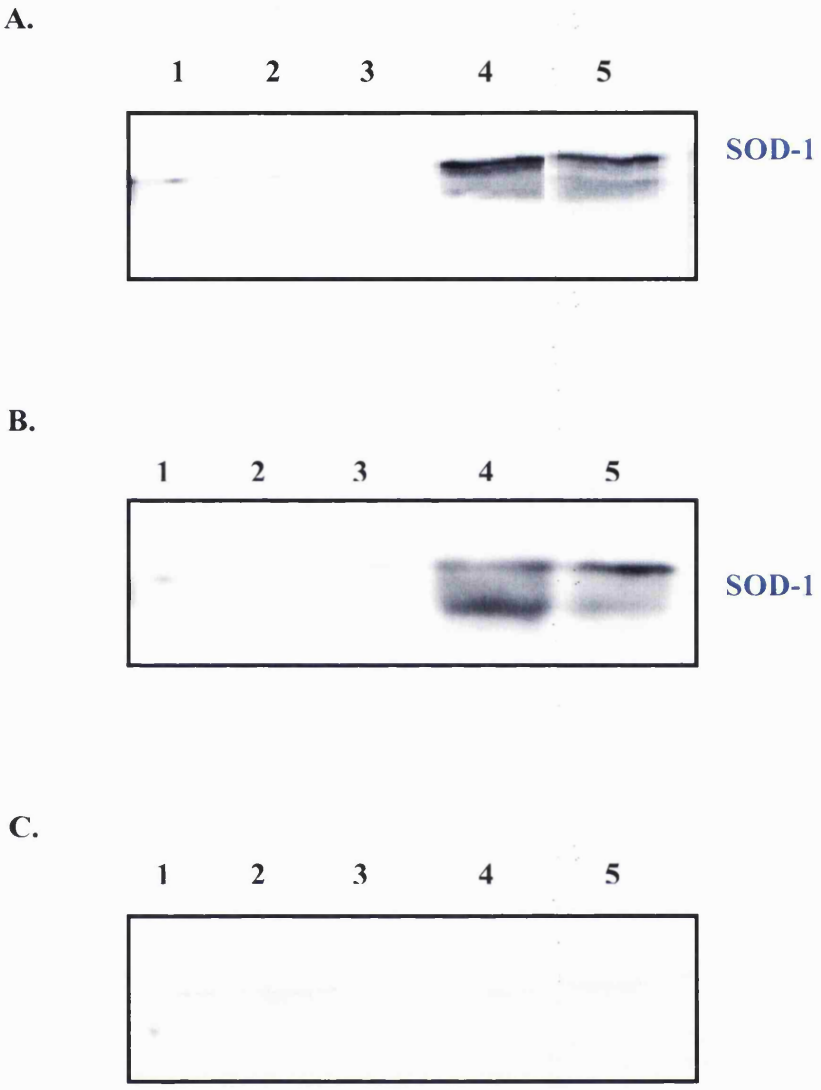
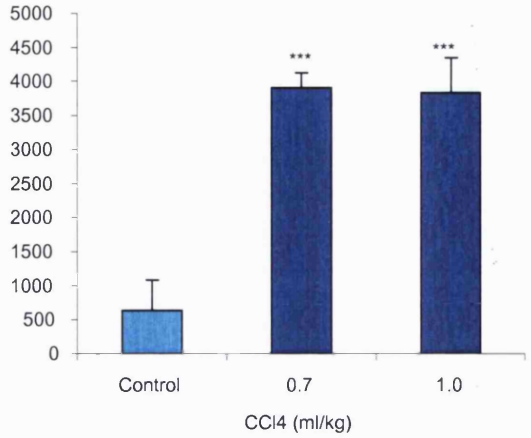


Figure 5.20 Western blots of urine samples collected during the induction of fibrosis. Samples for SDS-PAGE were prepared as described in Section 2.14 with each sample containing 14 µl of urine. **A.** Urine collected after three weeks of dosing twice a week with CCl_4. **B.** Urine collected after six weeks of dosing twice a week with CCl_4. **C.** Urine collected at the end of the six week recovery period. In all blots: lane 1 2, 3 = control urine and lanes 4 and 5 = urine from rats treated with CCl_4 at 0.7 ml/kg.

A.



B.

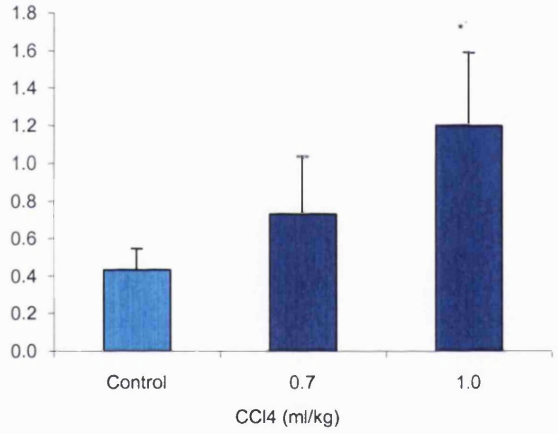


Figure 5.21 Mean SOD (A) and GLDH (B) levels in the urine of rats treated with CCl_4 at 0.7 and 1.0 ml/kg twice a week for six weeks. The SOD activity was measured as SOD units per litre of urine as described in section 2.18. GLDH activity was measured as described in Section 2.5. Values are means (error bars represent S.D.) of 6 animals. Values that differ from control by Student's t-test are shown, * $P < 0.05$, *** $P < 0.001$.

5.7 Discussion

Hepatic fibrosis is a common response to chronic liver injury in man from many causes, including alcohol and hepatitis (Friedman, 1993). Several rodent models of liver fibrosis/cirrhosis exist, including the repeated administration of allyl alcohol (AA), thioacetamide (TAA), DMN, and CCl_4. Hepatic fibrosis has been induced by repetitive administration of AA to rats twice a week for 8 weeks (Jung et al., 2000). The TAA rat model has been proven to closely resemble the major features of human cirrhotic pathology (Fontana et al., 1996; Sanz et al., 1997; Sato et al., 2000). However, the TAA model is a slowly developing one, and Sato et al. (2000) found that the fibrotic lesions only developed after treatment with TAA three days a week for 8-10 weeks. Al-Bader et al. (2000) also induced fibrosis after dosing with TAA for ten weeks. It has also been reported that cirrhosis develops after 3-4 months of TAA treatment (Fontana et al., 1996; Balkan et al., 2001; Bruck et al., 2001). A study by Jeong et al. (2001) compared the use of TAA and CCl_4 and found that severe cirrhotic changes were apparent in the liver at 9 weeks in CCl_4-treated rats, and at 30 weeks in the case of TAA-treated rats.

DMN is a potent hepatotoxin that also can cause fibrosis of the liver in rats. In a study by George et al. (2001) DMN was administered on 3 consecutive days of each week over a 3-week period. It was found that at the end of the 3 week period, collagen fibre deposition could be observed, together with severe centrilobular necrosis. Ueki et al. (1999) administered DMN on 3 consecutive days of each week and after the fourth week, histological examination revealed the formation of regenerative nodules separated by fibrous septa. The administration of DMN appears to induce a rapid deposition of collagen and the development of fibrotic lesions in the rat.

The CCl_4 model is widely used to induce fibrosis/cirrhosis. CCl_4 causes rapid liver damage progressing from steatosis to centrilobular necrosis and with long term administration, hepatic fibrosis is induced (Luckey and Petersen, 2001).

The present study (experiment 1), with CCl_4 administered at levels from 0.06 to 0.36 ml/kg, was carried out to define a dose of CCl_4 that would induce liver fibrosis and determine a model for subsequent analysis of urinary markers of fibrosis. The dose range used was chosen based on the results of the dose response study described in

Chapter 3, where CCl_4 was given at levels from 0.4 to 2.8 ml/kg. In this study liver injury (with increases in ALT, AST and GLDH and hepatocellular necrosis) had been induced after a single dose of 0.4 ml/kg CCl_4. Therefore, since the fibrosis studies would involve multiple dosing of CCl_4 it was decided to dose at levels below 0.4 ml/kg. This would also be a much lower dose level of CCl_4 to use than the levels known to be nephrotoxic. Nephrotoxicity had only been seen at dose levels of 1.2 ml/kg and higher (Chapter 3, section 3.3.6).

In the present experiment 1, at the end of the six week period of dosing, the liver weights were higher in all CCl_4-treated rats suggesting that liver injury had been induced. This increase in liver weight was related to the dose of CCl_4. The serum biochemistry results show significant increases in AST, ALT and GLDH suggesting significant liver damage after 6 weeks of dosing. The ALT activity was statistically increased over the control at the lowest dose administered (0.06 ml/kg CCl_4) and both ALT and GLDH were significantly different from control in the 0.12 ml/kg group. As the dose level of CCl_4 was increased, the levels of all three enzymes increased. Elevated ALT and AST levels nearly always indicate injury to the liver (Jones and Berk, 1979). In acute injury, the ALT values tend to be greater than those of AST activity, whereas chronic injury induces much greater AST activity than ALT. In this first experiment, the AST activity was much greater than ALT in both the 0.30 and 0.36 ml/kg treatment groups suggesting significant chronic injury to the liver. Park et al. (2000) found significant increases in serum ALT and AST after 4 weeks of dosing with 1ml/kg CCl_4 twice a week. Chronic treatment of CCl_4 by inhalation produced an increase in serum AST activity which was 36 times greater than control after 4 weeks of dosing (Giménez et al., 1994). However, studies by Jiang et al. (1992) in which rats were dosed intraperitoneally with 1 ml/kg CCl_4 only achieved a two-fold increase in ALT activity in the serum from rats treated with CCl_4 over control levels.

In Experiment 1, ALP (Figure 5.5A) and bilirubin levels (Figure 5.5C) were also increased in the serum from rats treated with CCl_4 even in the 0.06 ml/kg group although the results are only statistically significant in the case of ALP. Both serum ALP and bilirubin rise with increasing dose levels of CCl_4. Increased levels of ALP and bilirubin are generally due to cholestatic lesions occurring as a result of an impaired bile flow, which may be caused by hepatocellular dysfunction or biliary obstruction

(Zimmerman, 1978). The process of fibrosis may result in distortion of the structure of the liver lobule by dense bands of collagen, which may link vascular structures (Gaudio et al., 1997).

In Experiment 1, the amount of albumin present in the serum of rats treated with CCl_4 twice a week for six weeks (Figure 5.5B) was similar to control at all dose levels apart from the 0.30 and 0.36 ml/kg groups in which the levels were decreased ($P < 0.05$). Serum albumin levels tend to be reduced in chronic liver injury as a result of the impaired ability of the damaged liver cells to synthesize protein (Horne et al., 1973). Giménez et al. (1994) induced a decrease in serum albumin following 16 weeks of treatment with CCl_4 by inhalation. Studies by Hernandez-Munoz et al. (2001) in which liver cirrhosis was induced in rats, revealed that after 10 weeks of repeated dosing with CCl_4 serum albumin levels were significantly reduced when compared to control. These workers also found significantly increased serum bilirubin levels at 10 weeks of dosing.

Histopathology of the liver samples in Experiment 1 after 6 weeks of dosing showed fibrosis was present, but it was not a consistent response throughout all CCl_4-treated rats. Even at the highest dose used (0.36 ml/kg) the fibrosis was relatively mild. Histopathology of the livers from CCl_4-treated rats showed hepatocellular degeneration and necrosis, similar to that obtained following a single dose of CCl_4. Sirius Red staining of randomly selected liver samples indicated that collagen fibres were present but there was variation in the degree of fibrosis induced. Liver fibrosis was induced in rats by Matsuda et al. (1997) after dosing with 0.4 ml/kg CCl_4 on three consecutive days for 12 weeks but here the fibrotic tissue accumulated in the periportal, intralobular and pericentral regions.

Studies to induce cirrhosis have in some cases used high doses of CCl_4 for longer periods of time, and well-developed cirrhosis has been obtained by the chronic administration of CCl_4 for longer than 8 weeks (Hsu, 1998; Ferre et al., 1999; Masson et al., 1999b). Also many models of CCl_4-induced fibrosis/cirrhosis have included a pre-treatment with phenobarbitone (Masuda et al., 1994; Hashimoto et al., 1999).

CCl_4-induced fibrosis has been obtained in many studies by the administration of larger doses of CCl_4 or by dosing for longer periods. Luckey and Petersen (2001) induced significant fibrotic changes after 4 weeks of dosing rats with 1.0 ml/kg CCl_4 twice a week. However, the hepatic injury was more evident after 6 weeks of dosing with a greater deposition of extracellular matrix and the appearance of bridging fibrosis. Park et al. (2000) administered CCl_4 orally at a dose of 1.0 ml/kg twice a week for 4 weeks; this induced the extensive accumulation of connective tissue resulting in the destruction of the lobular architecture and formation of septa. Paradis et al. (1999) induced fibrosis by dosing at 2.0 ml/kg CCl_4 twice a week for 3 weeks. Iredale et al. (1998) administered 2.0 ml/kg body weight to male rats twice a week for 4 weeks and observed obvious nodular fibrosis with deposition of well-delineated fibrotic septa.

In the present Experiment 1, following the 6 week recovery period, the liver weights were still slightly heavier than control but the only statistical differences were at 0.18 and 0.24 ml/kg CCl_4 ($P < 0.05$). Therefore, this suggests that on removal of the toxic stimulus, the liver was able to begin to recover from the induced damage. At the 12 week post mortem the serum biochemistry results revealed that ALT and GLDH activities were similar to control in all dose groups. Whereas, the AST activities were significantly reduced when compared to the controls in the 0.24, 0.30 and 0.36 ml/kg groups, but comparable to control values at all the lower dose levels. There were no significant differences between the control and CCl_4-treated groups for the other serum parameters measured (i.e. ALP, albumin and bilirubin). Again, these results suggest that the liver has undergone a period of repair. The histology results for Experiment 1 showed that following the six week recovery period the livers had returned to normal and there was no evidence of fibrosis.

In Experiment 1, at the dose levels chosen, nephrotoxicity was not induced as determined by histopathology of the kidneys after six weeks of CCl_4 dosing, and this was also the case following the six weeks recovery period. Also serum urea levels were no different to the control in all dose groups, as were the creatinine levels (except in the 0.36 ml/kg group in which the serum creatinine for the CCl_4 group was significantly less than control). Therefore the histological findings, and the serum urea and creatinine results suggest the absence of kidney injury in this study after six weeks of CCl_4 dosing. Although the serum urea measurements after 12 weeks were higher for all the CCl_4-

treated groups than control, this increase was not significant at any dose level and the histopathology observations confirmed the absence of nephrotoxicity.

Therefore in Experiment 1, although liver fibrosis in the CCl_4-treated groups had been induced, the response was mild and inconsistent between the groups and even within treatment groups. It was therefore decided that it would be necessary to administer higher dose levels of CCl_4 in a follow-up study. Consequently, the second study was carried out with dose levels between 0.4 and 1.0 ml/kg rising in increments of 0.1 ml/kg. However, it was considered important to consider the possibility of kidney damage during this study since the dose of 1.0 ml/kg was close to our original threshold for nephrotoxicity from an acute dose of CCl_4, which was 1.2 ml/kg (Chapter 3).

The results of Experiment 2, in general terms were in parallel to those of Experiment 1 in that the liver weights and serum biochemistry results tended to follow the same patterns. ALT, AST and GLDH were increased at all dose levels of CCl_4 and a dose response relationship was also clearly apparent (Figure 5.12). At all dose levels (except in the 0.7 ml/kg group) the AST activity was greater than that of ALT indicating, again, that chronic injury had been induced. ALP activity was significantly greater in the CCl_4 groups than in the controls at all dose levels, as was the serum bile acid measurements. The bilirubin levels were also greater than control at all dose levels of CCl_4 administered. A study by Vollmar et al. (1999) revealed that after administering 0.15 ml/kg CCl_4/100g rat body (i.e. 1.5 ml/kg) weight twice a week for 8 weeks, marked increases were observed in serum AST and ALT values. Wang et al. (1996) induced liver fibrosis in both male and female Wistar rats after dosing by gavage with CCl_4 at 1.0 ml/kg every five days for either 3 weeks or 6 weeks. These workers found that ALT activity in females treated with CCl_4 for six weeks was 4 times greater than control activity. In these studies serum bilirubin was also measured and found to be significantly greater than control in the CCl_4-treated rats.

The histopathology results of Experiment 2, showed that nephrotoxicity was not induced in any of the CCl_4-treated rats even though the serum urea levels after six weeks of dosing (Table 5.3) were significantly increased in all CCl_4-treated groups compared to control. The serum creatinine measurements after six weeks of dosing were reduced in all CCl_4-treated groups with significant decreases at 0.6, 0.7, 0.8 and

1.0 ml/kg CCl_4. A reduction of serum creatinine generally results from a decrease in muscle mass or from a diet that is inadequate in protein (York, 2004). In this study either cause could be possible since the rats were likely to be eating less as the study progressed due to the effects of the CCl_4. Lopez-Lirola et al. (2003) found that in CCl_4-induced cirrhosis muscle atrophy was observed, however this was due to protein deficiency and not a result of the induced cirrhosis. However the serum creatinine levels measured after six weeks recovery period were similar to control levels for most CCl_4-treated rat groups. Conversely, the serum urea measurements remained increased in the 0.4, 0.5 and 0.6 ml/kg groups, but the levels were decreased when compared to control in all other CCl_4-treated groups. This reduction was statistically significant in the highest dose group (1.0 ml/kg).

Histopathological study of the liver from Experiment 2 revealed that fibrosis had been induced with capsular pitting in the CCl_4-treated rat livers. Liver sections were randomly selected and stained with Sirius Red. This special stain revealed the presence of perivascular fibrosis and delicate bridging between the centrilobular veins, periportal and capsular areas, but even at the higher dose levels used there was still some variation both between, and within, the treatment groups. Histopathology of liver at 12 weeks revealed the presence of mature collagen in the subcapsular regions, and fibrosis extending into the parenchyma along the sinusoids. Therefore this experiment clearly demonstrated that the induced fibrosis was not reversed after the six weeks recovery period.

The variation in the degree of fibrosis obtained during such studies with the CCl_4 fibrosis model is a well-known phenomenon. Proctor and Chatamra (1982), in an attempt to overcome this problem described a method whereby they weighed the animals every day following intragastric administration of CCl_4 and then used this weight to calculate the subsequent dose of CCl_4. Using this method they managed to induce cirrhosis in about 75 % of the rats dosed. In Experiment 1 and Experiment 2, rats were weighed twice a week just prior to receiving a dose of CCl_4; the dose of CCl_4 received by each animal was calibrated once each week to account for any weight changes in response to CCl_4. To obtain a significant amount of hepatic damage, it is necessary for the liver to be subjected to doses of CCl_4 where the tissue will not be able to fully recover before the next dose is administered (Proctor and Chatamra, 1982).

However, it is known that this dose of CCl_4 will be different for each animal due to an unpredictable variation in the hepatic response to the toxicant. The response will also vary for each individual over the course of a study as the animal ages. Also CCl_4 can reduce the amount of the metabolising enzymes (cytochrome P450) and therefore decreases the sensitivity of the liver to further doses. There is also no reliable method for monitoring the change in liver damage with each dose of CCl_4 (Proctor and Chatamra, 1983). For these reasons, it is often difficult to obtain a uniform and consistent degree of fibrosis or cirrhosis with the CCl_4 rat model (Proctor and Chatamra, 1982).

However, other groups have reported good fibrotic responses after dosing rats twice a week with CCl_4 at 0.2 ml/kg by intraperitoneal injection for 8 and 12 weeks (Ohishi et al., 2001). Here it was found that after eight weeks of dosing histopathological assessment of the livers revealed periportal fibrosis and fine fibres in the portal areas. By 12 weeks the fibrosis had expanded along the sinusoids with evidence of bridging between portal tracts. Ferré and colleagues (1999) induced liver cirrhosis in rats by the intraperitoneal injection of 0.5 ml/kg CCl_4 twice a week for 9 weeks. In another study (Rivera et al., 2001) rats were dosed intragastrically with CCl_4 once a week for nine weeks. The outcome was extensive collagen deposition and bridging between portal regions. However Cabré et al. (2000) carried out a study in which 0.5 ml CCl_4 was administered by intraperitoneal injection to rats weighing 210 ± 20 g twice a week for 9 weeks. By two weeks after the commencement of dosing fibrosis was achieved in all rats treated with CCl_4. In a similar study by Varela-Moreiras et al (1995), steatosis was achieved after three weeks of dosing and cirrhosis after nine weeks. It is therefore possible that in our own studies we would have achieved more significant fibrosis by dosing for a longer period of time or even with a larger dose of CCl_4.

McLean and colleagues (1969) adapted a CCl_4 model in the rat by introducing phenobarbital administration as an inducer of the cytochrome P450 system, thereby sensitising the liver to the subsequent toxic dose of CCl_4. Phenobarbital was added to the drinking water and after 10 to 14 days the first dose of CCl_4 was administered. Phenobarbital is a very potent liver stimulant and therefore this model has become widely used to induce cirrhosis more rapidly (Hashimoto et al., 1999). However, phenobarbital induces liver hypertrophy and hyperplasia and therefore the results of

such studies are affected by the initial weight of the liver and the length of time phenobarbitone is administered before the first dose of CCl_4 (Chatamra and Proctor, 1981). If these factors are taken into account then the phenobarbital/CCl_4 model of fibrosis/cirrhosis can produce relatively consistent results. Studies carried out to assess the role of phenobarbital conclude that it is essential for the rapid development of cirrhosis in the rat (Rozga et al., 1991). Horne et al. (1973) reported that in rats treated with a combination of phenobarbitone and CCl_4 for 9 weeks a very high percentage developed cirrhosis. Other groups describe the development of liver fibrosis using this combination within 3-4 weeks, and cirrhosis by 10 weeks (Rockey et al., 1992; Kokudo et al., 1992; Masuda et al., 1994). However, even with the use of the adapted fibrosis model, great variation occurs with the response of the animals to CCl_4 and it remains difficult to obtain a consistent model of fibrosis/cirrhosis (Masuda et al., 1994).

The purpose of carrying out the present studies was to develop a model of liver fibrosis and then analyse urine from fibrotic rats for the presence of protein urinary markers. The results from Experiment 2 showed that the administration of CCl_4 at higher dose levels than those in Experiment 1 produced a good model of fibrosis. The degree of fibrosis induced was not reversed even after a 6 week recovery period. Therefore urine samples from rats in this study were analysed for protein markers of liver fibrosis.

All urine samples were analysed by 1D SDS-PAGE as before and this revealed that the only protein band apparent in the CCl_4-treated urine, which could not be detected in control urine, was the SOD-1 band. SOD would be expected to appear in the urine following chronic administration of CCl_4 since the metabolism of the chemical is the same as for an acute dose and therefore the initial damage is induced via the generation of free radicals. The appearance of these free radicals would stimulate the up-regulation of free radical scavengers such as superoxide dismutase (SOD) and catalase. CCl_4 exposure induces hepatocyte injury, resulting in inflammation; this inflammatory process brings about the activation and proliferation of Kupper cells, which then release a cascade of cytokines. The chronic injury produced by repeated dosing with CCl_4 therefore, is due to the inflammatory response mediated by these cyokines (Orfila et al., 1999). Thus, during chronic injury inflammation can lead to hepatocyte necrosis resulting in rupture of the hepatocyte plasma membrane. This may lead to the release of intracellular enzymes into the plasma and therefore it is feasible that increased amounts

of the enzyme SOD may be released into the plasma and eventually be eliminated in the urine.

SOD-1 was identified in the urine from rats treated with CCl_4 after both three and six weeks of dosing (Figure 5.19). But SDS-PAGE analysis did not detect any SOD-1 in the urine samples collected at 3 weeks into the recovery period (week 9) nor at 6 weeks of the recovery period (week 12). This proved that on removal of the stimulus for fibrosis no new hepatocytes were being injured and therefore SOD-1 and other enzymes were no longer leaking from hepatocytes into the plasma. This is confirmed by the serum biochemistry results obtained after six weeks of the recovery period (week 12). At week 12, most of the serum parameters measured were similar to control values, whereas six weeks earlier at the height of injury the serum enzymes were significantly greater than the controls at all CCl_4 dose levels. Serum markers and SOD-1 in urine have been shown to recover during the 6 week recovery period, however during this period fibrosis does not recover. Therefore these serum markers and SOD-1 are markers of the liver injury that results in fibrosis but not of the established fibrotic state.

Hsiao et al. (2001) measured SOD activity in mouse liver tissue following CCl_4-induced fibrosis and found that the activity was reduced to approximately half the control level after eight weeks of dosing. In the present study (Experiment 2) the presence of the increased SOD-1 enzyme in the CCl_4 urine samples was verified by Western blotting with the SOD-1 antibody (Figure 5.20). SOD-1 was greatly increased in the urine from rats treated with CCl_4 and was barely visible in the control samples. After six weeks of no treatment Western blotting with the SOD-1 antibody revealed that SOD-1 levels were similar to control in all urine samples from CCl_4-treated rats. Urine samples were also analysed using the SOD activity assay (Figure 5.21A). SOD activity was approximately six times greater than control in both the 0.7 and 1.0 ml/kg groups. The SOD activity expressed in Figure 5.21A is the actual activity measured and is not in this study normalised for creatinine. Urinary biomarkers are generally normalised for urinary creatinine measurements since animals generally excrete similar volumes of creatinine. However during the present study the serum creatinine measurements for the CCl_4-treated rats were significantly decreased when compared to control (Table 5.3), which often indicates renal dysfunction or a change in muscle mass and may reflect changes in urinary creatinine.

The activity of GLDH was also measured in the urine samples collected after dosing with CCl_4 twice a week for six weeks (Figure 5.21B). This figure shows a clear dose response relationship, with GLDH activity in the urine increasing with the dose of CCl_4. GLDH activity is approximately three times greater in the urine from rats treated with repeated doses of CCl_4 at 1.0 ml/kg than in the controls. GLDH has a molecular weight of approximately 55 KDa and therefore is smaller in size than albumin, which is also present in all the rat urine samples. This ensures that any up regulation of the enzyme due to hepatocyte injury would result in the leakage of the enzyme from the injured cells, into the plasma, and therefore eventual elimination via the urine. GLDH is present at high concentrations in the liver, although it can also be found in the kidney and muscles. The activity of GLDH is much greater in the centrilobular region of hepatocytes than any other region and therefore it is a good marker of centrilobular necrosis (Moss and Henderson, 1999). Therefore the increase in GLDH in the rat urine following the repetitive dosing with CCl_4 is a good indicator of centrilobular liver injury.

The outcome of the present studies is that a model of fibrosis was developed and it was established that SOD-1 appears in the urine from the rats with liver fibrosis. The SOD activity was greatly increased in the urine from the CCl_4-treated rats (Figure 5.21A). However at both three and six weeks (week 9 and 12) after stopping CCl_4 treatment, SOD-1 could no longer be detected in the urine. This indicates that the hepatocytes are no longer being damaged and no further SOD-1 is being released into the plasma. At 12 weeks after the start of the fibrosis study collagen fibres could still be detected in the liver by histopathological analysis (Figure 5.18), indicating that reversal of fibrosis had not occurred. Perhaps, if the study had been continued for longer, and the liver analysed after a longer recovery time, then the fibrosis may have been reversed.

It was not possible to detect any other proteins apart from SOD-1 in the urine from rats with fibrosis using one-dimensional SDS-PAGE, but it is likely that other proteins appear in the urine following chronic injury induced by CCl_4. The resolving power of 1D SDS-PAGE is limited and often bands may mask the appearance of less abundant protein bands of similar molecular weight. Therefore, the use of 2D gel electrophoresis will allow better separation of protein bands and may reveal further proteins.

Chapter six

Detecting marker proteins by two dimensional gel electrophoresis

6.1 Introduction

Both one dimensional SDS PAGE and SELDI failed to reveal marker proteins other than Cu/Zn SOD in rat urine following the induction of hepatic fibrosis (Chapter 5). This was the marker protein found in urine in response to acute toxicity induced by CCl_4 (Chapters 3 and 4). The increase in Cu/Zn SOD in urine in response to acute CCl_4 treatment was dramatic as judged by Western blot and by activity measurements, therefore its detection by one dimensional SDS PAGE was perhaps not surprising. In an attempt to identify further novel markers in the CCl_4-induced model of fibrosis, an alternative, more sensitive method of protein identification was sought.

Two-dimensional gel electrophoresis (2-DGE) separates proteins according to isoelectric point (pI) in the first dimension and according to mass using SDS PAGE in the second dimension (see Section 1.7.1.1). This offers enhanced resolution of protein mixtures and can reveal proteins that may not separate on a 1D gel. This technique has been used extensively to detect the differential expression of proteins in cells and tissues in response to altered physiological states, pathological states, or in response to pharmacological intervention (Bárány et al., 1998; Celis and Gromov, 1999; Moller et al 2001).

The reproducibility of 2-DGE has been improved drastically as a result of immobilised pH gradients (IPG) (Bárány et al., 1998; Garfin, 2003) (See Section 1.7.1.1). The use of IPGs generates good resolution between variants with a pI difference of 0.001 pH units and produces a good correlation between the pI predicted from a database and that obtained on a 2D gel (Righetti and Bassi, 1997). IPG strips allow for a much greater loading capacity of the gel and allow the use of narrow pH gradients to further increase resolution of proteins in selected pI ranges (Celis and Gromov, 1999).

2-DGE has been applied successfully to the screening of potential biomarkers following hepatotoxicity. Kristensen et al. (2000) carried out proteomic analysis using 2-DGE of cellular and secreted proteins of normal and activated hepatic stellate cells. In this study stellate cells were activated either *in vitro* by cultivating quiescent stellate cells for 9 days or *in vivo* by injecting rats with CCl_4 for 8 weeks. They identified a total of 43 proteins with altered expression levels in *in vivo* and/or *in vitro* activated stellate cells. A study carried out by Fountoulakis et al. (2000) investigated protein expression levels

in mouse liver following toxicity induced by paracetamol. Mice were administered paracetamol at a dose level of 100 or 300 mg/kg or its non-toxic regioisomer 3-acetamidophenol at 300 mg/kg. In this study 2-DGE revealed that the expression levels of 35 proteins were altered in the mouse liver. Newsholme et al. (2000) induced hepatomegaly in rats by the administration of a substituted pyrimidine derivative. 2-DGE detected a significant change in at least 17 liver proteins compared to control, of these proteins 12 were increased and 5 decreased.

It was hoped that 2-DGE analysis of the urine samples in the rat model of fibrosis generated in Chapter 5 would be able to separate more proteins and perhaps reveal a liver specific marker for this condition. For comparison, urine samples from rats subjected to acute doses of CCl_4 in Chapters 3 and 4 were also analysed by 2-DGE.

6.2 Two dimensional gel electrophoresis of urine from rats with liver fibrosis

Figure 6.1 shows typical 2-D gels of urine from a control rat (A) and a rat in which liver fibrosis has been induced (B). Despite correcting the volumes of urine to ensure equal protein loading according to albumin content (as judged by 1-D SDS PAGE) it would appear that gel B has slightly more protein as indicated by the albumin streak at 66 kDa at the top of the gel in the middle. There are relatively few basic proteins running with high pI and the majority are running in the left hand side of the gels with pI values between 3 and 7. The major difference between the gels is the appearance in the fibrotic sample of the protein spots just below 20 kDa with pI between 4.5 and 7 (indicated by the red circle). This is undoubtedly Cu/Zn SOD and was confirmed by Western blot with the SOD-1 antibody (results not presented). The SOD-1 has not isofocussed into a single spot as some other proteins on the gel have. This may be an indication of multiple species of SOD-1 differing in their level of post-translational modification. Such modification has been suspected previously due to the migration on 1-D SDS PAGE with a molecular weight higher than that predicted by sequence.

Other proteins spots that appear or have increased following the induction of fibrosis are encircled in green. The areas encircled in green contain many densely stained spots which may be potential urinary markers of liver fibrosis. However in the urine from the fibrotic rat some proteins appear to have decreased in concentration when compared to the control urine. The area surrounded by the blue circle in gel B indicates protein spots that appear to have decreased in abundance following the induction of fibrosis. It seems clear that there are changes in the "urinary proteome" in rats in which hepatic fibrosis has been established. These proteins whose concentrations change represent potential markers for fibrosis. The identification of each by mass spectrometry (as performed previously for SOD-1 in Chapter 3) is now required.

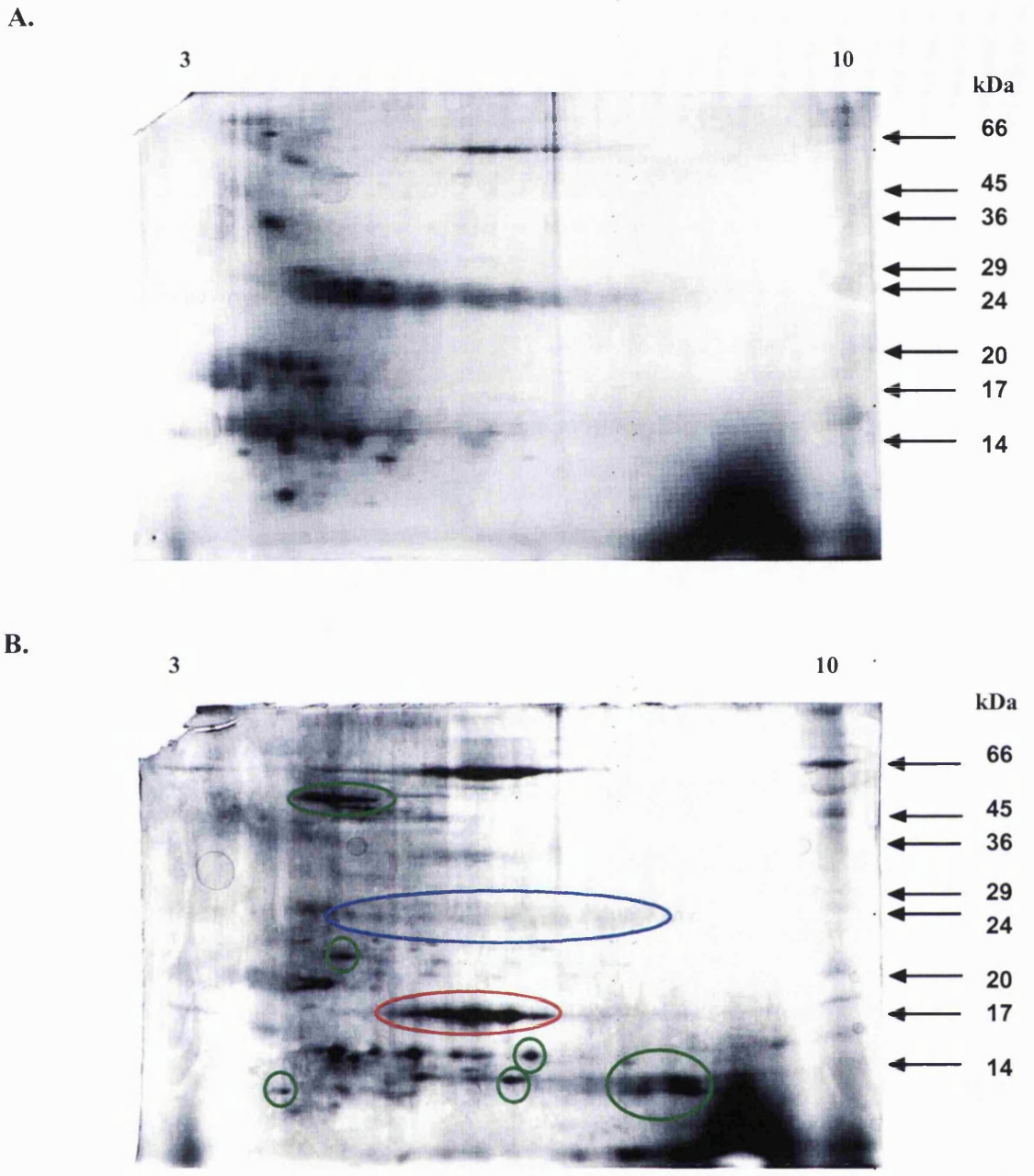


Figure 6.1 2-DGE of urine from a control rat and rat with liver fibrosis induced by repetitive CCl_4 dosing.
Female Wistar Han rats were dosed with 0 ml/kg (A. control) or 1.0 ml/kg (B. fibrotic) CCl_4 twice a week for six weeks. Rats were placed in metabolism cages immediately after the last CCl_4 dose and urine was collected for 24 hours. Urine samples were chosen from control rats and rats in which hepatic fibrosis had been demonstrated by histopathology (see Section 5.5.3). The urine samples were prepared for 2D gel electrophoresis as described in Section 2.19.1. Volumes were adjusted to achieve equal loading of albumin as judged from 1-D SDS PAGE. Urine samples were isofocussed on pH 3-10 NL IPG strips and the second dimension run on 15 % SDS PAGE. Gels were stained using silver stain as described in Section 2.13.

6.3 Two dimensional gel electrophoresis of urine from rats treated with a single dose of CCl_4

For comparison with the model of fibrosis, urine samples from rats following acute dosing with CCl_4 were also analysed by 2-DGE. Figure 6.2 A shows a typical 2D gel of urine from a control rat and Figure 6.2 B shows the urine from a rat treated with a single dose of 0.8 ml/kg CCl_4. This dose was chosen as it is low enough to avoid nephrotoxicity but high enough to ensure hepatic toxicity as shown in Chapter 3.

As seen before (Figure 6.1) virtually all urinary proteins run with pI values in the range of 3-7 (Figure 6.2). When gel B was compared with urine from a control rat (gel A) it was possible to identify proteins that have appeared or increased in concentration as a result of the CCl_4 treatment. Three regions in the gel containing such proteins are circled in blue. The spot circled in green contains the protein Cu/Zn superoxide dismutase (SOD-1) and this was confirmed by Western blotting with the SOD-1 antibody (Figure 6.4). Since most of the proteins isofocus in the lower pH range of the IPG strip, it seemed likely that enhanced resolution would be obtained if the pH range of the 2-DGE was narrowed.

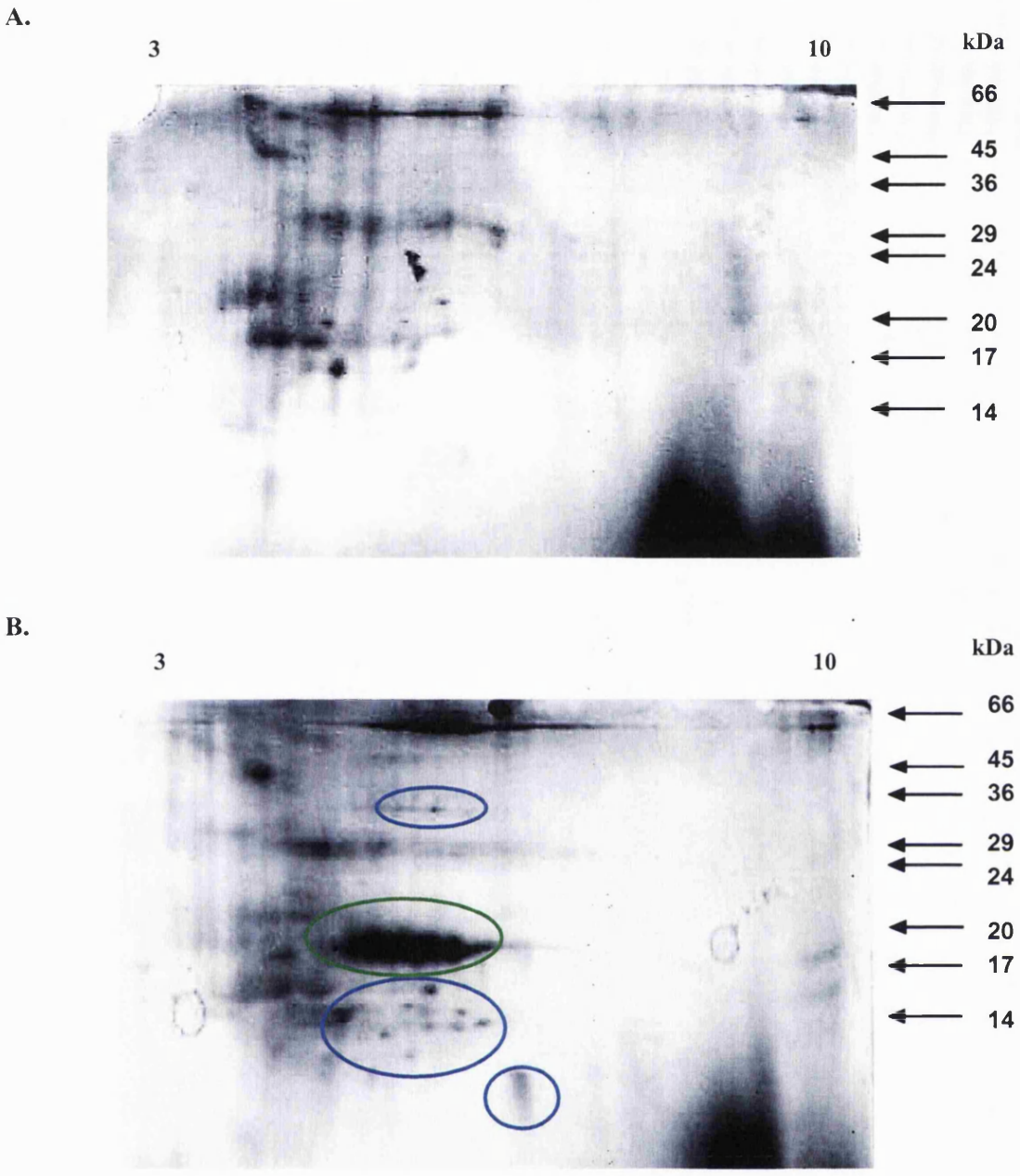


Figure 6.2 2-DGE (pH 3-10) of urine collected during the 24 hours after treating rats with a single acute dose of CCl_4.
Female Wistar Han rats were dosed with 0 ml/kg (A. control) or 0.8 ml/kg (B) CCl_4 and placed in metabolism cages where urine was collected for 24 hours. The urine samples were prepared for 2D gel electrophoresis as described in Section 2.19.1. Volumes were adjusted to achieve equal loading of albumin as judged from 1-D SDS PAGE. Urine samples were isofocussed on pH 3-10 NL IPG strips and the second dimension run on 17 % SDS PAGE. Gels were stained using silver stain as described in Section 2.13.

Figure 6.3 shows that in the narrower range of pH 3-6, the urinary proteins show much greater separation across the gel. When gel B (urine from a rat treated with CCl_4) is compared to the control urine sample (gel A) it is now possible to observe many protein differences. Proteins that appear or increase due to CCl_4 treatment are circled in blue. Cu/Zn SOD, as confirmed by Western blotting (results not presented) is again indicated by the green circle. One can conclude that as with the model of liver fibrosis (Figure 6.1) there are a number of proteins whose concentration in urine appears to change as a result of acute CCl_4-induced hepatotoxicity. Their detection has been aided enormously by the resolving power of 2-DGE coupled to detection by silver-staining. Their identification by mass-spectrometry and investigation as liver-specific markers is the next step of this investigation.

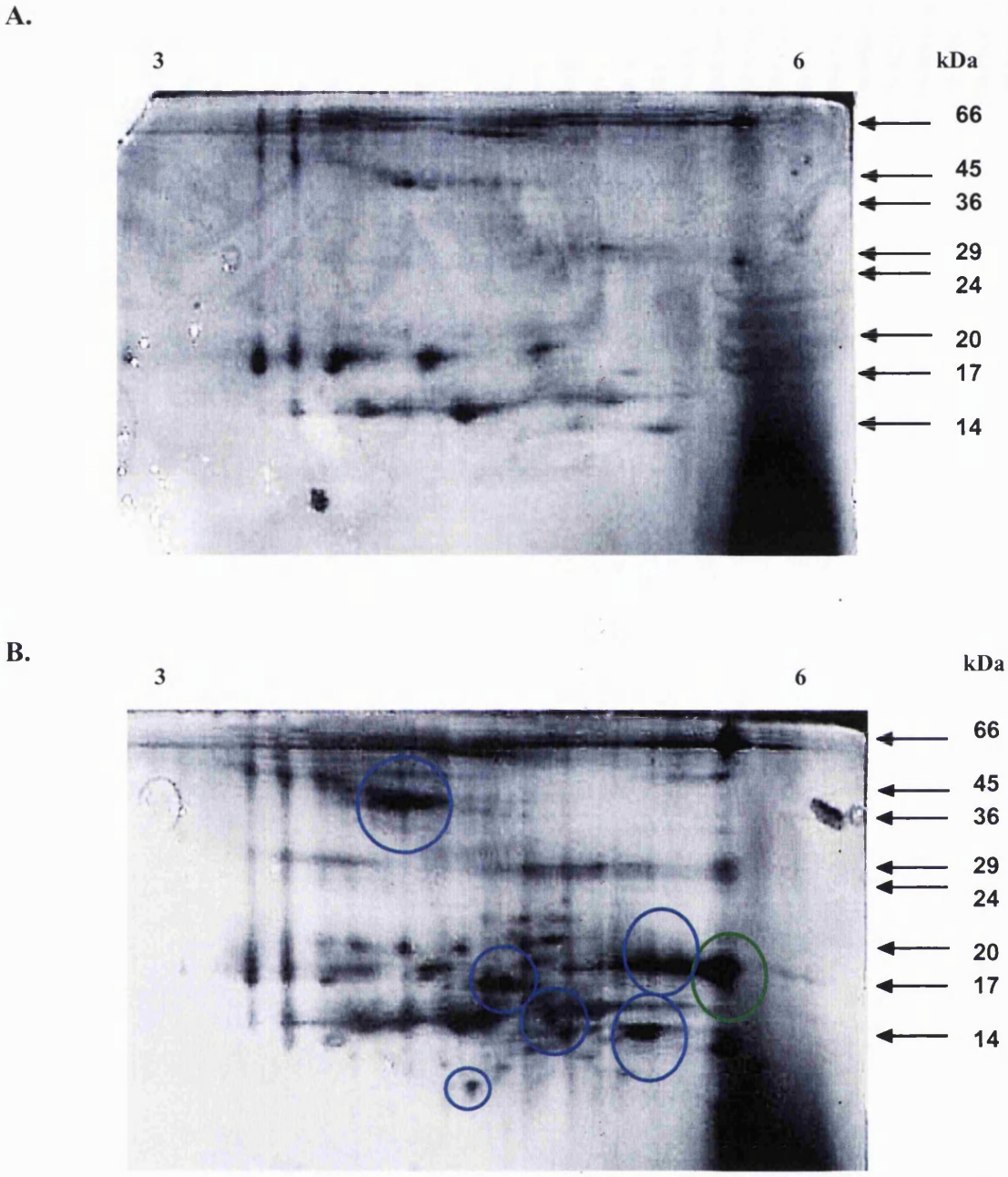


Figure 6.3 2-DGE (pH 3-6) of urine collected during the 24 hours after treating rats with a single acute dose of CCl_4.

Urine samples were collected and analysed as described in Figure 6.2 for control (A) or 0.8ml/kg CCl_4-treated rats (B) with the exception that they were isofocussed on pH 3-6 NL IPG strips.

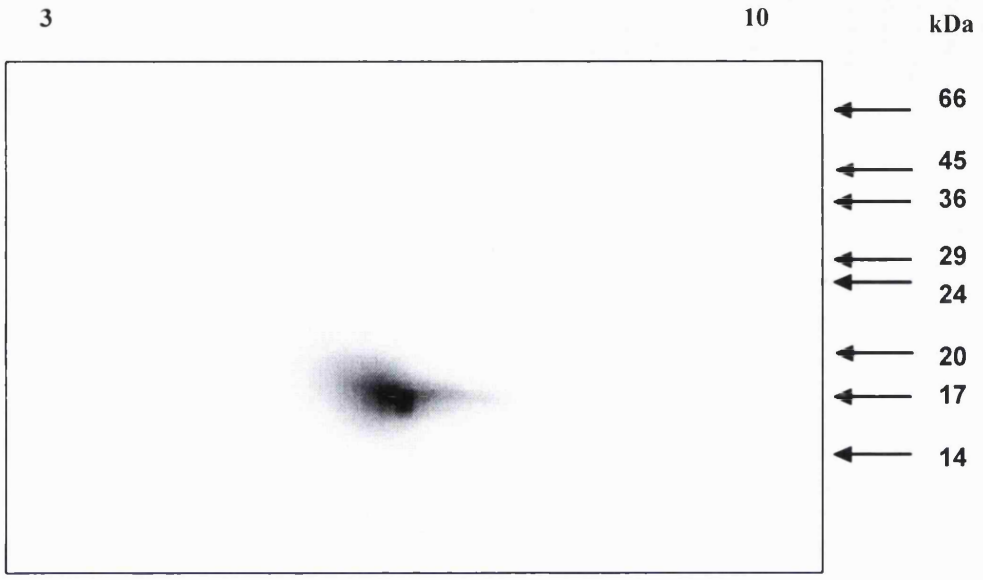


Figure 6.4 Western blot of a 2-DGE (pH 3-10) of urine collected during the 24 hours after treating rats with a single acute dose of CCl_4.
Female Wistar Han rats were dosed with 0 ml/kg (A. control) or 0.8 ml/kg (B) CCl_4 and placed in metabolism cages where urine was collected for 24 hours. The urine samples were prepared for 2D gel electrophoresis as described in Section 2.19.1. Volumes were adjusted to achieve equal loading of albumin as judged from 1-D SDS PAGE. Urine samples were isofocussed on pH 3-10 NL IPG strips and the second dimension run on 17 % SDS PAGE. Gels were stained using silver stain as described in Section 2.13. Western blotting was carried out using the SOD-1 antibody as described in Section 2.14.

6.4 Two dimensional gel electrophoresis of liver homogenates from rats after a single dose of CCl_4.

Figure 6.5 shows that in contrast to urine, the proteome of rat liver is distributed across the pH gradient with a number of basic proteins apparent that isofocussed at the higher end of the pH 3-10 gradient. Many proteins have either completely disappeared or have decreased in abundance in the liver following the administration of CCl_4. On comparison of liver homogenates from control (Fig 6.5A) and treated rats (Fig 6.5B) it can be seen that the abundance of some proteins does not change (eg. the three spots in the very middle of the gels). However, the areas circled in gel B represent proteins that appear to have decreased following treatment with CCl_4. The decrease in some of these proteins may be due to decreased expression or perhaps more likely due to liver necrosis and rupture of the cell membrane resulting in some proteins leaking from the cell. This has been observed previously for SOD-1 in liver homogenates (see Section 4.5.3). Identification of the proteins that disappear from the liver in response to CCl_4 treatment by mass spectrometric techniques is the obvious next step in the study. It would be particularly interesting to see whether any of these proteins match those that appear in the urine samples analysed by 2-DGE (Figures 6.2 and 6.3).

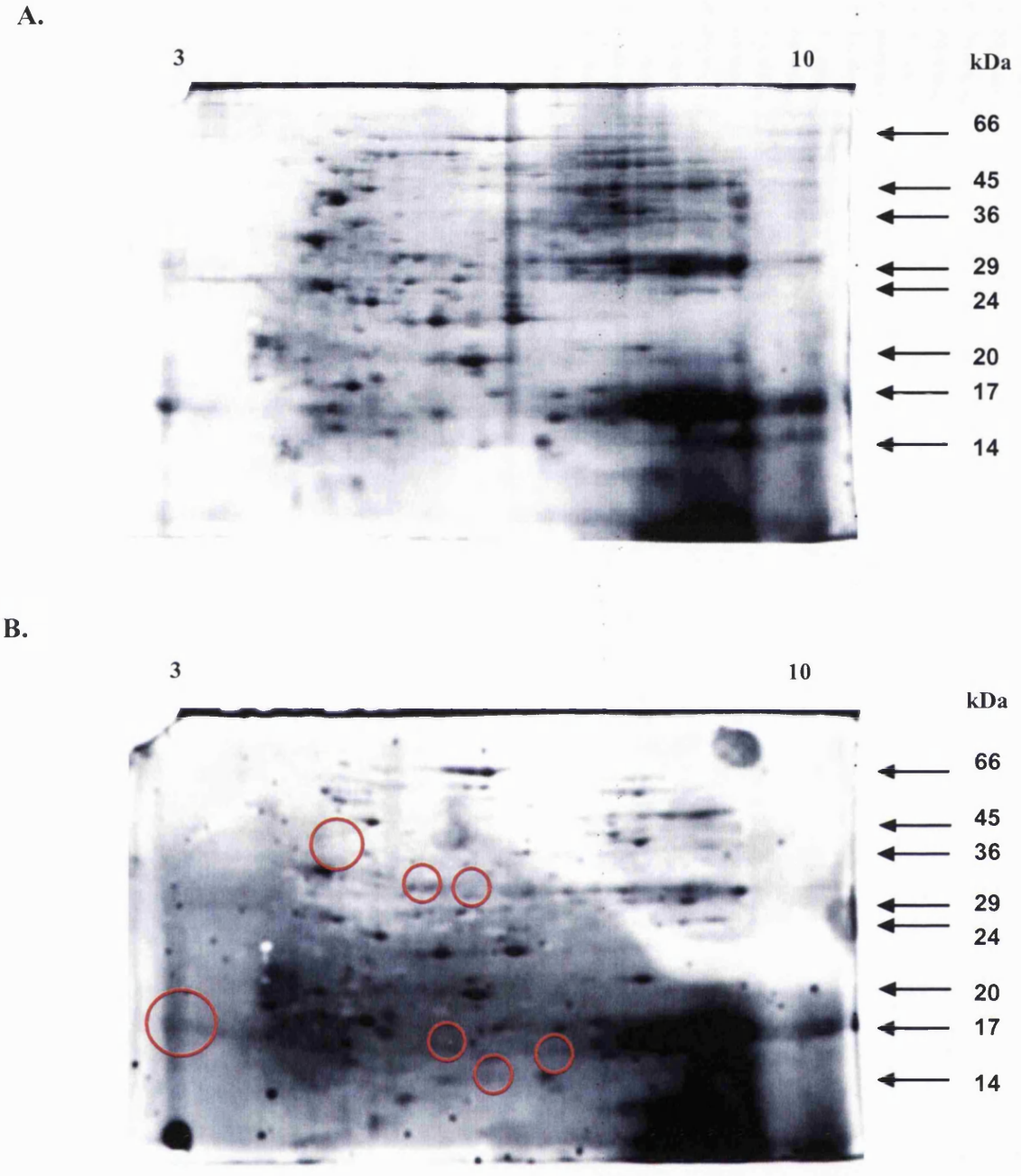


Figure 6.5 2-DGE (pH 3-10) of liver homogenates collected 24 hours after treating rats with a single acute dose of CCl_4.
Female Wistar Han rats were dosed with 0 ml/kg (A. control) or 0.8 ml/kg (B) CCl_4 and livers were collected 24 hours later at post mortem and stored in liquid nitrogen. Liver homogenates were prepared as described in Section 2.7 and equal protein concentrations of homogenate (100 µg) were loaded and isofocussed on pH 3-10 NL IPG strips and the second dimension run on 17 % SDS PAGE. Gels were stained using silver stain as described in Section 2.13.

6.5 Discussion

The only possible protein marker detected in urine samples from rats with hepatic fibrosis, or following acute CCl_4-treatment, using 1D SDS-PAGE was Cu/Zn superoxide dismutase (SOD-1). Two-dimensional gel electrophoresis achieved a much greater separation of urinary proteins. When 2-DGE was applied to the urine samples it became apparent that a far greater number of proteins were increased in concentration in urine from fibrotic rats or rats with hepatotoxicity induced by an acute dose of CCl_4. It is not possible to say without identification whether these proteins were the same in both models of toxicity. Some of them certainly migrated to similar areas in the 2-D gel (compare Figures 6.1 and 6.2).

As seen before, SOD-1 concentration in urine increased in response to fibrosis or acute CCl_4-treatment. This was confirmed by Western blotting with the SOD-1 antibody. The Western blot revealed that a small amount of SOD-1 could be detected using the SOD-1 antibody in control urine. However, Western blotting of the 2D gel of CCl_4-treated urine detected a much greater quantity of SOD, which appeared as a large broad spot on the blot. This verifies a very large increase in the amount of SOD-1 in the rat urine in these models. This was considerably greater than any increase in other proteins that could be distinguished as possible markers for liver injury or fibrosis.

The resolving power of 2-DGE was emphasized when the "urinary proteome" where most of the protein spots are confined to the acidic end of the gel (i.e. between approximately pH 4 and 6), was separated using IPG strips with a narrower (pH 3-6) range (Fig 6.3). This greater resolution showed even clearer differences in the protein make-up of urine from control and treated rats (compare Fig 6.3 A and B).

The proteins that appear in urine from fibrotic rats (Figure 6.1) or rats with acute hepatotoxicity (Figures 6.2 and 6.3) and were detected by 2-DGE have not been identified as yet. This is the important next step. Techniques that have already been applied for the identification of SOD-1 in this study such as in-gel digestion with trypsin and identification by mass spectrometry would seem an obvious route. Unfortunately, time has not allowed embarkation upon such experiments in the current work. It is possible that one or more of these urinary proteins may prove to be liver-

specific and a good urinary marker of liver injury and/or fibrosis. This would further confirm the potential use of urine as a non-invasive method of detecting hepatotoxicity.

2-DGE has become an important tool in the screening for toxicological biomarkers. Venkatraman et al. (2004) detected a total of 43 proteins with altered expression in rat liver following chronic ethanol exposure, of these 13 had increased and 30 had decreased expression levels. Chevalier et al. (2000) exposed rat primary hepatocyte cultures to the peroxisome proliferator nafenopin and using 2-DGE they identified 32 proteins with altered expression.

Myrick et al. (1993) used 2-DGE to detect possible urinary protein biomarkers of occupational exposure of humans to cadmium. Koliakos et al. (2003) identified urinary biochemical markers of early renal dysfunction that were associated with iron overload in beta-thalassaemia. Bandara et al. (2003a and b) successfully identified a potential biomarker of renal proximal tubular toxicity by 2-DGE analysis of plasma.

Two-dimensional gel electrophoresis is also a useful technique for the identification of biomarkers of disease. The method of analysis has been applied to human urine for the diagnosis of renal disease (Tracey et al., 1992; Oda et al., 1996). Lim et al. (2002) examined three tissue types from patients with hepatocellular carcinoma and found a significant change in the expression levels of 21 proteins. The 2-DGE analysis of peripheral blood mononuclear cells from patients with arthritis revealed an increase in the protein expression of 18 proteins (Dotzlaw et al., 2004).

In this study many of the proteins observed in the urine 2D gels appear as spots that are joined together in a horizontal row, such a pattern is likely to be the result of variable post-translational modifications (Thongboonkerd et al., 2002). Post-translational modification of SOD-1 has already been discussed as a possible explanation of the observation that SOD-1 runs on SDS PAGE at a molecular weight higher than that predicted from its sequence. This is supported by the observation that SOD-1 on 2-D gels appears as a row of spots. The SOD-1 subunit is known to have an acetylated amino terminus (Fridovich, 1982).

Studies by Thongboonkerd and colleagues (2002) compared the proteomes obtained for human urine after either ultracentrifugation or acetone precipitation prior to 2D

electrophoresis and found the proteomes obtained with each method were very different. Therefore, it is possible that changing the method of sample preparation used in the present study may produce different urinary rat proteomes following CCl_4-induced hepatotoxicity.

The application of 2D electrophoresis to liver homogenates obtained after treating rats with a single dose of CCl_4 proved that the administration of CCl_4 results in the loss of proteins from the liver. Figure 6.5 clearly shows that fewer proteins are apparent in the liver homogenate from CCl_4-treated rats than controls. This may result from liver necrosis and the breakdown of the hepatocelluar membranes thus allowing the liver proteins to leak into the plasma.

Analysis of tissue targets of toxicity by 2-DGE has been used successfully on many occasions to identify potential biomarkers. Iida et al. (2003) have documented global changes in expression of the mouse liver proteome in response to carcinogens. Another study has identified potential biomarkers of hydrazine-induced hepatotoxicity in rats (Kleno et al. 2004). The identification of protein changes in rat liver elicited by a group of model hepatotoxicants, methapyrilene, cyproterone acetate and dexamethasone offers a compelling argument in support of the use of 2-DGE and mass spectrometry for the identification of specific biomarkers (Man et al. 2002).

Thome-Kromer et al. (2003) identified 163 proteins in rat liver including Cu/Zn superoxide dismutase in a proteome study comparing rat liver with human cells. They used nine hepatotoxicants to induce variations in the rat liver proteome and identified a total of thirteen potential liver toxicity marker proteins, however they were not all liver-specific and included AST.

The present study has highlighted a number of protein changes in urine and liver following CCl_4 administration both as an acute single dose and chronically in the production of hepatic fibrosis. This confirms the potential that 2-DGE has in the identification of urinary markers of toxicity. Future work would require the identification of these proteins in order to decide if any truly represent protein markers of hepatotoxicity or hepatic fibrosis.

Chapter seven

Conclusion

The objective of this project was to identify protein markers of hepatotoxicity using non-invasive methods. A model of hepatotoxicity was developed in Han Wistar female rats by the administration of carbon tetrachloride (Chapter 3) and this was extended to the development of a model of hepatic fibrosis (Chapter 5). The non-invasive method chosen was urine analysis since this is a quick and effective means of determining toxicity, and samples are easily collected. Many biomarkers are already measured in urine, such as the measurement of urinary aldehydes, catecholamines and nitric oxide (Hermanns et al., 1998; Draper et al., 2000; Banerjee et al., 2003). Some previous studies have already successfully identified specific urinary protein markers of disease and toxicity (Dare et al., 2002; Kageyama et al., 2004). Urine samples were collected from our animal models and changes in the protein complement, the "urinary proteome", were analysed using a range of proteomic techniques including SELDI, 1-D SDS-PAGE, 2-DGE and immunochemistry.

It was established that a dose level below 1.2 ml/kg of CCl_4 was necessary in order to avoid nephrotoxicity, since above this level urine from rats contained blood, serum urea and creatinine levels were raised and histological examination of the kidneys revealed injury in the form of degeneration and necrosis of the proximal convoluted tubules. Maximum injury to the liver occurred at 24-36 hours after a single dose of CCl_4, as determined by the increase in activity of serum enzyme markers of liver injury (ALT, AST and GLDH) and by the histological findings of liver vacuolar degeneration, neutrophil infiltration and centrilobular necrosis.

SELDI analysis of urine samples using normal phase chips revealed the appearance of a protein of approximately 15.7 kDa in the urine of CCl_4-treated rats. This protein was not present in any of the control urine samples and as the CCl_4 dose increased, the intensity of the signal peak for this protein generally increased. 1D SDS-PAGE of the urine samples revealed the appearance of a protein band estimated to be 18.4 KDa in the urine of CCl_4-treated rats, which was not present in any of the control urine samples. Nano-ES-MS/MS identified this protein as Cu/Zn superoxide dismutase (SOD-1). SOD-1 is an important enzyme responsible for the scavenging of reactive oxygen species in aerobic organisms and in doing so protects them from oxidative damage (Fridovich, 1982). Studies revealed that SOD was detected in the urine of rats treated with CCl_4 at 0.8 ml/kg from 12 hours post-dosing up to and including 60 hours post-dosing. The time points at which SOD appeared in the rat urine corresponded to

changes in liver histology and increased serum enzyme markers of hepatotoxicity. At 12 hours post-dosing serum AST, GLDH and ALT activities were significantly greater in the CCl_4-treated rats than in control and remained elevated until about 60 hours post-dosing. Therefore the presence of SOD-1 in the urine correlated with changes in the serum AST, ALT and GLDH activity. The histopathological observations of vacuolar degeneration, neutrophil infiltration and centrilobular necrosis had returned to normal by 7 days post-dosing in most animals.

The pattern of appearance of the 15.7 KDa SELDI protein and the 18.4 KDa 1D SDS-PAGE protein was virtually identical throughout these studies suggesting that they were one and the same. As SOD-1 is a homodimer of two subunits with predicted mass of 15.8 kDa each it seems likely that the SOD-1 subunit runs anomalously on SDS-PAGE at 18.4 kDa.

Western blotting with a SOD-1 antibody detected trace amounts of SOD-1 in urine from control rats but confirmed that the amount of SOD-1 was greatly increased in the urine of CCl_4-treated animals. This was supported by the assay of SOD activity, which was much greater (52-fold) in urine samples from CCl_4-treated rats compared to controls. Increases in SOD activity and increases in immunodetectable SOD-1 protein were generally dose-dependent despite animal variation. The lowest dose at which increased SOD-1 could be detected by Western blot in the urine from rats treated with CCl_4 was 0.2 ml/kg. The lowest dose at which an increase in SOD activity was detected was 0.1 ml/kg CCl_4. The ability to observe and measure changes in SOD-1 at these low CCl_4 dose levels and the correlation between the appearance of SOD-1 in urine and the appearance of the serum enzyme markers and histopathology of liver injury suggests that it could be a sensitive urinary marker of hepatotoxicity.

One major misgiving concerning SOD-1 as a marker of hepatotoxicity is its liver-specificity because the enzyme is present in many organs throughout the body (Frederiks and Bosch, 1997). Tissue homogenates from liver, kidney, spleen, heart, lungs, skeletal muscle and small intestine were analysed for changes in SOD-1 following treatment with CCl_4 and Western blotting revealed that SOD-1 was decreased in the rat liver but there was no change in any other tissue analysed (Chapter 4). These results suggest that the appearance of the SOD-1 in the rat urine was due to its release from necrotic hepatocytes and it may well therefore be a potential marker of

hepatotoxicity. However this requires further investigation including changes in urinary SOD-1 in response to damage in other organs for example the heart, kidney and lungs. Experiments could be carried out to induce for example cardiac injury, by the administration of a compound such as doxorubicin. Urine samples from these rats could be collected and analysed using the same methods as for the studies carried out in this project. If SOD-1 was increased in the urine sample in response to the induced cardiac injury then we could confirm that the appearance of increased SOD-1 in the urine was not specific to liver injury. By repeating this experiment in other rat organs we may find that SOD-1 is a protein marker of non-specific organ injury. However, in this project SOD-1 was increased in the rat urine samples and this therefore means it is a non-invasive marker.

Further work is needed in order to determine if other hepatotoxicants such as phenobarbital, diethylnitrosamine and alpha-naphthylisothiocynate would also cause SOD-1 to be released into the urine from hepatocytes. This is important because these compounds have slightly different mechanisms of action. CCl_4 is metabolised by the cytochrome P450 system into the trichloromethyl free radical and this in turn produces further free radicals (Recknagel et al., 1977; Poyer et al., 1978). The role of SOD-1 is removal and protection from free radicals. Thus it is possible that the appearance of SOD-1 in urine is a reflection of the metabolism of CCl_4 and the mechanism of its production of hepatotoxicity via oxygen free radicals. If this was so then one might expect an increase in the expression of SOD-1 in liver in response to CCl_4 and this has been reported in studies using hepatocyte cultures. In the present study it was clearly a loss of SOD-1 from liver tissue that resulted in its appearance in urine and not a general increase in its expression.

CCl_4 was used to successfully produce a rat model of liver fibrosis (Chapter 5). The search for urinary protein markers using previous methods revealed only SOD-1. No other protein differences were observed when the urine samples were analysed using 1D SDS-PAGE. Therefore 2-DGE was used to increase the resolution of the protein components of the "urinary proteome". The 2D gels presented in Chapter 6 clearly suggest that there are many protein differences between the urine samples of control and fibrotic rats and between rats acutely dosed with CCl_4. For the most part these are in the form of the appearance of proteins in the urine in response to hepatotoxicity. In support

of this, 2-DGE analysis of liver extracts revealed the disappearance of proteins in response to CCl_4-treatment.

These proteins have not been identified but could easily be identified by in-gel digestion and mass spectrometry using similar methods to those that identified SOD-1. The protein spots could be cut out and in-gel proteolytic digestion carried out using trypsin. The resulting peptides could then be identified using mass spectrometry. Upon identification, it is possible that one or more of these proteins may be found solely in the liver and therefore their appearance in the urine would only reflect hepatotoxicity. In the event of such a finding further experiments would be needed to establish the sensitivity and specificity of the potential marker. A low dose study such as that described in Chapter four and an early time course study would determine if the protein appeared in the urine following low dose administration of CCl_4 and how soon after administration it could be detected. Further work would include inducing hepatotoxicity by the administration of other compounds such as those described above and analysing the urine for the appearance of the marker protein. If the protein was both liver-specific and sensitive then it could have great potential as a non-invasive marker for hepatotoxicity, especially since liver injury is currently assessed by the measurement of a combination of serum enzymes.

The increased appearance of SOD-1 in the urine following CCl_4-induced hepatotoxicity is a novel finding. This study makes it clear that the analysis of urine samples for protein biomarkers has great potential as a non-invasive method of determining hepatotoxicity. The studies so far indicate that SOD-1 is a potential sensitive urinary protein marker, further investigation could decide if the appearance of SOD-1 specifically indicates liver injury. The fact that SOD-1 can be detected following low doses of CCl_4 and therefore only mild injury, and that it appears in the urine only 12 hours after dosing and can be measured using non–invasive techniques may mean that even if not liver-specific, SOD-1 may be a good marker of general organ injury.

Chapter eight

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