The Assessment of Cardiac Biomarkers in Rat Models of Cardiotoxicity

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A thesis submitted for the degree of Doctor of Philosophy in Toxicology, University of London
ABSTRACT

Cardiotoxicity is an adverse effect of many drugs and chemicals. However, the assessment of toxic myocardial injury in preclinical animal studies for drug safety evaluation has not been optimised. At present, cardiac injury is generally assessed using serum enzymes and these changes are correlated with histopathological lesions in the heart. Nevertheless, it has recently been suggested that the assessment of cardiac injury using serum enzymes as biomarkers lacks sensitivity and it has therefore been proposed that new markers should be identified and validated; a proposed new serum marker is cardiac troponin I (cTnI). The aim of the present investigations was to develop models of cardiotoxicity in the Hanover Wistar rat using isoproterenol (ISO), allylamine (AA) and erucic acid (EA), to assess the usefulness of serum cTnI in comparison with the traditional markers of myocardial injury. Single dose studies with ISO and AA resulted in myocardial degeneration/necrosis, and vasculopathy was also present in the case of AA. These experiments demonstrated that serum cTnI was a sensitive and specific biomarker of acute cardiomyocyte injury. Administration of EA, by gavage or in the diet, induced myocardial lipidosis and necrosis. Serum levels of cTnI were generally elevated in association with myocardial necrosis, but not with lipidosis. It is concluded that cTnI is a sensitive and organ-specific marker of cardiotoxicity in the rat, but further validation studies are required with a wider range of cardiotoxic agents.
ACKNOWLEDGEMENTS

I wish to thank, firstly, my supervisors, John Turton, Bill Griffiths, Malcolm York and Tom Williams for all of their help and support over the past 4 years. Thank you goes especially to John, for his friendship, counsel and coffee! Thank you, Malcolm, for all of your guidance on clinical chemistry and for giving me an insight into what goes on in the pharmaceutical industry. Thank you to all the staff at GlaxoSmithKline for their help and hard work; particular thanks go to Clare Stamp and Silvia Guionaud, for all of their assistance, especially when I was driving them mad with endless, and often idiotic, telephone calls!

I wish to thank Dr. Sandra Kennedy and The School of Pharmacy for financial input, without whom this project would not have taken place.

Finally, I wish to thank my family and friends for all of their support, advice and unfaltering faith in me. I’ve finally done it!
PLAGIARISM STATEMENT

This thesis describes research conducted in the School of Pharmacy, University of London between October 2003 and October 2006 under the supervision of Dr. John Turton. I certify that the research described is original and that any parts of the work that have been conducted by collaboration are clearly indicated. I also certify that I have written all the text herein and have clearly indicated by suitable citation any part of this dissertation that has already appeared in publication.

Signature

Date 2008
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<thead>
<tr>
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<th>Full Form</th>
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<tbody>
<tr>
<td>AA</td>
<td>Allylamine</td>
</tr>
<tr>
<td>ACS</td>
<td>Acute coronary syndromes</td>
</tr>
<tr>
<td>ALP</td>
<td>Alkaline phosphatase</td>
</tr>
<tr>
<td>ALT</td>
<td>Alanine aminotransferase</td>
</tr>
<tr>
<td>ANP</td>
<td>Atrial natriuretic peptide</td>
</tr>
<tr>
<td>AST</td>
<td>Aspartate aminotransferase</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>AV</td>
<td>Atroventricular</td>
</tr>
<tr>
<td>BNP</td>
<td>Brain natriuretic peptide</td>
</tr>
<tr>
<td>CABG</td>
<td>Coronary artery bypass grafting</td>
</tr>
<tr>
<td>CHF</td>
<td>Congestive heart failure</td>
</tr>
<tr>
<td>CHOL</td>
<td>Cholesterol</td>
</tr>
<tr>
<td>CICR</td>
<td>Calcium-induced calcium release</td>
</tr>
<tr>
<td>CK</td>
<td>Creatine kinase</td>
</tr>
<tr>
<td>CK-MB</td>
<td>Creatine kinase-MB</td>
</tr>
<tr>
<td>COMT</td>
<td>Catechol-O-methyltransferase</td>
</tr>
<tr>
<td>CPA</td>
<td>Cyclophosphamide</td>
</tr>
<tr>
<td>CREAT</td>
<td>Creatinine</td>
</tr>
<tr>
<td>cTn</td>
<td>Cardiac troponin</td>
</tr>
<tr>
<td>cTnC</td>
<td>Cardiac troponin C</td>
</tr>
<tr>
<td>cTnI</td>
<td>Cardiac troponin I</td>
</tr>
<tr>
<td>cTnT</td>
<td>Cardiac troponin T</td>
</tr>
<tr>
<td>CVS</td>
<td>Cardiovascular system</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>DOX</td>
<td>Doxorubicin</td>
</tr>
<tr>
<td>EA</td>
<td>Erucic acid</td>
</tr>
<tr>
<td>EC</td>
<td>Excitation-contraction</td>
</tr>
<tr>
<td>ECG</td>
<td>Electrocardiogram</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme-linked immunosorbent assay</td>
</tr>
<tr>
<td>FD</td>
<td>Found dead</td>
</tr>
<tr>
<td>FNCC</td>
<td>Femoral nucleated cell count</td>
</tr>
<tr>
<td>Acronym</td>
<td>Description</td>
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<tr>
<td>---------</td>
<td>-------------</td>
</tr>
<tr>
<td>GFR</td>
<td>Glomerular filtration rate</td>
</tr>
<tr>
<td>GI</td>
<td>Gastrointestinal</td>
</tr>
<tr>
<td>GLD</td>
<td>Glutamate dehydrogenase</td>
</tr>
<tr>
<td>H&amp;E</td>
<td>Haematoxylin and eosin</td>
</tr>
<tr>
<td>HBD</td>
<td>$\alpha$-hydroxybutyric dehydrogenase</td>
</tr>
<tr>
<td>HEAR</td>
<td>High erucic acid rapeseed oil</td>
</tr>
<tr>
<td>H-FABP</td>
<td>Heart fatty acid-binding protein</td>
</tr>
<tr>
<td>ICD</td>
<td>Intercurrent death</td>
</tr>
<tr>
<td>IMDM</td>
<td>Iscove’s Modified Dulbecco’s Medium</td>
</tr>
<tr>
<td>IP</td>
<td>Intraperitoneal</td>
</tr>
<tr>
<td>ISO</td>
<td>Isoproterenol</td>
</tr>
<tr>
<td>IV</td>
<td>Intravenous</td>
</tr>
<tr>
<td>kDa</td>
<td>Kilodaltons</td>
</tr>
<tr>
<td>KIE</td>
<td>Killed <em>in extremis</em></td>
</tr>
<tr>
<td>LD</td>
<td>Lactate dehydrogenase</td>
</tr>
<tr>
<td>LEAR</td>
<td>Low erucic acid rapeseed oil</td>
</tr>
<tr>
<td>MHC</td>
<td>Myosin heavy chain</td>
</tr>
<tr>
<td>MI</td>
<td>Myocardial infarction</td>
</tr>
<tr>
<td>MSB</td>
<td>Martius’ Scarlet Blue</td>
</tr>
<tr>
<td>MW</td>
<td>Molecular weight</td>
</tr>
<tr>
<td>NAD</td>
<td>No abnormalities detected</td>
</tr>
<tr>
<td>NBF</td>
<td>Neutral buffered formalin</td>
</tr>
<tr>
<td>NEFA</td>
<td>Non-esterified fatty acids</td>
</tr>
<tr>
<td>NS</td>
<td>Not statistically significant</td>
</tr>
<tr>
<td>.OH</td>
<td>Hydroxyl radical</td>
</tr>
<tr>
<td>ORO</td>
<td>Oil Red O</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PD</td>
<td>p-phenylenediamine</td>
</tr>
<tr>
<td>PTAH</td>
<td>Phosphotungstic acid haematoxylin</td>
</tr>
<tr>
<td>RCF</td>
<td>Relative centrifugal force</td>
</tr>
<tr>
<td>SA</td>
<td>Sinoatrial</td>
</tr>
<tr>
<td>SC</td>
<td>Subcutaneous</td>
</tr>
<tr>
<td>SD</td>
<td>Standard deviation</td>
</tr>
<tr>
<td>-SH</td>
<td>Thiol</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>SMC</td>
<td>Smooth muscle cell</td>
</tr>
<tr>
<td>SR</td>
<td>Sarcoplasmic reticulum</td>
</tr>
<tr>
<td>sTnI</td>
<td>Skeletal troponin I</td>
</tr>
<tr>
<td>TEM</td>
<td>Transmission electron microscopy</td>
</tr>
<tr>
<td>TMPD</td>
<td>2,3,5,6-Tetramethyl-p-phenylenediamine</td>
</tr>
<tr>
<td>Tn</td>
<td>Troponin</td>
</tr>
<tr>
<td>TnC</td>
<td>Troponin C</td>
</tr>
<tr>
<td>TnI</td>
<td>Troponin I</td>
</tr>
<tr>
<td>TnT</td>
<td>Troponin T</td>
</tr>
<tr>
<td>TP</td>
<td>Total protein</td>
</tr>
<tr>
<td>TRIG</td>
<td>Triglycerides</td>
</tr>
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</table>
CHAPTER 1: INTRODUCTION

1.1 THE HEART

1.1.1 Background

The heart, along with the vasculature, comprises a major organ system of the body, the cardiovascular system (CVS). The CVS plays the important role of supplying the cells and tissues of the body with respiratory gases, nutrients, chemical messengers and metabolites, as well as the removal of waste products of cellular metabolism and foreign materials, such as invading micro-organisms. In addition, the CVS is responsible for the maintenance of the internal homeostasis of the body, as well as the regulation of body temperature and cellular pH (Veinot et al., 2001).

With the role the heart plays in the transport of blood, the organ is exposed to any metabolites that have been absorbed into the bloodstream. Hence, the heart may be susceptible to toxic injury. The severity of the toxic injury will be dependent upon the concentration of a cardiotoxic agent within the myocardium, and also the duration of exposure to the compound itself. Damage to the heart may be due to direct or indirect actions of a toxic agent on the tissue: direct effects on the CVS result in structural and/or functional changes, whereas indirect effects are secondary to changes occurring in other organ systems, such as the endocrine system (Ramos et al., 2001).

1.1.2 Anatomy of the heart

1.1.2.1 Gross anatomy of the heart

The heart is situated in the mediastinum, surrounded by a fibrous sac, the pericardium. The heart is divided by the interatrial and interventricular septums into the right and left halves; each consisting of an atrium and a ventricle. The anatomy of the human heart is shown is Figure 1.1. The right side of the heart represents the low-pressure system and deals with deoxygenated blood, while the left side carries oxygenated blood and is responsible for providing pressure to ensure sufficient organ perfusion (d’Uscio et al., 2000).
Oxygenated blood is brought to the heart from the lungs via drainage through the pulmonary veins into the left atrium. Blood then enters the left ventricle via the left atrioventricular (AV) (bicuspid) valve. Contraction of the left ventricle forces the blood around the body to the tissues via the aorta, arteries, arterioles and the capillaries. The venous system takes deoxygenated blood away from the tissues via the capillaries, venules and veins, where it eventually collects into the vena cavae (superior and inferior), which drain into the right atrium. The deoxygenated blood then passes into the right ventricle via the right AV (tricuspid) valve, which contracts, sending the blood to the lungs through the pulmonary arteries (Figure 1.1). In the rat heart (Figure 1.2), there are 2 precavae present in the vena cava: the right empties into the right atrium and the left joins with the azygous vein before draining into the right atrium (Sharp and LaRegina, 1998). The blood supply to the heart itself is achieved by the coronary arteries, which branch into the intramyocardial arteries that supply a rich capillary bed throughout the organ (Van Vleet et al., 1991).

1.1.2.2 Histology of the heart

The heart wall consists of 3 layers, the endocardium, the myocardium and the epicardium (Figure 1.3). The endocardium is the membrane which covers all the inner surfaces of the heart (and lines the blood vessels) and is composed of a single layer of squamous endothelial cells. The myocardium is the muscle layer of the heart, containing cardiomyocytes, blood vessels and nerves, the epicardium is a thin, serous membrane covering the outer surface of the heart. The epicardium consists of a single layer of mesothelial cells resting on a basal lamina, and a thin layer of loose connective tissue (areolar tissue). The pericardial space contains pericardial fluid, which acts as a lubricant as the heart moves within the pericardium (Rhodin, 1977).

1.1.2.2.1 The myocardium

The myocardium is formed of cardiac muscle cells (cardiomyocytes) and a connective tissue interstitium, with the cardiomyocytes comprising approximately 1 third of the cells within the muscle layer in man (Veinot et al., 2001). The well-vascularised interstitium is formed mainly of fibroblasts, with a small number of other cells, such as adipocytes and leukocytes (Van Vleet et al., 1991). In sections examined for histology, the thickness of the myocardium is dependent on the pressures present in each chamber: the atria are thin and the ventricles are thick. In addition, the left ventricular
myocardium is thicker than that of the right ventricle, due to the higher pressures in the systemic (rather than the pulmonary) circulation.

1.1.2.2 The cardiomyocytes

The cardiomyocytes are the energy-generating cells within the myocardium and are specialised, elongated, striated muscle cells (myofibres), which contain 1 or 2 centralised nuclei, a continuous limiting plasma membrane (the sarcolemma) and numerous contractile myofibrils surrounded by sarcoplasm (Netter, 1969). The contractile elements, constituting approximately 50% of the cell, form into myofibrils that are separated by the interfibrillar matrix, which contains the mitochondria, the sarcoplasmic reticulum (SR) and T tubules, the Golgi apparatus, glycogen and lysosomes (Van Vleet et al., 1991). The cardiomyocytes are linked together by the intercalated discs, at which the membranes of adjacent cardiomyocytes are connected by desmosomes and linked by gap junctions (Figure 1.4). The intercalated discs allow the propagation of the cardiac action potential (Forbes and Sperelakis, 1985).

The atrial and ventricular myocytes are generally similar in structure and function; however atrial myofibres are smaller than ventricular cardiomyocytes and the sarcoplasmic reticulum and T tubule system is less well-developed (Veinot et al., 2001).

1.1.2.2.3 The myofibrils

There are 2 types of myofilaments present in the cardiomyocytes, the thick and thin elements, composed of myosin and actin (and associated proteins), respectively. The orderly alignment of the myosin and actin form the contractile units, the sarcomeres (Figure 1.5), which give cardiac muscle (and skeletal muscle) a striated appearance. The thin filaments are attached at their plus ends to the Z disc at each end of the sarcomere; while the capped minus ends extend towards the middle of the sarcomere, where they overlap with the thick filaments. The accessory proteins ensure the uniformity of the organisation, length and spacing in the sarcomere. Tropomodulin caps and stabilises the minus ends of the thin filaments, nebulin stretches between the Z disc (composed of capZ and α-actinin) and the minus end of the thin filament to dictate the length of the sarcomere, and titin acts as a molecular spring to maintain the thick filaments in the centre of the sarcomere (Alberts et al., 2002; Panaviene et al., 2007).
The SR is a specialised form of smooth endoplasmic reticulum necessary in the transmission of the cardiac impulse and also in the uptake, storage and release of calcium ions for muscle contraction (Van Vleet et al., 1991). At the Z discs, T tubules extend inward from the sarcolemma, making contact with the SR. The SR is also in close contact with the contractile apparatus of the cell (Scheuermann, 1993). Following the generation of a cardiac action potential, the T tubules spread the impulse through the myofibre, resulting in the release of calcium ions from the SR via the opening of calcium release channels (Veinot et al., 2001). Calcium is then available for myocyte contraction, as described below.

1.1.3 Physiology of the heart

1.1.3.1 The action potential and the cardiac impulse

Unlike skeletal muscle, cardiac tissue is able to contract without external neural or hormonal stimuli. The pacemaker activity of the heart is generated in the sinoatrial (SA) node by a specialised cell population adapted to the generation of a rhythmic electrical impulse (Kléber and Rudy, 2004). Cells within the SA node spontaneously depolarise and repolarise to generate an action potential, which electrically excites the sarcolemma. The wave of depolarisation travelling from the SA node spreads rapidly across the atria and to the atrioventricular (AV) node. It is conducted via the gap junctions at the intercalated discs (Forbes and Sperelakis, 1985). Conduction is slowed through the AV node, so as to allow contraction of the atria prior to ventricular contraction (Martini, 2006). The action potential then passes along the interventricular septum with the AV bundle (bundle of His) to the Purkinje fibres, which stimulate the ventricles to contract. In this way, the heart beats in a highly controlled fashion.

1.1.3.2 Excitation-contraction coupling

When the wave of depolarisation reaches the sarcolemma of a cardiomyocyte, the drop in membrane potential opens L-type calcium channels to allow a small influx of calcium into the cardiomyocyte, which then binds to the ryanodine receptors on the SR to release large amounts of Ca$^{2+}$ (Fabiato, 1983). This process, termed calcium-induced calcium release (CICR), underlies the process of excitation-contraction (EC) coupling in
the heart (Lukyanenko et al., 1996). Conversely in skeletal muscle contraction, EC coupling is not controlled by CICR; rather it is effected by voltage-gated calcium release (Gordon et al., 2000).

1.1.3.3 Innervation of the heart

Nervous control of the heart is regulated by the sympathetic and parasympathetic branches of the autonomic nervous system. The release of noradrenaline from the sympathetic fibres results in increased automaticity, positive inotropy and positive chronotropy; whereas the vagal parasympathetic system, through release of acetylcholine, causes decreased automaticity, negative inotropy and inhibition of the AV conduction (Rang et al., 1999).

1.1.3.4 The endocrine function of the heart

Secretory granules were first identified in the atrial cardiomyocytes by Jamieson and Palade (1964), and have been shown by others to contain atrial natriuretic peptide (ANP) (Vuolteenaho et al., 1985; Pucci et al., 1992). Furthermore, it has been demonstrated that ventricular cardiomyocytes also may secrete natriuretic peptide; namely brain natriuretic peptide (BNP) (De Lemos et al., 2003). The natriuretic peptides play a role in the regulation of fluid volume, blood pressure and electrolyte balance (Apple and Jaffe, 2006). Hence, the heart also has an endocrine function in addition to its role in circulation. ANP and BNP are released in response to atrial and ventricular stretch, respectively, caused by volume overload (Clerico et al., 2006). The peptides cause vasorelaxation, inhibition of aldosterone secretion in the adrenal cortex, and inhibition of renin secretion in the kidney, as well as natriuresis and a reduction in intravascular volume, and these effects are augmented by antidiuretic hormone antagonism (Cho et al., 1999).

1.1.3.5 Myocardial metabolism

Cardiomyocytes have a high metabolic rate and rely on the production of high energy phosphates (adenosine triphosphate, ATP, and creatine phosphate) by the mitochondria from energy sources such as lactate, glucose, fatty acids and triglycerides. Under normal aerobic conditions and at normal workloads, the heart generates energy primarily by the
terminal (β-) oxidation of free fatty acids, whereas glucose metabolism is a much less important source of energy (Taegtmeyer, 1994).

### 1.1.4 Muscle contraction

Myocyte contraction is mediated by changes in intracellular calcium concentration, with myosin ATPase acting as an energy source, in the presence of magnesium ions (Isaacs, 1998). CICR results in a rise in intracellular calcium, which binds to troponin C (TnC), part of a 3 protein complex bound to tropomyosin, situated on the thin filament (Figure 1.6). The troponin complex is comprised of 3 subunits: TnC, the calcium binding subunit, TnI, the inhibitory subunit, and TnT, the subunit bound to tropomyosin (Metzger and Westfall, 2004). The binding of calcium to TnC results in a rearrangement of the troponin complex, so that tropomyosin is removed from its blocking position and the actin binding sites are revealed for myosin attachment (Moss et al., 2004) (Figure 1.7). This allows the actin and myosin filaments to interact, resulting in shortening of the sarcomere and muscle contraction; this process is referred to as the sliding filament theory (Cooke, 2004).

Relaxation of the cardiomyocytes requires a reduction in the intracellular calcium concentration, and in mammalian ventricular myocytes, there are 4 systems responsible for this process: the SR calcium ATPase (which actively pumps calcium back into the SR), the sarcolemmal sodium-calcium exchange (which pumps calcium out of the cell), the sarcolemmal calcium ATPase (which actively extrudes calcium from the cell) and the mitochondrial calcium uniporter (which sequesters calcium into the mitochondria) (Frank et al., 2003). The relative role of each system varies with species; in the rat, approximately 92% of calcium removal is via the SR calcium ATPase (Bers et al., 1996).

### 1.2 TOXIC RESPONSES OF THE HEART

The heart is particularly vulnerable to injury due to the limited proliferative capacity of cardiomyocytes (Bicknell et al., 2007). Compensatory hypertrophy of remaining cardiomyocytes is a hallmark of cardiac remodelling following myocardial damage (Scheuermann, 1993). Moreover, following cardiac injury, cardiac fibroblasts are also involved in tissue remodelling in repair, resulting in fibrosis and the formation of scar tissue. Therefore, if cells are irreversibly damaged and lost, there is little or no
regeneration of the myocardium and the cardiomyocytes are replaced by scar tissue. This will therefore generally impair myocardial function, because the tissue will no longer be contractile. Nevertheless, there is some emerging evidence that there may be a small population of cardiac stem cells which may cause some cardiac regeneration following damage; however more research is needed in this highly controversial area (Leri et al., 2005; Ahuja et al., 2007; Anversa et al., 2007).

The types of injury which may occur in the heart may be divided into 2 categories: structural and functional changes. Functional changes involve alterations in myocardial contractility, while structural changes are the morphological responses to cardiac injury and thus affect the organisation of the cardiomyocyte itself, and may be detected by microscopic examination.

### 1.2.1 Functional changes

The most important alterations to cardiac function generally arise from alteration to membrane function, in particular in ion transport and in the energy-producing systems of the cell, i.e. the mitochondria (Van Vleet et al., 1991).

#### 1.2.1.1 Arrhythmias and changes in contractility

An arrhythmia is defined as any variation from the normal rhythm of the heartbeat, which may be an abnormality of the rate, regularity, or site of impulse origin or the sequence of activation (Dorland, 2003). Compounds which directly influence the initiation or propagation of the cardiac impulse by disrupting the ionic gradient and fluxes are mainly involved in the induction of arrhythmias (Van Vleet et al., 1991). For example, ethanol is known to inhibit the sodium-potassium pump (Habuchi et al., 1995), which will cause a decrease in membrane potential, leading to depolarisation and contractions. Grayanotoxin, found in rhododendrons, opens fast sodium channels, and this also leads to depolarisation (Yuki et al., 2001), whereas tetrodotoxin blocks fast sodium channels, which results in cardiac arrest (Lazdunski and Renaud, 1982).

Myocardial contractility is dependent on intracellular calcium concentration, as described earlier. Heavy metals, such as lanthanum, manganese and nickel, block calcium channels, resulting in a negative inotropic effect (Van Vleet et al., 1991). In contrast, the cardiac glycosides, such as digoxin, partially inhibit the sodium-potassium...
exchanger, which allows more calcium to enter the cell, increasing the intracellular concentration, causing a positive inotropic effect (Schoner and Scheiner-Bobis, 2007).

1.2.2 Structural changes

In cardiotoxicity, the correlation between functional alterations and morphological changes is not strong. For example, bradycardia, tachycardia and arrhythmias are not always accompanied by pathological lesions (Gopinath et al., 1987). Lesions observed following toxic injury include multifocal myocardial necrosis, focal myolysis, vacuolation, and varying degrees of inflammatory and fibrotic changes. Histopathological changes may be sublethal (i.e. they may be reversible) or lethal, which results in cell death. Sublethal alterations include vacuolar degeneration, myofibrillar degeneration, fatty degeneration, pigment deposition, atrophy and hypertrophy; cardiomyocyte cell death may occur by apoptosis or necrosis. Table 1.1 presents some compounds which may cause the sublethal types of injury.

1.2.2.1 Vacuolar degeneration

Vacuolar, or hydropic degeneration, is the appearance of large clear fluid-filled vacuoles within the myocardial fibres. Vacuolar degeneration is a result of distension of the T tubules and SR, with resulting lysis of the contractile material (Van Vleet and Ferrans, 1986). Vacuolar degeneration is a histopathological change characteristic of anthracycline cardiotoxicity, and has been observed in man (Lefrak et al., 1973) and in experimental animals, including the rat (Villani et al., 1991), the rabbit (Jaenke, 1974) the dog (Solcia et al., 1981), the pig (Van Vleet et al., 1979) and the monkey (Sieber et al., 1980).

1.2.2.2 Myofibrillar degeneration

Myofibrillar degeneration, also known as myolysis or myocytolysis, is characterised by pale, eosinophilic cytoplasm with a loss of cross-striations (Van Vleet et al., 1991). Affected cardiomyocytes show a loss of intact myofibrils, with scattered masses of free thick and thin filaments, clumps of Z band material and accumulations of cytoskeletal filaments (Van Vleet and Ferrans, 1986). The loss of myofibrils results in a loss of contractility and resilience of the myocardium, which may lead to ventricular dilatation and heart failure (Isaacs, 1998). Myofibrillar degeneration has been reported to occur
following plasmocid treatment in the rat (D'Agostino, 1963) and furazolidone administration in poultry (Webb and Van Vleet, 1991). Myofibrillar degeneration is a common observation following sympathomimetic agent toxicity (Rona et al., 1959a; Fineschi et al., 1997). Oehmichen et al. (1990) studied the diagnostic significance of myofibrillar degeneration in a number of forensic pathology specimens, and found that the histopathological change may be seen in cases of acute morphine intoxication, hanging, strangulation, drowning, acute haemorrhagic shock and lethal acute brain injury.

1.2.2.3 Fatty degeneration

To the pathologist, the simple accumulation of neutral lipid in the cardiomyocyte cytoplasm is considered to be a degenerative, but reversible change (Isaacs, 1998). Lipid droplets may appear as empty vacuoles under the microscope, due to the solubility of the lipid in the solvents used in slide preparation. Myocardial lipidosis, or fatty degeneration, may be induced in a number of ways, including the overnight fasting of obese Zucker rats (Young et al., 2002), the feeding of a low potassium diet (French, 1952), the feeding of a high fat, choline deficient diet (Wilgram et al., 1954) and the feeding of erucic acid (EA), which is a component of rapeseed oil (Abdellatif and Vles, 1970). Cardiac lipidosis has, in addition, also been causally linked to cardiac dysfunction (Zhou et al., 2000); however it appears that this only occurs when the fatty infiltration is a permanent pathological change.

1.2.2.4 Pigment deposition

Lipofuscinosis is a spontaneous condition, which increases in incidence and severity with age. When such changes occur in the heart, it is referred to as brown atrophy (Anderson, 1985). Brown atrophy may also occur in cachexia and has been observed as a toxic response in the rat following the administration of Brown FK and chloroquine (Van Vleet and Ferrans, 1986).

1.2.2.5 Cardiac atrophy

Cardiac atrophy is a decrease in the size, strength, weight, and activity of the heart. Atrophy may be a compensatory response, as a result of decreased demand upon the heart muscle (Ito et al., 2003). Angiotensin-converting enzyme inhibitors are used to
treat hypertension by inducing vasodilation and decreasing peripheral resistance, thereby decreasing demand on the heart (Rang et al., 1999). The reduced circulatory demand may therefore cause atrophy of the myocardium.

1.2.2.6 Cardiac hypertrophy

Cardiac hypertrophy is an enlargement of the heart muscle mass, through enlargement of individual cardiomyocytes. This may be a physiological adaptation as is seen in exercise training (Fagard, 2003), a toxicological response to thyroxine (Craft-Cormney and Hansen, 1980), long-term isoproterenol (ISO) administration (Kizaki et al., 2005), potassium channel agonists, noradrenaline, vasodilating agents and calcium channel blockers (Isaacs, 1998), or myocardial repair (Scheuermann, 1993).

1.2.2.7 Apoptosis

Apoptosis is characterised by cell shrinkage, membrane blebbing, nuclear condensation and DNA fragmentation (Haunstetter and Izumo, 1998). Apoptosis, unlike necrosis, is an active process, and the switch between apoptosis and necrosis is dependent on intracellular ATP levels (Kang, 2001). Cardiomyocyte death by apoptosis is associated with ischaemic injury (Takemura and Fujiwara, 2006) and also in anthracycline-induced cardiac injury (Koh et al., 2002).

1.2.2.8 Necrosis

The term “necrosis” refers to the phase of degradation of cell components following irreversible cell injury in vivo. The most frequent sites of necrosis within the heart are in the left ventricular myocardium and subendocardium, often due to disturbances in vascular perfusion, a change characteristically observed with ISO administration (Rona et al., 1959a; Gopinath et al., 1987). The characteristic features of cardiomyocyte necrosis are increased eosinophilia, with nuclear pyknosis or karyorrhexis (Isaacs, 1998). Gradually, the cellular debris is removed by phagocytosis. A zone of intense inflammation often surrounds the necrotic area which activates complement and other pathways, leading to the inflammatory response (Trump et al., 2001).

There are 2 types of cardiomyocyte necrosis, coagulation necrosis and contraction band necrosis. Coagulative necrosis of the myofibres is characterised by glycogen depletion,
mitochondrial swelling, mild intracellular oedema, margination of nuclear chromatin, holes in the sarcolemma and relaxation of the myofibrils (Van Vleet et al., 1991). The necrotic cells tend to retain their cellular outline and the cardiomyocyte, devoid of its nucleus, appears as a mass of coagulated, pink-staining, homogeneous cytoplasm (Chandrasoma and Taylor, 1991). Coagulation necrosis occurs in areas of ischaemia, where there is no reperfusion of the tissue, such as in the central area of an infarct. In contrast, contraction band necrosis is characterised by hypercontraction of the myofibrils, with aggregated clumps of disorganised thick and thin filaments, associated with areas of sarcoplasm relatively devoid of myofibrils. Contraction bands form when cardiomyocytes die while being perfused and are found at the periphery of infarcts and during metabolic cell injury caused by catecholamines (Ganote, 1983).

Spontaneous foci of necrosis in the myocardium in the rat are a relatively common occurrence, and are generally referred to as an age-related change, particularly in the Sprague Dawley strain (Kemi et al., 2000).

1.2.2.9 Fibrosis

Cardiomyocyte necrosis is generally followed by leukocytic invasion and phagocytosis of the sarcoplasmic debris. Subsequent to these processes is a proliferation of fibroblasts, resulting in the deposition of connective tissue, which appears grossly as white scarring within the myocardium (Van Vleet et al., 1991).

1.3 BIOMARKERS OF TOXIC CARDIAC INJURY

A biological marker, or biomarker, may be defined as “a characteristic that is measured and evaluated as an indicator of normal biological processes, pathogenic processes or pharmacological responses” (Biomarker Definitions Working Group, 2001). In the heart, following structural damage to the cardiomyocyte, there will be a leakage of cellular constituents into the cardiac interstitium, followed by drainage into the lymphatics and microvasculature, and eventually the intracellular macromolecules may be measured and used in the detection of myocardial tissue injury (Antman, 2002).

The ideal biomarker for cardiac injury in the preclinical and clinical setting must meet a number of criteria (Table 1.2); however it must be pointed out that no single cardiac marker satisfying all the ideal criteria yet exists. The ideal cardiac biomarker should
have high specificity for cardiac tissue, so that the diagnosis of myocardial damage is absolute. Cardiac troponin I (cTnI), for example, is found only in myocardial tissue (Bodor et al., 1995) and is therefore a specific biomarker of cardiac injury. The creatine kinase isoenzyme MB (CK-MB) on the other hand, is a commonly used cardiac marker, but a low concentration of the isoenzyme is also found in skeletal muscle; therefore skeletal muscle damage may result in an increase in CK-MB activity in the blood (Adams et al., 1993a). If the marker is not expressed in the circulation of the healthy individual, then any slight deviation from the base line, indicating a mild injury, may be detected (Dolci and Panteghini, 2006). A high tissue/serum(plasma) ratio ensures sensitivity.

The requirement that marker release should be in proportion to the magnitude of the cardiac damage allows the use of cardiac biomarkers in the estimation of the size and the severity of injury (Kemp et al., 2004). For example, Aartsen et al. (2000) showed that the 4 h heart fatty acid-binding protein (H-FABP) plasma concentration was significantly correlated to the size of infarct in coronary artery-ligated mice as assessed 7 days after surgery. In addition, Ooi et al. (2000) found correlations between ante-mortem serum levels of CK-MB, cTnI and cardiac troponin T (cTnT) and post-mortem histological cardiac findings in man.

The molecular size of the biological marker will have an effect on release kinetics, as in general, small molecules are released at a faster rate than large molecules (Adams, 1999). Likewise, the intracellular compartmentalisation of the cellular constituents will also have an effect on the time course of release following injury. For example, a cytosolic protein will be released more rapidly than a structural protein, as only membrane disruption is required to bring about cytosolic leakage. However, for structural proteins, there must be both sarcolemmal disturbance and a dissociation and/or degradation of the subcellular contractile apparatus (Mair, 1997). Some cardiomyocyte cytoplasmic proteins (e.g. myoglobin, lactate dehydrogenase, LD) are released when there is reversible or irreversible damage to the sarcolemma; whereas the release of troponins, which are structural proteins, is a better indicator of actual cell death (Chesebro, 1997). However, due to a large molecular mass, the cytoplasmic CK-MB is only released when there is irreversible damage to cardiomyocytes (Wu, 1999).

A biomarker must persist in the circulation long enough to allow detection, without masking any recurring (e.g. a reinfarction) or underlying chronic injury. For example,
following MI in man, CK-MB activity is elevated for 2 to 3 days (Kemp et al., 2004). Although the cardiac troponins (cTns) have relatively short half-lives, the continuing liberation of the protein from the contractile apparatus explains the prolonged elevation of the cTns following cardiac injury (Adams, 1999). Figure 1.8 shows the time course of appearance of various cardiac markers in man after acute myocardial infarction (MI).

1.3.1 Aspartate aminotransferase

The appearance of AST in the serum of patients with acute MI was first reported by LaDue et al. (1954), who showed that serum AST activity was elevated a few hours after acute MI, peaked at 2 to 3 days and returned to baseline control levels within a week of injury. However, AST is not cardiac specific, and is found in significant quantities in a number of tissues including the liver, skeletal muscle and in erythrocytes (Wu, 1999; Dolci and Panteghini, 2006) and therefore the marker is not recommended for the diagnosis of cardiac injury in man or in experimental animal studies (Evans, 1991; Panteghini et al., 1999).

1.3.2 Creatine kinase

Creatine kinase (CK) is an important enzyme involved in cellular energetics and muscle metabolism. The CK reaction is essential for the rapid resynthesis of ATP when muscles are working, as it catalyses the reversible transfer of a phosphate residue in a reaction between ATP and creatine. There are 2 cytosolic subunits of CK: M (standing for muscle) and B (standing for brain). These subunits combine to form 3 dimeric isoenzymes, CK-MM, CK-BB and CK-MB, with molecular weights of between 80 and 86 kDa (Walliman et al., 1992). Cytosolic CK exhibits significant binding to myofibrils in both heart and skeletal muscle. A mitochondrial form of CK also exists, and this enzyme is also a dimer consisting of possibly identical subunits (Mair, 1997). The measurement of “total CK” in a patient serum or plasma sample refers to the collective activity of the 3 isoenzymes.

The CK isoenzymes display some tissue specificity, with CK-MM being the predominant form in skeletal muscle and CK-BB the major type in the brain and in other tissues (Wallace et al., 2004). CK-MB is found mainly in the myocardium, with trace amounts present in skeletal muscle. Although the distribution and amounts of the CK isoenzymes within tissues are generally specific, this may alter during development.
(Ingwall et al., 1980) and in cardiac disease (Nascimben et al., 1996). Indeed, the accumulation of B-containing CK isoenzymes (CK-MB and CK-BB) within the myocardium has been proposed as a marker for cardiac hypertrophy (Ye et al., 2001).

Both CK-MM and CK-MB are present in the myocardium; however the majority of CK activity in both cardiac and skeletal muscle is due to the presence of CK-MM. Fredericks et al. (2001) measured the CK-MB content in heart and skeletal muscle in various laboratory animals, and expressed CK-MB content as a percentage of total CK. It was reported that in skeletal muscle tissue, CK-MB content was less than 3 % in rat, dog, pig and monkey, and 7 % in human tissue. In the myocardium, the CK-MB/total CK ratio was more than 20 % in rat and human, and 8 %, 3 % and 5 % for dog, pig and monkey, respectively. Welsh et al. (2002) determined CK-MB concentrations in the left ventricular myocardium of post-mortem hearts of male patients of varying ages and found an 86.0-fold greater concentration of CK-MB in the myocardium of the older population (with or without cardiac disease) compared to that of the younger group (with no cardiac disease). Nascimben et al. (1996) studied CK-MB concentrations in normal and diseased human myocardium and demonstrated that total CK activity was reduced significantly in the left ventricle of failing hearts compared with healthy hearts, but CK-MB activity was increased approximately 7-fold in the failing hearts compared with control (27.3 % in failing hearts compared with 2.4 % in healthy individuals, p<0.0005). This increase in CK-MB activity is a compensatory mechanism in response to decreasing CK-MM activity in the diseased myocardium. These results agree with those of Voss et al. (1995), who suggested that CK-MB is upregulated following myocellular damage as a protective and reparative mechanism.

CK-MB is also present in some other tissues such as uterus, prostate and small intestine (Wallace et al., 2004), but again only in trace amounts. Therefore, CK-MB was, in the past, the isoenzyme marker of choice for the diagnosis of myocardial injury. Additionally, as mentioned previously, the large molecular mass of CK-MB means that it is only released when there is irreversible damage to the myocyte.

1.3.2.1 Creatine kinase-MB release in myocardial injury

Following acute MI and irreversible minor myocardial damage in man, the time profile of CK-MB release is monophasic in nature (Wu, 1999). In acute MI, release occurs within 1 to 3 h of symptom onset; evidence of pathological serum levels are seen at 6 to
10 h and serum levels peak on average at 24 h (Apple, 1992). However, peak serum levels occur earlier with non-Q wave infarctions (at 15 h following the onset of symptoms) than with Q wave infarcts (at 28 h) (Puleo and Roberts, 1988).

Upon release, CK-MB is taken up into the lymphatics and bloodstream. Degradation by proteolysis occurs in the reticuloendothelial system, and is not affected by changes in heart rate or cardiac output, or in hepatic or renal disease (Chesebro, 1997). However, the appearance (and thus, the disappearance) of myocardial proteins in the circulation is known to be dependent on myocardial infarct perfusion (Christenson et al., 1990). CK-MB levels generally return to baseline levels by 36 to 72 h (Adams et al., 1993a).

1.3.2.2 Alterations in levels of creatine kinase-MB in non-cardiac injury and false elevations

Although CK-MB is present in non-cardiac tissues in only trace amounts, damage to skeletal muscle and other tissue injury may elevate circulating levels. CK-MB may be elevated in acute muscle disease, chronic muscle disease and after intense exercise (Adams et al., 1993b). For example, Silverman et al. (1974) found that the CK-MB activity in the skeletal muscle of patients with Duchenne muscular dystrophy was increased to more than 10 % of the total CK activity (compared with 7 % in healthy individuals, as reported by Fredericks et al., 2001). The CK-MB release after rigorous exercise is associated with skeletal muscle necrosis (Adams et al. 1993a). Furthermore, CK-MB elevations have been reported by Char et al. (1998) to occur in rhabdomyolysis. Gloor et al. (1977) showed that intramuscular injections of the drugs benzcoctamine, diazepam and pethidine in dogs led to an increase in serum total CK activity, whereas the drugs administered by intravenous (IV) injection caused no such increases. Microscopic examination of the muscle tissue showed pathological changes, suggesting that the striated muscle was the source of the CK. Therefore, the observed CK elevations were a result of muscle injury.

False elevations in CK-MB have been shown to occur in patients with renal insufficiency (McLaurin et al., 1998) and patients on renal dialysis may have increased CK-MB levels (Chesebro, 1997). CK-MB levels may also be raised in hypothyroidism, as a result of myopathy (Adams et al. 1993a). Hypothyroidism decreases the rate of clearance of CK from the reticuloendothelial system, whereas hyperthyroidism enhances clearance (Chesebro, 1997). CK-MB levels may also be elevated in malignant
conditions. Annesley and McKenna (1983) report on a case of colon carcinoma where the patient had high circulating levels of CK-MB. A second case of a possible neoplasia-derived elevation in CK-MB was described by Isotalo et al. (1999) in a patient with metastatic alveolar rhabdomyosarcoma. In both cases, cardiac injury was ruled out and it was postulated that the malignant cells were the source of the CK-MB expression.

Some drugs have also been shown to cause false elevations in CK-MB activity, although not due to the route of administration. Compounds causing false elevations in CK-MB activity include benzodiazepines, tricyclic antidepressants, high dose aspirin and pyridoxine (vitamin B₆) (Chesebro, 1997).

1.3.3 Lactate dehydrogenase

The lactate dehydrogenases (LDs) are cytosolic tetrameric enzymes which exist as a number of isoenzymes. The L-LDs catalyse the reversible oxidation of L-lactate to pyruvate in the final stages of glycolysis. There are 5 isoenzymes composed of either muscle (M) or heart (H) subunits, and a sixth composed of C subunits. Although LD is located almost ubiquitously, with highest activities in skeletal muscle, liver, heart, kidneys, brain, lungs and erythrocytes (Mair, 1997), the existence of isoenzymes gives some aspect of tissue specificity. The 5 isoenzymes are named according to their mobility during electrophoretic separation: LD₁ consists of four H subunits, LD₅ of four M subunits, with LD₂, LD₃ and LD₄ being hybrids of the two main subunits (Evans, 1991).

1.3.3.1 Lactate dehydrogenase release in myocardial injury

LD₁ is the principal isoenzyme in the heart, but is also present in the brain, pancreas, kidney, stomach and erythrocytes (Adams et al., 1993a). In humans, there is normally more LD₂ than LD₁ in serum; however during myocardial damage, the LD₁ isoform is released and there is a reversal in the LD₂/LD₁ ratio, known as the ‘LD flip’ (Chu et al., 2002). In animals however, the circulating isoforms and ranges may vary. For example, in rats, the predominant isoenzyme in normal plasma is LD₅. Therefore, when myocardial damage has occurred, a significant increase in LD₁ levels may be needed before there is noticeable alteration in the total LD value (Evans, 1991). Nevertheless, in
man and in all experimental animal species, increased circulating levels of LD1 and LD2 is generally supportive of myocardial injury (Walker, 2006).

During acute MI, LD activity begins to increase 6 to 12 h from onset of chest pain. Levels peak 1 to 3 days later, and return to normal 8 to 14 days after the cardiac event (Mair, 1997). Smit et al. (1987) showed that the LD enzymes are cleared from the circulation by the reticuloendothelial system, in particular the Kupffer cells. Due to its broad diagnostic window, LD activity has been used mainly to diagnose acute MI patients who present late (48 to 72 h) after the onset of symptoms (Chesebro, 1997). Therefore the long time course of LD release is advantageous in the diagnosis of acute MI when CK-MB levels have returned to normal (Mair, 1997).

Although LD1 has been shown to be useful in the diagnosis of acute MI and myocardial damage, it is not a cardiac-specific enzyme, and circulating levels may be elevated following damage to any of the tissues where LD1 is located (i.e. brain, kidneys, pancreas, stomach or erythrocytes). In addition to the diagnosis of MI, the elevation of LD1 has been used to diagnose some haemolytic disorders, such as megaloblastic, pernicious and haemolytic anaemia (Panteghini et al., 2006). LD1 is also a tumour marker for testicular germ cell tumours (von Eyben, 2003). As LD1 is contained within erythrocytes, haemolysis may cause false positives in the diagnosis of myocardial injury, and levels must be corrected by measurement of free plasma haemoglobin (Ladi et al., 1990).

Therefore, due to its lack of tissue specificity and long time course of release, LD isoenzyme profiling is no longer recommended in the diagnosis of cardiac injury (Wu et al., 1998).

### 1.3.4 Myoglobin

Myoglobin is a haem-carrying protein located in the cytoplasm of cardiac and skeletal muscle myocytes, making up approximately 2% of the total muscle protein (Kemp et al., 2004). The relatively small size of myoglobin (17816 Da in man, Anderson, 1972) ensures a rapid release of the biomarker from the cardiomyocyte; however the cytoplasmic release of the protein means that it may be elevated in the circulation following reversible damage to the myocardial cells. In addition, the amino acid sequence of myoglobin in cardiac and skeletal muscle is identical and therefore the protein lacks specificity and will be increased following both myocardial and skeletal
muscle damage. Following acute MI in man, myoglobin appears in the circulation at 1 to 3 h following symptom onset and returns to base line levels at around 16 to 24 h (Chu et al., 2002). Therefore, the diagnostic time window of myoglobin is relatively short. In addition to concentrations being raised subsequent to myocardial damage, serum myoglobin levels are elevated in skeletal muscle and neuromuscular disorders, following strenuous exercise and after exposure to several toxins and drugs (Plebani and Zaninotto, 1998). Circulating levels of myoglobin are also raised in patients with renal insufficiency, due to a reduced renal clearance (Azzazy and Christenson, 2002).

In animals, myoglobin may be used as a marker of myocardial damage; however myoglobin structure varies greatly between species, so the differing amino acid sequences alter the immunoreactivity of the protein, which may prevent the use of particular screening methods, such as a radioimmunoassay in certain species (Evans, 1991).

1.3.5 Heart fatty acid-binding protein

Heart fatty acid-binding protein (H-FABP) is a member of a multigene family of relatively abundant cytoplasmic low molecular weight proteins, the fatty acid-binding proteins (FABPs), involved in the intracellular transport of fatty acids for energy metabolism (Glatz and van der Vusse, 1996). H-FABP has been identified in the heart, the kidney, the intestine skeletal muscle, aorta, adrenals, placenta and brain (Veerkamp and Maatman, 1995). Nevertheless, H-FABP has some potential for the diagnosis of myocardial injury, as the concentration in cardiac muscle is much greater than in skeletal muscle (Alhadi and Fox, 2004).

1.3.5.1 Heart fatty-acid binding protein release in myocardial injury in man

H-FABP was first demonstrated to be released from the injured myocardium by Glatz et al. in 1988, who showed that leakage of the protein occurred during the reperfusion of isolated rat hearts after 60 min of experimentally-induced ischaemia. Following on from this study, a number of reports have demonstrated that H-FABP is an early and sensitive marker of the injured myocardium in man (Tanaka et al., 1991; Tsuji et al., 1993; Ishii et al., 1997).

Tanaka et al. (1991) evaluated the usefulness of serum H-FABP measurements in patients with acute MI. Peak serum H-FABP levels occurred between 5 to 10 h
following the onset of symptoms suggestive of acute MI, whereas maximum CK-MB concentrations were seen between 10 to 15 h. Serial serum H-FABP levels were measured by Tsuji et al. (1993) in 63 cases of acute MI, immediately following symptom onset. Serum H-FABP values were demonstrated to be elevated above normal in 91 % of the samples tested within 3 h of symptom onset, and in 100 % of samples tested at 3 to 6 h. Elevations in serum H-FABP were also reported to occur much earlier than increases in serum levels of CK-MB. At 0 to 3 h following the onset of symptoms, serum CK-MB levels were increased in 20 % of the samples tested, and at 3-6 h in 65 % of samples. In a study of 165 patients at admission within 6 h of the onset of chest pain, Ishii et al. (1997) showed that the sensitivity and predictive accuracy of a serum H-FABP value of >12 µg/L in the diagnosis of acute MI were significantly higher those of serum myoglobin. However, increased concentrations of H-FABP were also observed in patients with unstable angina and in skeletal muscle injury, highlighting the lack of specificity of the marker.

The measurement of H-FABP as a marker of perioperative damage in cardiac surgery has also been investigated by some workers. Following coronary artery bypass grafting (CABG) in 10 patients, Hayashida et al. (2000) showed that plasma H-FABP peak levels occurred much quicker (at 1.4 h) than peak levels of CK-MB (which occurred at 2.5 h) or cardiac troponin T (cTnT; at 6.6 h). Furthermore, peak values of H-FABP correlated with those of CK-MB (r=0.51) and cTnT (r=0.60). Petzold et al. (2001) also investigated the utility of plasma H-FABP determinations in 32 patients after CABG. At 60 min following aortic declamping, 55 % of patients showed peak plasma H-FABP concentrations, whereas only 16 % had reached peak CK-MB or cardiac troponin I (cTnI) values at this time point. Peak H-FABP levels correlated well with peak CK-MB (r=0.44) and peak cTnI (r=0.55). Liu et al. (2005) investigated the use of plasma H-FABP measurements following single and double valve replacement surgery in 56 patients. Following reperfusion, the appearance of peak plasma H-FABP values occurred within 2 h, and this was significantly earlier (P<0.01) than the appearance of CK-MB (at 4 h) or cTnI (at 6 h). Therefore, the studies described above indicate that H-FABP may be a useful early indicator of myocardial damage following cardiac surgery.
1.3.5.2 Heart fatty-acid binding protein release in myocardial injury in experimental animal studies

No reports have been identified in the literature investigating the utility of H-FABP in experimental models of drug- or compound-induced cardiotoxicity; however 3 reports were identified studying H-FABP release into the circulation following coronary artery ligation, which is a well-established animal model of ischaemia and MI.

Sohmiya et al. (1993) totally ligated the left anterior coronary artery in dogs for 2 h to induce ischaemia, and then released the ligation. Blood samples were taken prior to ligation, every 30 min during the 2 h occlusion period, and at periods up to 8 h following reperfusion. Plasma levels of H-FABP increased rapidly after reperfusion, peaking to a mean value of 188.8 µg/L at 20 to 45 min (pre-ligation plasma H-FABP levels were 15.3 µg/L). Plasma H-FABP values remained elevated above the pre-ligation control levels at 7 h following reperfusion. Furthermore, cardiac infarct size correlated closely with the amount (in µg/L) of H-FABP released.

Partial coronary artery ligation in mice resulted in significant increases in H-FABP plasma concentrations above the control level within 2 h following surgery, with peak levels occurring at 4 h (Aartsen et al., 2000). Plasma values of H-FABP returned to baseline at 24 h, and plasma concentrations correlated well with cardiac infarct size.

In the rat, peak plasma H-FABP levels occurred at 15 min after reperfusion following the induction of myocardial ischaemia through ligation of the left anterior coronary artery; plasma values were still elevated after 60 min of reperfusion (Knowlton et al., 1989). Myocardial tissue levels of H-FABP following 60 min of ischaemia and 60 min of reperfusion were decreased by more than 50 %, reflecting the loss of the protein from the cytoplasm of damaged cardiomyocytes.

1.3.6 Troponins

The troponins are a complex of structural proteins located on the thin filament of the contractile apparatus that regulate the calcium dependence of muscle contraction in both skeletal and cardiac muscle (McDonough et al., 1999).

TnT, TnI and TnC exist as isoforms in different types of striated (skeletal and cardiac) muscle. The isoforms result from the transcription of genes specific for the respective
3 isoforms of TnT and TnI have been identified: 1 in slow-twitch skeletal muscle, 1 in fast-twitch skeletal muscle, and 1 form in cardiac muscle (Cummins and Perry, 1978). Therefore, the cardiac isoforms of TnT and TnI, cardiac TnT (cTnT) and cardiac TnI (cTnl), have different amino acid sequences to skeletal TnT and TnI (Wilkinson and Grand, 1978) and thus are specific to the heart. cTnl contains a 31-amino acid sequence at the N terminus that differentiates it from the skeletal muscle Tnl isoforms (Apple, 1999). However, the protein structures of cTnT and cTnl are highly conserved between species (Pearlstone et al., 1986; Katus et al., 1992; Malouf et al., 1992). There are only 2 isoforms of TnC, one found in fast-twitch skeletal muscle, and the other in both slow-twitch skeletal muscle and in cardiac muscle (Parmacek and Leiden, 1989). Therefore, cardiac troponin C (cTnC) has not been investigated further for use in the assessment of myocardial toxicity, as it is not possible to differentiate slow-twitch skeletal muscle and cardiac muscle injury with the measurement of cTnC.

1.3.6.1 Troponin release in myocardial injury

1.3.6.1.1 Troponin release in cardiac disease

Following acute MI in man, raised serum cardiac Tn (cTn) levels are evident in the circulation within 2 to 4 h of the onset of symptoms, and peak at approximately 18 to 24 h (Jaffe et al., 2006). Levels remain at detectable concentrations for 5 to 9 days (Larue et al., 1993). It is thought that the initial release of the free cytoplasmic component is the reason why the cTns may be detected so early after myocardial damage. In addition, the release kinetics of cTns in man are characteristically biphasic, and it is believed that the initial release is from the cytoplasmic pool and the second from the breakdown of the structural components of the cells; moreover the degradation of the structural pool is a much slower process, which accounts for the reasonably long half-life of the cTns (Babuin and Jaffe, 2005). Studies on the characterisation of cTn subunit release following acute MI in humans has shown that cTnT is released as cTnT-I-C (i.e. as a complex of cTnT, cTnl and cTnC) and as free cTnT; while cTnl is released as a complex of cTnT-I-C and cTnl-C (cTnl complexed with cTnC) and also as free cTnl (Katrukha et al., 1997; Wu et al., 1998). This obviously has implications in the design of antibodies for the detection of cardiac troponins, which must be able to recognise both the complexed and the free forms of the proteins. Cardiac Tn measurements are
also of use as adverse prognostic indicators of acute coronary syndromes (ACS), such as unstable angina, and also in the detection of cardiac trauma, myocarditis, pericarditis, amyloidosis and sepsis (Antman et al., 1996; Babuin and Jaffe, 2005).

Preclinical studies on the use of cTn measurement have shown that cTnT and cTnI behave similarly across species as biomarkers of non-drug-induced myocardial injury (Walker, 2006). For example, in canine and rat models of MI, cTnT concentrations were shown to be increased within 1 h of surgery at levels 1000- to 10000-fold above baseline control values, and levels were highly correlated with infarct size within 3 h of injury (O'Brien et al., 1997). However, in a coronary artery ligation model in the mouse, cTnT levels assessed at 4 h post-surgery did not correlate well with infarct size measured at 7 days post-operation (Aartsen et al., 2000). In addition, cTnT and cTnI have been demonstrated to be released during ischaemia in the isolated rat heart model (Vorderwinkler et al., 1996; Bertinchant et al., 1999).

1.3.6.1.2 Troponin release in drug-induced injury

In addition to cTn release following myocardial damage due to disease, the cTns have been used in the detection of cardiac injury in drug- and toxic agent-induced cardiac injury in man and in experimental studies. For example, Lipshultz et al. (1997) showed that low-level cTnT elevations were detected in 60% of paediatric cancer patients treated with doxorubicin (DOX) chemotherapy and there was a significant correlation between cTnT elevations and subsequent electrocardiogram (ECG) abnormalities (left ventricular dilation and wall thinning). Levels of cTnT were used to assess cardiotoxicity in adults receiving anthracycline (i.e. DOX or daunorubicin) chemotherapy for haematological malignancies by Auner et al., 2003) and it was shown that cTnT monitoring may reveal delayed subclinical myocardial damage even after minor anthracycline exposure. Sympathomimetic agents have also been demonstrated to cause increases in circulating cTn levels. For example, in women given tocolytic therapy (IV fenoterol and verapamil), elevations in cTnT were evident 3 days following therapy (Adamcová et al., 2005). In addition, Hollander et al. (1998) showed that cTnI was the most specific marker (compared with CK-MB or myoglobin) for the detection of cocaine-induced myocardial necrosis. cTn elevations have also been demonstrated in man following acute colchicine poisoning, following snakebites and after jellyfish stings (Gaze and Collinson, 2005).
cTn measurements have been widely used in the assessment of cardiotoxicity in experimental animal studies. The well-described model of DOX-induced cardiac injury has been used frequently to characterise cTn release following cardiotoxicity. Herman et al. (1998) gave male spontaneously hypertensive rats 1.0 mg/kg/week DOX for up to 12 weeks and demonstrated elevations in serum cTnT after 7 doses of DOX, when only minimal histological alterations of cardiomyocytes were observed. In addition, there was decreased immunostaining of cTnT in the DOX-treated hearts, indicating the loss of the protein from damaged cardiomyocytes. In a study in 2003, Bertinchant et al. evaluated the use of cTnT and cTnI in DOX-induced cardiomyopathy in male Wistar rats. Animals were given 1.5 mg/kg DOX weekly for up to 8 weeks, and after 5 and 8 weeks of DOX dosing, cTnT levels were significantly increased in comparison with controls; cTnI values were increased also, but not significantly. However, it was not clear whether there was any correlation between cardiac troponin levels and histopathological evidence of cardiac injury. Koh et al. (2004) administered DOX IV weekly at 2.0 mg/kg for 8 weeks to male Wistar rats and showed that serum cTnT levels were significantly increased in the week 10 and week 12 blood samples (2 and 4 weeks post-dosing, respectively), and there was an inverse correlation between cTnT levels and % fractional shortening, highlighting the sensitivity of the biomarkers in the prediction of cardiac injury.

In addition, there are some studies evaluating the usefulness of serum cTn measurements in the assessment of ISO-induced cardiotoxicity in the rat. Bleuel et al. (1995) measured cardiac troponin T (cTnT) levels in the serum after 2 SC injections of ISO, 7 h apart, at 4000 µg/kg in male Crl: CD (SD) BR rats. Serum concentrations of cTnT were raised significantly at 6 h after the first injection and persisted for 48 h after the first injection. Histological examination of the hearts of the drug-treated rats showed acute myofibrillar degeneration 24 h after the first injection. It was therefore concluded that serum cTnT levels correlated well with the histological alterations associated with ISO-induced cardiac injury. The diagnostic usefulness of serum cTnT and cTnI levels were compared by Bertinchant et al. (2000) in the detection of early myocardial damage induced by ISO. Wistar rats were treated with a single dose of ISO at 4000 µg/kg SC. Serum cTnT and cTnI levels were measured at 0, 2, 4, 6 and 24 hours after drug administration, with hearts taken for histology at 2, 4, 6, 24 and 48 h post-dosing. Both cTnT and cTnI levels were significantly increased above control values at 2, 4 and 6 h, with levels returning towards base line control levels by 24 h post-dosing.
Histopathological evidence of myocardial injury was first seen at 4 h post-dosing; nevertheless, serum levels of both cTnI and cTnT correlated well with the extent of myofibrillar degeneration assessed by histological examination, thus confirming the usefulness of cTnT and cTnI in the assessment of ISO-induced myocardial damage in the rat. York et al. (2007) gave female Hanover Wistar rats single intraperitoneal (IP) dose of ISO at 50000 μg/kg and showed that at 1 h post-dosing, all rats (n=4) had positive serum cTnI and cTnT response; however the earliest microscopic changes were observed at 4 h post-dosing. The utility of cTn determination has also been investigated in animal studies using cardiotoxic compounds such as orciprenaline (Bertsch et al., 1997), cyclophosphamide (CPA) (Mythili et al., 2004, 2005, 2006) and ethanol (Patel et al., 2001).

1.3.6.2 Alterations in troponin levels in non-cardiac injury and false elevations

Although the cTns are specific to the heart, there are some non-cardiac conditions which lead to increases in their circulating values. The most common cause of death in chronic renal failure tends to cardiovascular, and coronary artery disease is also common (Jaffe et al., 2006). Li et al. (1995) measured cTnT levels in 82 patients with chronic renal failure and found that cTnT elevation was relatively frequent in chronic renal failure in the absence of an acute ischaemic event, especially in diabetic patients. However, cTnI does not appear to be raised in renal disease (Donnino et al., 2004).

cTnI is not expressed in healthy or diseased human skeletal muscle (Bodor et al., 1995); although Punukollu et al. (2004) showed that cTnI was elevated in rhabdomyolysis, and a raised cTnI concentration was associated with higher morbidity. Bodor et al. (1997) demonstrated that the mean myofibrillar cTnT concentrations were 10 mg/g in cardiac tissue, 0.8 mg/g in normal skeletal muscle, 0.7 mg/g in the skeletal muscle of polymyositis patients and 4.37 mg/g in Duchenne muscular dystrophy patients.

Various factors may interfere with the cTn assays, and common causes of false-positive cTn tests include heterophilic antibodies, rheumatoid factor and fibrin clots (Roongsritong et al., 2004).
1.3.7 Natriuretic peptides

The natriuretic peptides, ANP and BNP, are involved in the regulation of fluid volume, blood pressure and electrolyte balance (Apple and Jaffe, 2006), as they are released in response to atrial and ventricular stretch, respectively, caused by volume overload (Clerico et al., 2006). Thus, BNP has been investigated in man for the detection of congestive heart failure (CHF). However, elevated BNP values also identify patients with ACS who are at higher risk, and also predict an adverse outcome in stroke, obstructive sleep apnoea, diabetes, left ventricular hypertrophy and stable adverse coronary artery disease (Kemp et al., 2004; Jaffe et al., 2006). In experimental animals, BNP may also be used to assess cardiac injury. Jiang et al. (2005) induced myocardial necrosis in male Sprague Dawley rats by the administration of 20000 μg/kg ISO by subcutaneous (injection) on 2 daily occasions and showed that myocardial BNP expression was significantly increased in ISO-treated rats. Moreover, in the rat coronary artery ligation model of MI, plasma BNP levels at day 3 post-operation correlated significantly with infarct size determined at 1 week, 1 month, 3 months or 6 months after surgery (Mączewski and Mackiewicz, 2007).

1.4 AIMS OF THIS PROJECT

The aim of this project was to assess the utility of cardiac biomarkers in models of drug- and compound-induced cardiac injury in the rat. For this, 3 compounds were employed: ISO, allylamine (AA), and EA. Work was also completed using repeat dosing schedules with the anticancer drugs, CPA and DOX; however, due to constraints of thesis length, the work is not reported, but is being prepared for publication.
Figure 1.1. Diagrammatic section of the human heart (anterior view). The arrows indicate the direction of blood flow. AV = atrioventricular. Adapted from Widmaier et al. (2005).
Figure 1.2. Diagrammatic section of the rat heart (posterior view). From Rowett (1989).
Figure 1.3. Diagrammatic representation of the layers of the heart wall. Areolar tissue is also known as loose connective tissue. From Martini et al. (2006).
Figure 1.4. Diagrammatic representation of the cardiomyocytes and intercalated discs. From Martini et al. (2006).
Figure 1.5. Diagrammatic representation of the organisation of the sarcomere. From Alberts et al. (2002).
Figure 1.6. Diagrammatic representation of actin and associated proteins present on the thin filament of striated muscle. TnT, TnC and TnI = troponin T, troponin C and troponin I, respectively. From Matthews and van Holde (1990).
Figure 1.7. Diagrammatic representation of the regulation of muscle contraction. 

(A) Relaxed muscle, low intracellular calcium concentration; the arrangement of actin, tropomyosin and troponin in the thin filament is such that most myosin headpieces are blocked from contact with the thin filament. (B) Calcium binding, high intracellular calcium concentration. The binding of calcium to TnC results in a rearrangement of the thin filament components so that the myosin binding sites on the actin molecule are made available. TnT, TnC and Tnl = troponin T, troponin C and troponin I, respectively. From Matthews and van Holde (1990).
Figure 1.8. Time course of the appearance of cardiac markers in the blood after acute myocardial infarction in man. CK = creatine kinase. CKMB = creatine kinase-MB isoform. CV = coefficient of variation. AMI = acute myocardial infarction. From Jaffe et al. (2006).
### Table 1.1. Agents which may cause sublethal structural changes in the heart.

<table>
<thead>
<tr>
<th>Structural Change</th>
<th>Agents</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vacuolar degeneration</td>
<td>Anthracycline anticancer drugs</td>
</tr>
<tr>
<td>Myofibrillar degeneration</td>
<td>Sympathomimetic agents, e.g. isoproterenol, cocaine Plasmocid Furazolidone Acute morphine intoxication</td>
</tr>
<tr>
<td>Fatty degeneration</td>
<td>Erucic acid</td>
</tr>
<tr>
<td>Fatty degeneration</td>
<td>Ethanol</td>
</tr>
<tr>
<td>Pigment deposition</td>
<td>Brown FK</td>
</tr>
<tr>
<td>Pigment deposition</td>
<td>Chloroquine</td>
</tr>
<tr>
<td>Cardiac atrophy</td>
<td>Angiotensin-converting enzyme inhibitors</td>
</tr>
<tr>
<td>Cardiac hypertrophy</td>
<td>Thyroxine</td>
</tr>
<tr>
<td>Cardiac hypertrophy</td>
<td>Potassium channel antagonists</td>
</tr>
<tr>
<td>Cardiac hypertrophy</td>
<td>Noradrenaline</td>
</tr>
<tr>
<td>Cardiac hypertrophy</td>
<td>Vasodilators</td>
</tr>
<tr>
<td>Cardiac hypertrophy</td>
<td>Calcium channel blockers</td>
</tr>
</tbody>
</table>
Table 1.2. Characteristics of an ideal cardiac biomarker.

| Specificity | Absent from non-myocardial tissue  
|            | Absent under normal physiological conditions  
| Sensitivity | High concentration in myocardium after myocardial injury  
|            | Ability to detect as abnormal with even minimal elevation  
|            | Appear in quantities that are directly proportional to the extent of myocardial damage  
| Release    | Small size to allow rapid release following cardiac damage  
|            | Long half-life in blood for a convenient diagnostic time window  
| Analytical | Cost-effective  
|            | Rapid turnaround time  
|            | Precision  
|            | Accuracy  
| Other      | Bridge preclinical and clinical situations  
|            | Noninvasive/accessible  

Adapted from Kemp et al. (2004), Wallace et al. (2004), Dolci and Panteghini (2006).
CHAPTER 2: MATERIALS AND METHODS

2.1 ANIMALS AND ANIMAL HUSBANDRY

Hanover Wistar rats (Harlan, Bicester, Oxon OX25 1TP, UK) were used in all experimental procedures. Animals were allowed to acclimatise for at least 7 days before studies commenced and were housed in communal cages in groups of 2-6, with access to diet (extruded Global Rodent Diet, Harlan Teklad, Bicester, Oxon OX25 1TP, UK) and mains drinking water ad libitum. Environmental conditions were, temperature: 20 °C ± 2 °C; light: regular 12-hour light/dark cycle, lights on at 07.00 h; relative humidity: 40-60 %. All animal procedures were conducted under local Ethical Committee guidelines and approval for Home Office project and personal licences, and followed UK Home Office (1989) “Code of Practice for the Housing and Care of Animals Used in Scientific Procedures”.

In the studies involving the collection of urine, animals were housed individually in metabolism cages (Techniplast, Kettering, Northamptonshire NN15 6XR, UK) designed to separate and collect faeces and urine for a maximum period of 24 hours. Animals did not have access to diet, however mains drinking water was provided ad libitum.

During the experiments, animals were observed daily and sometimes more frequently and clinical signs recorded. Body weights of animals were measured at least twice a week during periods of compound administration and in periods of non-dosing. If animals began to lose weight, or were displaying overt signs of compound-induced toxicity, and it was considered that they would not recover, they were killed in extremis (KIE). On a small number of occasions, animals were found dead (FD) at the morning inspection. All animals KIE or FD are referred to as “intercurrent death” (ICD) animals.

2.2 FEEDING GROUND DIETS AND MEASUREMENT OF DIET CONSUMPTION

In the dietary studies on EA, LEAR and HEAR oil, the amount of diet consumed by each group was monitored throughout the treatment period. A cage of animals (n=5) was given either 100 g or 150 g of fresh diet daily in heavy bowls (mortars) which were placed in the corner of the cage. The following morning, the bowl was taken from the cage, debris in the diet removed by sieving, and the diet remaining weighed. The
difference between the weight of the diet of the previous day and the current day gave the weight of diet consumed per cage per day. This value was divided by the number of rats in the cage to give a diet consumption value measured in g diet consumed/rat/24 hours. The relative diet consumption was also calculated, measured in g diet consumed/kg body weight/24 hours. This was calculated using the group mean body weight for each cage of animals. All consumption measurements were for 24 h periods. Following the weighing of the diets, a fresh 100 g or 150 g was replaced in the cage.

2.3 MEASUREMENT OF WATER CONSUMPTION

In the studies where water consumption was monitored, full water bottles were weighed daily. The difference in weight (in g) from the current day and the previous day was the measure of the ml of water consumed per cage per day. This value was divided by the number of rats in the cage to give a water consumption value in ml/rat/24 hours. All water consumption measurements were for periods of 24 hours.

2.4 DRUGS AND CHEMICALS

2.4.1 Isoproterenol

DL-Isoproterenol hydrochloride (ISO; Sigma-Aldrich Company Ltd, Poole, Dorset BH12 4QH, UK) was dissolved in deionised water or phosphate buffered saline (PBS) at concentrations of 0.48 to 6250 μg/mL. Solutions were prepared fresh to avoid oxidation (Yates and Dhalla, 1975). An oxidation product of the catecholamines, adrenochrome, is known to cause ultrastructural myocardial changes (Singal et al., 1982). Control animals were treated with deionised water or PBS (vehicle).

2.4.2 2,3,5,6-tetramethyl-p-phenylenediamine

2,3,5,6-tetramethyl-p-phenylenediamine (TMPD; Aldrich/Sigma-Aldrich) was suspended in vegetable oil at a concentration of 6.088 μmol/mL (2.268 mg/mL). Solutions were prepared fresh to avoid auto-oxidation of TMPD (Draper et al., 1994). Control animals were treated with vegetable oil (vehicle).
2.4.3 Allylamine

Allylamine hydrochloride (AA; TIC/Fluorochem Ltd, Old Glossop, Derbyshire SK13 7RY, UK) was dissolved in deionised water at concentrations of 5 to 70 mg/mL. Due to the hygroscopic nature of AA, the compound was stored and weighed out in a dry air environment. Control animals were treated with deionised water (vehicle).

2.4.4 Erucic acid

Low erucic acid rapeseed oil (LEAR oil; erucic acid content <2 %; Pura Foods Ltd, London E14 0JH, UK) and high erucic acid rapeseed oil (HEAR oil; erucic acid content approximately 45 %; John L. Seaton and Co Ltd, Hessle, East Yorkshire HU13 0EG, UK) were administered by gavage or were mixed with ground diet (Global Rodent Diet, Harlan Teklad) to produce “LEAR oil diet” and “HEAR oil diet”. Sunflower oil (Tesco Stores Ltd, Cheshunt, Hertfordshire EN8 9SL, UK) was mixed with ground diet to produce “Sunflower oil diet”. Purified erucic acid (EA; Fluka/Sigma-Aldrich) was mixed with LEAR oil (“spiking”) to give an EA content of approximately 45 % and administered by gavage (“Spike oil”) or in the diet (“Spike oil diet”).

Oils were mixed with ground diet to give an oil content (w/w) of 20 %. Therefore, diets were composed as follows:

- Control ground diet: Ground Global Rodent Diet.
- Sunflower oil diet: Ground Global Rodent Diet mixed with sunflower oil, 20 % (w/w).
- LEAR oil diet: Ground Global Rodent Diet mixed with LEAR oil, 20 % (w/w).
- Spike oil diet: Ground Global Rodent Diet mixed with Spike oil, 20 % (w/w).
- HEAR oil diet: Ground global rodent diet mixed with HEAR oil, 20 % (w/w).
Diets were mixed for 20 minutes using a food mixer (Kenwood Chef Classic KM400; Kenwood Ltd, Havant, Hampshire PO9 2NH, UK) on the lowest speed setting. Diets when mixed were stored at 4°C.

2.5 IN-LIFE BLOOD SAMPLING

Where in-life data was required during the study period, a sample of blood was taken from the lateral tail vein. Animals were placed in a recovery chamber (VetTech Solutions Ltd, Congleton, Cheshire CW12 4TR, UK) at 37-40°C, for approximately 10 minutes (but no longer than 15 minutes) to allow visualisation of the tail vein. Individual animals were restrained using a tube type animal holder (VetTech Solutions), and a 21 or 23 gauge Butterfly needle (Venisystems, Abbott Laboratories Ltd, Queenborough, Kent ME11 5EL, UK) was used to withdraw blood, dependent upon the weight of animal.

2.6 AUTOPSY PROTOCOL

Animals were killed by an intraperitoneal (IP) injection of Euthatal (pentobarbitone sodium, 200 mg/ml; Merial Animal Ltd, Harlow, Essex CM19 5TG, UK), or by exsanguination under deep isoflurane (Abbott Laboratories Ltd) anaesthesia (3.5 % isoflurane in O₂ at 2 litres/minute) using an inhaled anaesthetics trolley (VetTech Solutions).

A sample of blood was removed either from the inferior vena cava (isoflurane anaesthesia) or abdominal aorta (Euthatal injection). For haematological analysis, a 0.5 mL blood sample was anticoagulated with 1.5 mg/mL dipotassium ethylene diaminetetraacetic acid (EDTA) (Teklab, Sacriston, Durham DH7 6LG, UK) and placed on a mixer (Spiramix; Denley Instruments Ltd, Billingshurst, West Sussex RH4 9EY, UK). For plasma clinical chemistry analysis, blood was collected into lithium heparin tubes (Microtainer PST LH, Becton Dickinson, Franklin Lakes, New Jersey, USA). For serum clinical chemistry analysis, blood was collected into serum separator tubes (Microtainer SST, Becton Dickinson). Organs of interest were removed from each animal, depending upon the study and the compound being tested, and placed in histological fixative.
If required, a femoral nucleated cell suspension was prepared for the assessment of the femoral nucleated cell count (FNCC). The right hind limb was removed and the surrounding muscle stripped from the femur. Sharp scissors were used to remove the epiphyses, and the contents of the femur aspirated into 5 ml Iscove's Modified Dulbecco’s Medium (IMDM; Gibco Cell Culture/Invitrogen, Paisley PA4 9RF, UK) supplemented with 10 % foetal calf serum (PAA Laboratories GmBH, A-4041 Pasching, Austria) to give a marrow cell suspension.

2.7 ANALYSIS OF BLOOD AND MARROW SUSPENSIONS

Blood samples in EDTA and bone marrow suspensions in IMDM were analysed by flow cytometry on an Advia 120 haematology analyser (Bayer Diagnostics, Newbury, Berkshire RG14 1JA, UK) using rat-specific software. Parameters measured included: red blood cell count (RBC), haemoglobin (Hb), haematocrit (HCT), mean cell haemoglobin concentration (MCHC), mean cell volume (MCV), mean cell haemoglobin (MCH), reticulocyte count (RETIC), platelet count (PLT), white blood cell count (WBC), lymphocyte count (LYMPH), neutrophil count (NEUT), and monocyte count (MONO). The FNCC was obtained using the basophil channel of the Advia 120.

2.8 CLINICAL CHEMISTRY

2.8.1 Plasma clinical chemistry

Heparinised blood in Microtainer PST LH tubes was placed on a mixer (Spiramix) for 5 minutes. Tubes were centrifuged for 5 minutes at 3,500 relative centrifugal force (RCF). Plasma was removed and immediately stored at -80 °C until analysis. Plasma total creatine kinase (CK) and total lactate dehydrogenase (LD) were analysed on the Advia 1650 (Bayer Diagnostics). LD isoenzyme (LD1-5) and CK isoenzyme (CK-MB, CK-BB, CK-MM) activities were analysed on the Advia 1650 (Bayer Diagnostics), whereas the relative percentages of each isoenzyme were measured by electrophoresis on the SAS-1 plus/SAS-2/Platinum electrophoresis system (Helena Biosciences Europe, Sunderland Enterprise Park, Sunderland SR5 3XB, UK).
2.8.2 Serum clinical chemistry

Blood was allowed to coagulate in Microtainer SST tubes for 2.5 hours. Tubes were centrifuged for 5 minutes at 2,500 RCF. The serum was removed and stored at -80°C until analysis. Serum samples were analysed on the Advia 1650 (Bayer Diagnostics). Parameters measured included: urea, creatinine (CREAT), total protein (TP), albumin, total cholesterol (CHOL), triglycerides (TRIG), non-esterified fatty acids (NEFA), glucose, lactate, calcium, sodium, potassium and chloride levels. The activities of the following enzymes were also determined: alanine aminotransferase (ALT), aspartate aminotransferase (AST), total CK, total LD, glutamate dehydrogenase (GLD) and aldolase. Heart fatty acid-binding protein (H-FABP) was measured by a solid-phase enzyme-linked immunosorbent assay (ELISA) method (Hycult biotechnology, 5400 AA Uden, The Netherlands).

Serum cardiac troponin I (cTnl) levels were measured by automated immunochemiluminescence on the ACS: 180S kit (Bayer Diagnostics), the Centaur CP (Bayer Diagnostics) or on the Beckman Access. The limits of detection for these assays are 0.03, 0.006 and 0.006 µg/L, respectively. Serum cardiac troponin T (cTnT) levels were measured by automated immunochemiluminescence on the Elecsys 2010 (Roche Diagnostics, Burgess Hill, West Sussex RH15 9RY, UK).

2.9 ANALYSIS OF URINE

Urine samples were screened semiquantitatively by dipstick analysis (Multistix 10 SG; Bayer Diagnostics). Parameters measured were: leucocytes, nitrite, urobilinogen, protein, pH, blood (haemoglobin), specific gravity, ketones, bilirubin and glucose. Urine samples were also measured for H-FABP, TP and CREAT using the methods described above (Chapter 2.8.2). TP was also normalised for CREAT values to give a "corrected TP" (TPc).

2.10 HISTOLOGICAL EXAMINATION OF TISSUES

At autopsy, organs were removed, weighed and placed in 10 % neutral buffered formalin (NBF; Pioneer Research Chemicals, Colchester, Essex CO2 8HX) for a minimum of 14 days. Lungs were removed and inflated with formalin prior to
submersion. Testes were placed in a modified Davidson’s fluid (Latendresse et al., 2002) composed of 30% of 38% formaldehyde (BDH Laboratory Supplies, Poole, Dorset BH15 1TD, UK), 15% of 100% ethanol (Fisher), 5% of glacial acetic acid (BDH) and 50% of deionised water. After 24 hours, testes were transferred into 70% methanol (Fisher). Sternums were fixed in 10% NBF for a minimum of 2 days, and then decalcified in 100% formic acid for a minimum of 2 days. Sternums were then trimmed of excess tissue and decalcified again for a minimum of 3 days prior to processing. Tissues were processed routinely and embedded in paraffin, sectioned at 3 μm and stained with haematoxylin and eosin (H&E).

Hearts were transversely cut to generate 3 levels: base (atria and basal ventricles), mid-ventricular region, and ventricular apex. The 3 levels were trimmed to generate a single block containing a longitudinal section of both atria/basal ventricles, a transverse section of the left and right mid-ventricular region and a longitudinal section of the left and right ventricular apex (Isaacs, 1998). Selected sections of hearts were stained with Martius’ Scarlet Blue (MSB), for connective tissue; and phosphotungstic acid haematoxylin (PTAH), Heidenhain’s iron haematoxylin, for the detection of the cross-striations formed by the cardiac myofilaments; or immunostained with an anti-cTnI antibody (goat anti-human cTnI; BiosPacific, Emeryville, California 94608, USA). Oil red O (ORO) stain was also used on selected sections of tissues (heart, skeletal muscle, liver) to confirm the presence of neutral lipid.

Selected sections of formalin-fixed hearts were washed overnight in Millonig’s phosphate buffer. Tissue samples were then secondary fixed in 1% Millonig’s buffered osmium tetroxide (Agar Scientific, Stansted, Essex CM24 8DA, UK), dehydrated in graded alcohols and then infiltrated/embedded into Agar 100 low viscosity resin (Agar Scientific). Semi-thin (survey) sections of approximately 1μm were cut and stained with toluidine blue (Agar Scientific) to determine areas of interest. From the toluidine blue survey sections, ultra-thin sections (60 to 90 nm) were cut using a Leica Ultracut R ultramicrotome (Leica Microsystems Ltd, Knowlhill, Milton Keynes, Buckinghamshire MK5 8LB, UK), stained with uranyl acetate and lead citrate (Leica), and examined using a Hitachi H7500 transmission electron microscope (Hitachi-High Technologies, Finchampstead, Wokingham, Berkshire RG40 4QQ, UK), operated at 60 kV.
CHAPTER 3: PRELIMINARY STUDIES ON THE CARDIOTOXICITY OF ISOPROTERENOL

3.1 INTRODUCTION

3.1.1 Background

Isoproterenol (ISO) was the first pure β-adrenoceptor agonist to be synthesised (Sears and Lötvall, 2005). The drug was developed in the 1940s and quickly became widely used for the relief of the symptoms of asthma (Waldeck, 2002). However, although ISO is a very potent β-agonist, with almost no action on α-adrenoceptors, the drug does not distinguish between β₁ and β₂ receptors, i.e. it is non-selective. As the drug stimulates β₁ receptors in the heart, ISO has significant extra-pulmonary side effects, such as tachycardia, arrhythmias and palpitations. Due to this, the drug is no longer used in the UK and USA for the relief of asthma symptoms (British National Formulary, 2006); however, ISO has become widely used in toxicological studies as a model drug to induce cardiac muscle injury with resulting myocardial ischaemia and the formation of infarct-like lesions (Rona et al., 1959a; Handforth, 1962; Judd and Wexler, 1974; Bleuel et al., 1995).

3.1.2 Cardiotoxicity of isoproterenol

3.1.2.1 Cardiotoxicity in man

Aelony et al. (1975) presented a case study of a 39 year old asthmatic woman who inhaled around 675 mg of ISO from a pressurised bronchodilator aerosol over a 3 day period. Electrocardiogram (ECG) readings were suggestive of acute myocardial infarction (MI), and showed ST segment elevation and inverted T waves, and the diagnosis of myocardial necrosis was confirmed by the presence of elevated serum levels of lactate dehydrogenase (LD), aspartate aminotransferase (AST), creatine kinase (CK) and α-hydroxybutyric dehydrogenase (HBD).
3.1.2.2 Cardiotoxicity in experimental animal studies

The cardiotoxicity of ISO was first investigated in detail in the rat by Rona et al. (1959a) and Chappel et al. (1959), with the majority of subsequent work being carried out in this species; however myocardial lesions have also been produced by ISO administration in the dog (Rona et al. 1959b), the monkey (Maruffo, 1967), the cat (Rosenblum et al., 1965), the hamster (Handforth, 1962), the turtle (Ostådal et al., 1968) and the frog (Carlsten et al., 1982).

3.1.2.2.1 Cardiotoxicity in the rat

The group of Rona and colleagues were the first to describe the use of ISO to induce myocardial necrosis in the rat (Rona et al. 1959a; Chappel et al. 1959). When ISO was administered by subcutaneous (SC) injection on 2 occasions (24 h apart) to male and female Wistar rats at dose levels of 80 μg/kg and above, cardiac lesions were present in 100 % of animals killed at 24 h post-dosing (Chappel et al., 1959). Cardiac changes were characterised by hyaline necrosis, inflammatory cell infiltration, interstitial oedema, swelling of the fibroblasts and early fibroblast proliferation; lesions were localised mainly in apex and papillary muscle. At doses of ISO of 5250 μg/kg and higher, animals appeared very weak following drug administration, and were prostrate with their front legs extended and had laboured breathing (Chappel et al., 1959). In a follow-up study, Rona et al. (1959a) showed that an ISO dose level of 40 μg/kg by the SC route was sufficient to produce myocardial lesions in the male Wistar rat, and the LD$_{50}$ of a SC dose of ISO was 680000 μg/kg (i.e. 680 mg/kg).

The time course of cardiac injury was studied by Rona et al. (1961) following a single SC injection of ISO at 85000 μg/kg to male Wistar rats. At 30 min post-dosing, the structure of the myocardium was generally preserved; however at 2 h, myofibres displayed increased eosinophilia, and there were areas of mast cells, histiocytes and lymphocytes in the epicardial portion of the heart. By 8 h post ISO administration, there was significant myofibre eosinophilia and neutrophil infiltration, with interstitial oedema. At 24 h after the SC ISO injection, focal myocardial necrosis was evident, with significant neutrophil infiltration and pronounced interstitial oedema. Dosing ISO as 2 doses of 85000 μg/kg SC 24 h apart to male Wistar rats, Rona and Kahn (1969) followed the longer term histological changes in the heart. The myocardial necrosis peaked at around 48 h post-dosing, and at 72 h, necrotic debris were being removed by
histiocytes, and the interstitial oedema regressed. There was a proliferation of fibroblasts and by 5 days post ISO administration (i.e. 5 days after the 2nd dose), fibroblast mitoses were evident. At 7 days, reticulin and collagen fibres became apparent, suggesting fibrosis. Pick et al. (1989) used a much lower dose of ISO (1000 μg/kg) on 2 occasions (SC injection, 24 h apart) in the male Wistar rat, and showed that at day 1 (after a 2 day course of ISO), there was disruption of the collagen matrix, but the cardiac muscle fibres remained intact. New collagen fibres were evident at day 2 post-dosing and developed further over time. By day 8, thick (rather than thin) collagen fibres were the most frequent, and were present in areas which were previously devoid of collagen, this suggested therefore the presence of scar tissue.

A number of electron microscopy studies have been performed to assess ultrastructural cardiac changes after a single injection of ISO very early after drug administration. Following a single SC administration of ISO at 25000 μg/kg to male Wistar rats, Kutsuna (1972) observed swelling of the endoplasmic reticulum and a reduction in the density of the mitochondrial matrix at 4 min post-dosing. At 8 min, cardiac myofilaments appeared disorganised and there was some distortion of the mitochondria. The normal array of the myofibrils had disappeared from 30 to 60 min post ISO administration, and there was proliferation of fibroblasts at 6 h. Csapó et al. (1972) reported that following the intraperitoneal (IP) injection of a single dose of ISO at 85000 μg/kg to male Sprague Dawley rats, myofibrils became disorganised and the Z bands irregular at 6 min post-dosing. At 10 min, there was considerable disorganisation of the myofibrils and contraction bands appeared, signifying the start of cardiomyocyte necrosis.

ECG studies on ISO-induced cardiotoxicity show changes consistent with myocardial ischaemia and/or necrosis. Laboratory-bred piebald rats (both sexes) were given ISO by SC or IP injection at dose levels ranging from 5000 to 80000 μg/kg for periods between 2 days and several weeks (Hill et al., 1960). Rats showed ECGs with inverted T waves, indicative of myocardial necrosis (John and Fleisher, 2006), and at autopsy, all ISO-treated animals had small foci of subendocardial necrosis. Rajadurai and Stanely Mainzen Prince (2007) administered 2 SC doses of ISO (24 h apart) at 85000 μg/kg to male Wistar rats and studied the ECG patterns of drug-treated rats. ISO-treated rats showed a marked elevation in the ST segment, indicating myocardial ischaemia (John and Fleisher, 2006).
3.1.3 Serum changes in isoproterenol-induced cardiotoxicity

The induction of myocardial necrosis by ISO in the rat causes alterations in a number of serum parameters, as well as pathological changes in the heart itself. The administration of ISO to male and female Sprague Dawley rats at 500000 μg/kg (i.e. 500 mg/kg) SC resulted in a decrease in serum total protein (TP) levels, during the necrotic phase of cardiac injury (Wexler et al., 1968). In addition, serum concentrations of non-esterified fatty acids (NEFA) and glucose levels were increased during necrosis. Wexler and Kittinger (1963) also showed that serum cholesterol was increased after a single SC injection of ISO at 500000 μg/kg to male Sprague Dawley rats. Finally, levels of serum lactate have been shown to increase in response to a single SC administration of ISO at 500000 μg/kg in the male Sprague Dawley rat (Lutmer and Wexler, 1971).

ISO administration also may have an effect on the serum enzymes which have traditionally been used in the diagnosis of cardiac injury. Wexler and Kittinger (1963) showed the serum activities of AST, ALT and LD increased in response to a single SC dose of ISO at 500000 μg/kg (500 mg/kg) to male Sprague Dawley rats at 24 h post-dosing. Wenzel and Chau (1966) found that a single SC injection of ISO at a dose level of 50000 μg/kg (50 mg/kg) caused an increase in serum AST levels, with the peak at 2 days post-dosing in the male Sprague Dawley rat. Serum CK activity increased within 1 h of drug administration when ISO was given as a single SC dose at 500000 μg/kg to male Sprague Dawley rats, concurrent with the start of myocardial necrosis (Wexler, 1970), and peaked at 6 h post-dosing.

The measurement of isoenzymes of CK and LD has given some degree of specificity in the diagnosis of cardiac injury induced by ISO. Barrett et al. (1988) administered 1000 μg/kg ISO by IV injection to male Wistar rats and showed levels of serum total LD activity were not increased significantly in response to ISO injection; however LD1 values were significantly (P<0.001) raised by ISO administration at 8 h post-dosing. The study of Barrett et al. (1988) also showed non-significant elevations in serum CK and AST activities. Significant (P<0.05) increases in serum LD1, LD2 and CK-MB values were observed at 2 h post-dosing by Preus et al. (1988), who gave a single SC injection of 5000 μg/kg ISO to male Wistar rats. Focal myocardial injury could only be observed by light microscopy from 6 h post ISO administration.
3.1.4 Factors affecting isoproterenol-induced cardiotoxicity

There are a number of factors which may influence the cardiotoxicity observed following ISO administration. The dose level of ISO chosen will obviously affect the severity of cardiotoxicity. A single SC injection of 40 μg/kg is sufficient to cause myocardial necrosis in the male Wistar rat (Rona et al., 1959a).

The route of administration also has an effect on the degree of cardiotoxicity induced by ISO. It is known that in man, an IV dose of ISO is the most effective, due to first pass metabolism and inactivation of the drug by the oral and inhaled routes (Davies, 1982). In the rat, SC dosing of ISO brings about a greater response than IP administration. The Registry of Toxic Effects of Chemical Substances lists the LD<sub>50</sub> for ISO in the rat to be 128000 μg/kg by IP injection and 600 μg/kg by the SC route.

The time point of sampling following ISO administration will influence the histopathological cardiac changes observed. Rona et al. (1961) saw increased eosinophilia of myofibres 2 h following ISO administration, with focal myocardial necrosis at 24 h post-dosing. Furthermore, Rona and Kahn (1969) observed fibroblast proliferation and mitoses at 5 to 7 days post ISO dosing.

The sex of the rat has been shown to influence the degree of ISO-induced cardiotoxicity, with males exhibiting a slightly greater sensitivity to the drug than age-matched females (Chappel et al., 1959; Rona et al., 1959c). The greater sensitivity of male animals has been attributed to their faster rate of growth, and therefore their weight (Kahn et al., 1969). There is a correlation between the weight of the animals treated and the severity of myocardial necrosis (Rona et al., 1963). Young rats with a low body weight were much less sensitive to the cardiotoxic effect of ISO compared with larger rats (Rona et al., 1959c). In addition, Balazs et al. (1962) showed that animals with a higher proportion of body fat had a higher sensitivity to the cardiotoxic effects of ISO, showing that body weight and body fat affect sensitivity.

Finally, there are some strain differences in the sensitivity to ISO, with Sprague Dawley animals being somewhat less sensitive to ISO than rats from the Long Evans or Wistar strains (Rona et al., 1959c).
3.1.5 Isoproterenol toxicity to other target organs

At very high doses of ISO, lesions may be observed in organs other than the heart. When ISO was administered to male Wistar rats at dose levels of 1700000 (1700 mg/kg) µg/kg and above, extra-cardiac histopathological lesions consisted of centrilobular liver necrosis and haemorrhage, and cortical ischaemia/necrosis of the kidney (Rona et al., 1959a). Wexler and Kittinger (1963) administered 2 SC doses of ISO at 500000 µg/kg, 24 h apart, to male and female Sprague Dawley rats and demonstrated pulmonary oedema, severe hydrothorax, and hypertrophy of the thymus and the adrenal glands within 2 days of ISO administration.

3.1.6 Aims of the present studies

The aims of the present studies were to assess the cardiotoxicity of ISO in the Hanover Wistar rat following a single dose of the drug, to identify the threshold dose that would cause an elevation in serum clinical chemistry parameters, and in particular an increase in cardiac troponin I (cTnI), and also define the threshold dose for the induction of histopathological lesions in the heart. In addition, the route of administration of ISO (IP, SC) was investigated over a 5 h post-dosing period to find whether this had any effect on the degree of cardiotoxicity observed; levels of cTnI and other biochemistry parameters were also investigated in relation to the 5 h post-dosing period.

A preliminary (abstract) report on these investigations has been published (Brady et al., 2005).
3.2 MATERIALS AND METHODS

3.2.1 Experimental protocols

3.2.1.1 Experiment 1; dose ranging study, 1 – 25000 µg/kg

OBJECTIVE: To investigate whether myocardial lesions can be induced by a single intraperitoneal administration of isoproterenol at dose levels from 1 to 25000 µg/kg.

3.2.1.1.1 Animal handling

ISO dosing solutions were prepared at concentrations of 0, 0.5, 2.4, 4.3, 21.5, 43.0, 198.8, 307.5, 1537.5, 2500.0 and 6250.0 µg/ml, as described in Chapter 2.4.1. The vehicle was deionised water. Female Hanover Wistar rats (n=130; mean weight 151.3 g) were treated with a single IP dose of ISO at dose levels of 0 (deionised water, vehicle controls, n=30), 1, 5, 10, 50, 100, 500, 1000, 5000, 10000 or 25000 µg/kg (n=10/group). Due to the large size of the study, it was sub-divided into 3 smaller experiments, and 10 control animals were killed in each smaller experiment (Experiment A: 0, 1, 5 and 10 µg/kg ISO; Experiment B: 0, 50, 100 and 500 µg/kg ISO; Experiment C: 0, 1000, 5000, 10000 and 25000 µg/kg ISO). Animals from each control and ISO-treated group (n=10) were autopsied at 2 h (n=5) and 24 h (n=5) post-dosing for clinical chemistry and histopathological investigations. Each group of n=5 animals occupied 1 cage.

3.2.1.1.2 Sample handling

At autopsy, animals were killed by IP injection of Euthatal (Chapter 2.6) and the hearts removed, weighed and placed in formalin fixative (Chapter 2.10). Serum was prepared and clinical chemistry analysis performed on all animals in all groups (Chapter 2.8.2). Parameters measured were: lactate, glucose, NEFA, potassium, ALT, AST, total CK, total LD, glutamate dehydrogenase (GLD), aldolase and cTnI. Levels of cTnI were measured on the Bayer ACS: 180S system. Sections of hearts were examined histologically from all animals in all groups. Heart lesions were scored for severity on a scale of 0 to 5: 0 = no abnormalities detected (NAD), 1 = minimal, 2 = mild, 3 = moderate, 4 = marked and 5 = very marked.
3.2.1.2 Experiment 2; route of administration study

OBJECTIVE: To investigate whether the route of administration (intraperitoneal and subcutaneous) is related to the severity of the cardiotoxicity induced by isoproterenol

3.2.1.2.1 Animal handling

ISO dosing solutions were prepared at concentrations of 0, 1366.7 (IP dosing) and 1383.3 (SC dosing) µg/ml (as described in Chapter 2.4.1). The vehicle was deionised water. Female Hanover Wistar rats (n=60; mean weight 83.9 g) were treated with a single IP (n=30) or SC (n=30) dose of ISO. Of the (n=30) IP-dosed animals, 5 were dosed with deionised water (vehicle control) and 25 with ISO at 5000 µg/kg; control animals were autopsied at 0 h and n=5 ISO-treated rats were autopsied at 1, 2, 3, 4 and 5 h post-dosing. Each treatment group of n=5 rats occupied 1 cage. The same procedure was used for the n=30 SC-dosed animals.

3.2.1.2.2 Sample handling

Sample handling was as for Experiment 1 (dose ranging study, 1 – 25000 µg/kg) except that hearts were not assessed histopathologically.

3.2.2 Statistical analysis

Data from the 2 studies were analysed using one-way analysis of variance (ANOVA) followed by Dunnett’s test for post hoc multiple comparison. In cases of violation of the assumptions for parametric testing, the Kruskal-Wallis test was used in combination with Dunn’s post-test. Statistical analysis was performed using GraphPad Prism version 3.00 for windows (GraphPad software, San Diego, California, USA).
3.3 RESULTS

3.3.1 Experiment 1; dose ranging study, 1 – 25000 µg/kg

OBJECTIVE: To investigate whether myocardial lesions can be induced by a single intraperitoneal administration of isoproterenol at dose levels from 1 to 25000 µg/kg.

3.3.1.1 Clinical signs and body weight changes

Rats were treated with a single IP injection of ISO at dose levels of 0 (vehicle-treated; deionised water) to 25000 µg/kg. During the study, animals remained healthy and no clinical signs of toxicity were observed. The group mean body weights of rats at the time of ISO dosing, and at 24 h time point are presented in Table 3.1. ISO administration did not appear to have an adverse effect on mean body weight gain. For example, the control group gained a mean of 2.9 g in the 24 h period following ISO dosing, the 500 µg/kg ISO group gained 0.8 g, and the 25000 µg/kg animals gained 2.6 g.

3.3.1.2 Serum clinical chemistry

3.3.1.2.1 2 h post-dosing

Results are summarised in Table 3.2. In general, at 2 h post-dosing, the administration of ISO at dose levels from 1 to 25000 µg/kg did not appear to affect group mean serum levels of potassium, ALT or AST. However, serum lactate levels were significantly raised in the 1000 µg/kg (P<0.05) and the 5000 µg/kg (P<0.001) ISO dose groups. These increases were 22.5 and 32.0 % above the mean control value, respectively. The 1000 µg/kg ISO group mean serum glucose concentration was decreased significantly (P<0.05) below the control result, however this was the only change observed in this parameter at any ISO dose level. There was some evidence of a dose-related trend for the elevation of NEFA values in the ISO-treated groups, at doses of 1000 µg/kg ISO and above. These increases were statistically significant (P<0.001) at 10000 and 25000 µg/kg ISO (increases to 133.9 % and 180.4 % above the control level, respectively).
There was some evidence that ISO administration elevated serum levels of CK; however none of the increases were statistically significant (Table 3.2). The CK control (0 µg/kg) group mean was 878.2 U/L. At 100 µg/kg ISO, the mean CK value was 1111.0 U/L, and at 25000 µg/kg, 1126.0 U/L; these increases were 26.5 and 28.2 % above the control value, respectively. The 25000 µg/kg dose group mean LD activity was raised significantly (P<0.001) at 2 h post-dosing, to 34.3 % above the control level. It is of note that at 500 µg/kg, the group mean GLD level was decreased to 0.88 U/L (NS; control value 2.48 U/L); at 25000 µg/kg, the appropriate value was 4.14 U/L (NS). Serum aldolase levels in the 10000 and 25000 µg/kg dose level groups were also increased significantly (P<0.001) compared with control values (increases to 33.0 and 40.0 % above the control).

Group mean serum levels of cTnl at 2 h post ISO dosing were increased above base line control values (0.030 µg/L) at ISO dose levels of 10 µg/kg and above (Table 3.3). The increases were to some extent dose-related and statistically significant in the 500, 10000 and 25000 µg/kg ISO groups (P<0.05, P<0.001 and P<0.001, respectively). The group mean cTnl levels in these groups were 2.200, 4.820 and 2.986 µg/L, representing 72.3-, 159.7- and 98.5-fold increases above the control (0 µg/kg, vehicle-treated) group mean of 0.030 µg/L.

3.3.1.2.2 24 h post-dosing

At 24 h post-dosing, the group mean serum NEFA and ALT results were not affected by the administration of ISO (Table 3.4). Levels of lactate, however, were significantly decreased below the mean control value in the 1 and 5 µg/kg ISO dose groups (P<0.001 and P<0.05; decreases to 68.5 % and 72.6 % of the control level, respectively). In the 1 µg/kg ISO dose group, the group mean serum glucose concentration was raised significantly (P<0.001) and was an increase of 22.1 % above the base line control value; however in the higher dose levels, ISO appeared to decrease glucose values: at 25000 µg/kg, the group mean glucose value was reduced to 7.24 mmol/L, which was 83.9 % of the control result (P<0.05). Serum potassium levels were increased above the mean control value at ISO dose levels of 1, 5, 500, 5000, 10000 and 25000 µg/kg, however the increase was only significant at the highest dose level (P<0.001).
Levels of serum AST were significantly reduced (P<0.001) in the lowest ISO dose level group (1 μg/kg) at 24 h post-dosing (Table 3.4). Levels of serum CK at the lowest ISO dose levels (1 and 5 μg/kg) were decreased significantly (P<0.001) in comparison with the mean control value (982.8 U/L); however at 25000 μg/kg ISO, the serum CK activity was significantly increased to 1304.0 U/L (P<0.05). Likewise, the mean serum LD values for 1 and 5 μg/kg were decreased significantly (P<0.05) whereas the result for the 25000 μg/kg group was a significant increase (P<0.001). A very similar pattern of results was obtained for the serum aldolase levels. The 50 μg/kg ISO group mean GLD activity was reduced to 0.86 U/L, which was 26.1 % of the control result (3.30 U/L); this decrease was statistically significant (P<0.05), however no other alterations in serum GLD results were observed at the 24 h autopsy time point in the other ISO dose level groups.

At 24 h post ISO administration, mean serum levels of cTnI remained elevated above the mean baseline control value (0.030 μg/L) in the 500, 10000 and 25000 μg/kg ISO dose groups, the increases were 0.7-, 1.9- and 2.8-fold, respectively (NS) over the mean control level (Table 3.4).

3.3.1.3 Gross pathology and histopathology

At autopsy, no macroscopic abnormalities were observed in any of the rats in the various ISO treatment groups at either time point (2 h and 24 h post-dosing).

The administration of a single IP dose of ISO at the higher dose levels had a significant effect on the group mean relative heart weights at both the 2 h and 24 h post-dosing autopsies (Figure 3.1A, 3.1B). At 2 h post-dosing, the group mean relative weight of the control (0 μg/kg ISO, vehicle-treated) animals was 3.86 g/kg (Figure 3.1A). There was evidence of increased relative weight at ISO dose levels of 1000 μg/kg and above. The mean relative heart weights at these higher dose levels were increased above the control mean value as follows: 1000 μg/kg, an 8.0 % increase (NS); 5000 μg/kg, 15.3 % (P<0.001); 10000 μg/kg, 23.8 % (P<0.001) and 25000 μg/kg, 23.8 % (P<0.001) (Figure 3.1A). At the lower ISO dose levels (1, 5, 10 and 50 μg/kg), there appeared to be a reduction in group mean relative heart weights (NS). At 24 h post-dosing (Figure 3.1B), the group mean relative heart weights of rats given ISO dose levels of 500 μg/kg, 1000 μg/kg, 5000 μg/kg, 10000 μg/kg and 25000 μg/kg appeared to be increased over the
mean control level; as follows: 500 μg/kg, 7.5 % increase (NS); 1000 μg/kg, 15.2 % increase (P<0.001); 5000 μg/kg, 12.0 % increase (NS); 10000 μg/kg, 26.2 % increase (P<0.001) and 25000 μg/kg, 23.5 % increase (P<0.001). As at 2 h post ISO dosing, there appeared to be a similar pattern of reduced relative weights at the lower ISO dose levels (1, 5, 10 and 50 μg/kg ISO, NS).

There was no histopathological evidence of cardiac injury at any ISO dose level at 2 h post-dosing. The incidence and severity of microscopic findings in the heart observed at the 24 h autopsy point is summarised in Table 3.5. At 24 h, the administration of ISO resulted in chronic myodegeneration, graded as minimal to moderate, in a proportion of animals at dose levels of 10 μg/kg and above. Chronic degeneration was characterised by multifocal areas of myocardial degeneration/necrosis and myocyte loss, with replacement by mononuclear cells and fibroblasts (Figure 3.2). Lesions observed at 24 h increased in incidence and severity with increasing dose levels of ISO. Hearts from control rats were histologically normal.

3.3.2 Experiment 2; route of administration study

OBJECTIVE: To investigate whether the route of administration (intraperitoneal and subcutaneous) is related to the severity of the cardiotoxicity induced by isoproterenol

3.3.2.1 Clinical signs

Female Hanover Wistar rats were given a single IP or SC dose of ISO at 5000 μg/kg. There were no clinical signs of toxicity observed following the administration of the drug by either dose route.

3.3.2.2 Serum clinical chemistry

3.3.2.2.1 Intraperitoneal dosing

Results are summarised in Table 3.6. The administration of ISO at 5000 μg/kg by IP injection did not affect serum levels of lactate, potassium, ALT or GLD. However, at 3 h and 4 h post-dosing, the group mean serum glucose concentration was decreased
significantly (P<0.05 and P<0.001, respectively) compared with the mean control value; mean levels were also decreased at 1, 2 and 5 h post-dosing but the reductions were not significant.

At 1 h post-dosing, the group mean serum NEFA level was increased significantly (P<0.001) above the base line control value. The control NEFA result was 0.342 mmol/L; at 1 h post-dosing, the group mean level was 0.892 mmol/L (a 160.8 % elevation above the mean control value) (Table 3.6). Levels were also raised (NS) at the other time points post-dosing. The group mean serum AST values were increased significantly (P<0.001) in the ISO-treated animals at 2 to 5 h post-dosing. Levels of CK were increased in the rats given a single IP injection of ISO at 5000 μg/kg at 1, 2, 3, 4 and 5 h post-dosing, increases above the control value of 46.0 % (NS), 138.1 % (P<0.05), 115.0 % (P<0.05), 122.6 % (P<0.05) and 103.2 % (NS), respectively. Serum LD activities were raised from 1 to 5 h post ISO administration; increases were statistically significant at 2 (P<0.05), 3 (P<0.001), 4 (P<0.05) and 5 h (P<0.05) post-dosing. Group mean aldolase activities were elevated above the mean control value from the 2 h autopsy time point; increases were statistically significant at 3 (P<0.001) and 5 h (P<0.05) post-dosing (Table 3.6).

Serum mean levels of cTnl following a single IP dose of ISO at 5000 μg/kg were elevated above the mean control value (0.044 μg/L) from the 1 to the 5 h post-dosing time points (Table 3.7). However, the increases were not significant at any time point. At 1 h, the group mean serum cTnl concentration was 0.784 μg/L, a 16.8-fold increase over the mean control value. Serum cTnl levels appeared to peak at approximately 2 h post-dosing; at 2 h the cTnl group mean was elevated to 2.366 μg/L, 52.8-fold above the control value of 0.044 μg/L. At 5 h post ISO administration, the group mean cTnl result remained increased above the control value (0.704 μg/L compared with the control value of 0.044 μg/L, a 15-0 fold increase). The average of the cTnl mean fold increases at 1 to 5 h was 17.96.

3.3.2.2.2 Subcutaneous dosing

The SC administration of ISO at 5000 μg/kg did not affect group mean serum values of lactate, potassium, ALT, GLD or aldolase (Table 3.8). Serum glucose concentrations, however, were decreased at all time points from 1 to 5 h post-dosing. Levels of serum
glucose fell to 70.5 (P<0.001), 72.1 (P<0.001), 80.7 (P<0.05), 81.1 (NS) and 77.1 % (P<0.05) of the control value at the 1, 2, 3, 4, and 5 h post-dosing autopsy time points, respectively.

The group mean serum NEFA levels were increased at all ISO post-dosing time points, with statistical significance at 1 (P<0.001), 2 (P<0.001) and 3 h (P<0.05) post ISO administration, where the increases were 311.5, 175.8 and 115.9 % above the mean control value, respectively (Table 3.8). Serum AST activities appeared to be raised above the control value at all time points post-dosing; however the increases were NS at any time. The group mean serum CK values began to increase at 1 h post-dosing, and appeared to peak around 2 h (P<0.05) post-dosing, when the level was 120.9 % above the control value. Serum LD levels were increased at all time points post dosing; however no increases were statistically significant.

Following a single SC injection of ISO at 5000 μg/kg, group mean serum cTnI values were increased at all time points post-dosing, however the elevations were NS at any time (Table 3.9). At 0 h (vehicle control), the mean serum cTnI level was 0.042 μg/L. At 1 h post-dosing, the group mean serum cTnI was increased to 2.352 μg/L, a 55.0-fold increase above the control value. The cTnI concentration appeared to peak at approximately 3 h post-dosing, when the group mean value was 8.590 μg/L, an increase of 203.5-fold above the control value of 0.042 μg/L. The mean levels at 4 and 5 h post-dosing were below the mean levels at 3 h post ISO administration. At 4 h the mean serum cTnI value was 1.888 μg/L and at 5 h the mean value was 1.700 μg/L; these results were 44.0- and 39.5-fold higher than the mean control value, respectively. The average of the cTnI mean fold increases at 1 to 5 h was 86.18.
3.4 DISCUSSION

ISO is a synthetic catecholamine and a potent β-adrenoceptor agonist which has been shown to induce myocardial ischaemia and necrosis in a number of experimental animal species, including the rat (Chappel et al., 1959), the monkey (Maruffo, 1967) and the dog (Rona et al., 1959b). ISO causes myocardial necrosis through an exaggerated pharmacological effect: stimulation of β₁ receptors in the heart results in increased cardiac output, excitability and rate of contraction; β₂ agonism results in vasodilatation, which may lead to a fall in blood pressure (Sweetman, 2006). A reduction in blood pressure in conjunction with increased heart rate may result in areas of tissue hypoxia and ischaemia.

The aims of the present studies were to assess the cardiotoxicity of ISO in the Hanover Wistar rat following a single dose of the drug, to identify the threshold dose of cardiotoxicity. In addition, the route of administration of ISO was investigated to find whether this had any effect on the degree of cardiotoxicity observed.

In Experiment 1 (dose ranging study, 1 – 25000 µg/kg), female Hanover Wistar rats were administered a single dose of ISO IP at dose levels of 0 (vehicle-treated control), 1, 5, 10, 50, 100, 500, 1000, 5000, 10000 or 25000 µg/kg and animals sampled at both 2 h and 24 h post-dosing. There were no clinical signs of toxicity observed in ISO-treated animals, and there were no major alterations in body weight observed in the 24 h post-dosing period. No animals died in the present study, indicating that a dose level of 25000 µg/kg was well tolerated.

ISO induced a number of changes in serum clinical chemistry parameters (Table 3.2, 3.4). Levels of serum lactate were significantly raised at 2 h post-dosing in the 1000 µg/kg (P<0.05) and 5000 µg/kg (P<0.001) dose groups (Table 3.2); at 24 h, the 1 µg/kg and 5 µg/kg lactate concentrations were significantly decreased (P<0.001 and P<0.05, respectively) (Table 3.4). Serum lactate levels were shown by Lutmer and Wexler (1971) to be increased 2 h following the SC administration of ISO to male Sprague Dawley rats at a dose level of 500000 µg/kg and levels had almost returned to control values by 24 h post-dosing. The accumulation of lactate (lactic acid) in a tissue, resulting in increased levels in the serum, may occur through anaerobic metabolism, in which lactate is a by-product. Lactic acidosis (type A, hypoxic) is associated with decreased tissue oxygenation (Sacks, 2006) and therefore, in the present study,
increased lactate levels may have reflected some degree of hypoxia in the heart and in other organs.

The group mean serum glucose concentrations were significantly reduced (P<0.05) at the 2 h time point in the 1000 μg/kg ISO group (Table 3.2); at 24 h, levels were increased significantly (P<0.001) in the 1 μg/kg ISO dose group, but were decreased significantly (P<0.05) at 25000 μg/kg (Table 3.4). These results differ from those of Wexler et al. (1968), who showed that ISO treatment in Sprague Dawley rats of both sexes at 500000 μg/kg SC resulted in elevated serum glucose levels at 24 h post-dosing, during the “active process” of myocardial necrosis.

Serum NEFA concentrations were increased at the 2 h autopsy point at ISO dose levels of 1000 μg/kg and above, with statistical significance at 10000 and 25000 μg/kg (P<0.001). Wexler et al. (1968) found that serum NEFA levels rose significantly following the single SC injection of ISO at 500000 μg/kg to male and female Sprague Dawley rats, and elevations were concomitant with the “active stages” of necrosis in the heart (at 24 h post-dosing). In the present study, the group mean NEFA concentrations were raised at the 2 h time point (Table 3.2), when no histopathological changes were observed; however NEFA levels had returned to control values at the 24 h autopsy point (Table 3.4), when there was clear evidence of chronic myocardial degeneration. As a non-selective β-adrenoceptor agonist, ISO causes lipolysis through β3 stimulation (Rang et al., 1999). The heart derives a significant proportion of its energy supply from free fatty acids derived by lipolysis from adipose tissue (Taegtmeyer, 1994). Lipolysis generates free fatty acids, and therefore serum NEFA may be used as a measure of lipolysis, and thus as an indirect measure of compound exposure. In the present study however, free fatty acids may also play a role in ISO-induced cardiotoxicity, and have been demonstrated to significantly augment the degree of myocardial necrosis observed with ISO in the rat (Mohan and Bloom, 1999). Excess levels of fatty acids in the cardiomyocytes can be deleterious, and may result in oxidative stress and lipid peroxidation (Vik-Mo and Mjøs, 1981). Lipolysis also increases myocardial oxygen demand (Mjøs, 1971), which may in turn lead to tissue hypoxia and ischaemia.

At 24 h post-dosing, the 25000 μg/kg ISO group mean potassium concentration was significantly raised (P<0.001) compared with the control value (Table 3.4). Hyperkalaemia may occur following metabolic acidosis (through the redistribution of
potassium), but may also be evident in cases of hypoxia (Klutts and Scott, 2006). With treatments involving β2 agonists in the dog, an initial reduction in serum potassium levels may be seen at around 2 h post-dosing, due to increased cellular uptake, reflecting pharmacological activity; normally by 24 h post-dosing, there is a redistribution of the electrolyte, and hyperkalaemia may be observed (York, 2007). Therefore, in the present study, the increase in serum potassium at 25000 μg/kg ISO at 24 h (Table 3.4) may be viewed as a treatment-related effect.

At the higher ISO dose levels (10000 and 25000 μg/kg), there was some evidence that serum activities of CK, LD and aldolase were increased at the 2 h post-dosing time point (Table 3.2); at 24 h, levels remained elevated and were statistically significant at 25000 μg/kg ISO (CK, P<0.05; LD, P<0.001; aldolase, P<0.001) (Table 3.4). Wexler (1970) showed that serum CK levels were raised significantly at 1 h following the treatment of male Sprague Dawley rats with a single SC dose of ISO at 500000 μg/kg, concurrent with necrosis, peaking at 6 h post drug administration. A dose of 500000 μg/kg ISO SC also resulted in increased levels of LD in male Sprague Dawley rats at 24 h post-dosing (Wexler and Kittinger, 1963). CK and LD have traditionally been employed to assess cardiotoxicity in experimental animal studies (Evans, 1996), however neither marker is specific for cardiac tissue. Increases in serum aldolase values may be an indication of skeletal muscle disease or injury (Panteghini et al., 2006), but aldolase is thought to be a non-specific marker for generalised tissue damage (York, 2007). Thus, in the present study, elevations in serum aldolase may be possibly attributed to tissue damage at the site of injection, as well as being the result of ISO-induced cardiotoxicity.

At 2 h post-dosing, there were elevations in mean serum cTnl values at ISO dose levels of 10 μg/kg and above, with statistical significance at 500 (P<0.05), 10000 (P<0.001) and 25000 (P<0.001) μg/kg (Table 3.3). By the 24 h autopsy time point, cTnl levels were returning to baseline control values; however there were still elevations in the mean values in the 500, 10000 and 25000 μg/kg ISO dose groups (NS at all dose levels) (Table 3.4). cTnl is a specific and sensitive marker of cardiomyocyte injury (Gaze and Collinson, 2005), and therefore in the present study, the presence of increased cTnl in the serum of ISO-treated rats is an indication of ISO-induced cardiomyocyte degeneration and necrosis.
ISO treatment induced a significant increase in relative heart weights at 2 and 24 h post-dosing in the higher ISO dose levels (Figure 3.1). An increase in the relative weight of the heart may be caused by cardiac hypertrophy; indeed repeated isoproterenol administration induces this change (Kizaki et al., 2005); however hypertrophy is a chronic condition and an increase in myocardial mass is only likely to develop over a longer period of time than 2, or even 24, h. Therefore, in the present study, it is suggested that the significant increases in the group mean relative heart weights in ISO-treated rats is due to the pathological changes occurring in the heart in response to ISO administration, namely neutrophil, mononuclear cell and fibroblast infiltration, which was observed at 24 h post-dosing.

In the present study, the administration of ISO at dose levels of 10 µg/kg and higher resulted in chronic myodegeneration, characterised by myocardial degeneration/necrosis and myocyte loss, at 24 h post drug administration (Figure 3.2; Table 3.5). The time course of cardiac injury induced by ISO was studied by Rona et al. (1961) after treatment of male Wistar rats with a single SC dose of ISO at 85000 µg/kg. At 2 h post-dosing, there was increased myofibre eosinophilia, and by 24 h, focal myocardial necrosis was present, with neutrophil infiltration and interstitial oedema, peaking at 48 h. Therefore, in the present study, the time course of cardiac injury differed slightly from that observed by Rona et al. (1961), as no changes were observed at 2 h post-dosing; however the dose levels used in the present study are considerably lower than the 85000 µg/kg dose level used by Rona et al. (1961), and it is possible that at such lower dose levels, the time course of cardiac injury may alter.

When individual cTnl results from the 2 h autopsy (Table 3.3) are compared with the individual animal pathological lesion scores (severity grades) at 24 h (Table 3.5), a definite threshold ISO dose level of 10 µg/kg can be identified, at which cTnl values begin to be increased and where chronic myocardial degeneration begins to occur, but below which no cardiomyocyte damage is observed. These results are tabulated in Table 3.10. At the 2 h autopsy, the serum cTnl group mean was first increased at 10 µg/kg ISO with a mean level of 0.086 µg/L compared with the control result of 0.030 µg/L; at this ISO dose level, the 24 h mean pathological lesion score (severity grade) was also increased for the first time (a mean score of 0.4 compared with the control mean score of 0.0). The ISO dose level identified here of 10 µg/kg is significantly lower than the threshold dose of 40 µg/kg by the SC route found by Rona et al. (1959a) that would
produce myocardial lesions. In addition, Herman et al. (2006) showed that cardiac troponin T (cTnT) levels were elevated in male Sprague Dawley rats receiving 8 μg/kg ISO within 3 h of drug administration; however no microscopic cardiac lesions were identified at this dose level. Unfortunately, it was not possible to directly and statistically compare individual cTnI values with individual animal pathology scores in the present study, as the cTnI elevations were determined at the 2 h time point (Table 3.3), and at this time there was no evidence of histopathological change in the heart.

In conclusion, when ISO was administered as a single IP injection at doses of 10 μg/kg and above, increases in serum cTnI levels at 2 h post-dosing and histopathological lesions of cardiac injury at 24 h were observed. The measurement of the more traditional markers of cardiac injury, such as CK and LD, were demonstrated to be relatively insensitive in comparison to cTnI determinations. These patterns of response are indicative of ISO-induced acute cardiomyocyte injury.

In Experiment 2 (route of administration study), female Hanover Wistar rats were treated with a single dose of ISO at 5000 μg/kg by the IP or SC route, with animals killed at 0, 1, 2, 3, 4 and 5 h post-dosing. No clinical signs of toxicity were observed in treated animals.

When the findings from the 2 dose routes of ISO administration are compared, it can be seen that changes in serum glucose, NEFA, AST, CK and aldolase levels occurred when ISO was given by IP or SC injection (Table 3.6, 3.8). In addition, levels of serum cTnI were increased at all time points using both dose routes (Table 3.7, 3.9).

Serum glucose levels were decreased at all time points following the IP or SC injection of ISO at 5000 μg/kg (Table 3.6, 3.8). The largest reduction in serum glucose in the IP-dosed animals occurred at 4 h post-dosing, where the group mean serum glucose concentration was 7.40 mmol/L which was 71.4 % (P<0.001) of the control value. For animals given ISO by the SC route, the largest decline in serum glucose was seen at the 1 h post-dosing, where the group mean glucose level was 7.32 mmol/L which was 70.5 % of the control result (P<0.001). Therefore, there appeared to be no clear difference in the degree of the serum glucose response to ISO using the IP and SC dose routes. The present study was carried out in the morning, with time “0” being at approximately 8 am. Rats are nocturnal, and generally eat during the night, therefore at 8 am, it is possible that circulating glucose levels were high and concentrations then reduced over
time, i.e. the decrease did not reflect a drug-induced change. ISO, as a catecholamine, stimulates glycogenolysis through β2 stimulation in the liver (Crook, 2006). It is possible that ISO administration at 8 am (0 h) induced an initial breakdown of liver glycogen stores to raise serum glucose levels; however the increase in blood glucose concentration would then result in insulin release, to return glucose levels to "normal". Furthermore, in the daylight hours following ISO administration, animals would not be eating, and the variations in serum glucose observed may be a reflection of variation in liver glycogen content. Therefore, the alterations in serum glucose levels identified in Experiment 2 may not be related to the pathogenesis of drug-induced cardiac injury. However, an improvement to the present study design would have been to fast animals overnight prior to drug administration, to negate any variations in serum glucose and liver glycogen levels; or to use concurrent control animals at each sampling time point from 1 to 5 h post-dosing.

The group mean serum NEFA levels were increased by the administration of ISO by IP or SC injection (Table 3.6, 3.8). At 1 h post-dosing in the IP-dosed animals, the ISO-treated group mean NEFA result was increased to 0.892 mmol/L which was 160.8 % above the mean control level (P<0.001); levels appeared to peak at this time point, but were also raised above the control value (0.342 mmol/L) at all other time points (NS at any time). In animals treated with 5000 µg/kg ISO by SC injection, the group mean serum NEFA concentration at 1 h was elevated to 1.292 mmol/L which was 311.5 % above the control value of 0.314 mmol/L (P<0.001) and remained significantly elevated at 2 h (P<0.001) and 3 h (P<0.05) post-dosing; at 4 and 5 h the level was also raised (NS at either time). Thus, the administration of ISO by SC injection appeared to result in a larger NEFA response, suggesting a greater level of exposure to the drug.

Serum levels of AST were increased by the IP and SC injection of ISO (Table 3.6, 3.8). Using the IP dose route, the group mean AST activity was increased at all time points post-dosing, with statistical significance at 2, 3, 4 and 5 h post-dosing (P<0.001 at all time points). The greatest mean AST level was at 3 h, when the group mean value was increased to 122.0 U/L, giving an elevation of 59.7 % over the mean control figure of 76.4 U/L. Group mean AST levels were also elevated at all time points using the SC route of administration (Table 3.8); however these increases were not statistically significant. The largest increase in the serum AST following SC dosing also occurred at 3 h post-dosing, when the group mean was elevated to 127.6 U/L which was 62.3 %
over the mean control value of 78.6 U/L. Therefore, although there were some differences in the significance of the increases in group mean AST values, the responses showed a similar pattern of changes using both the IP and SC dose routes. Measurement of AST has been employed in the assessment of cardiotoxicity in some animal studies and the assay was originally used in man to detect MI (Wallace et al., 2004); however the marker is not specific or sensitive, and does not add greatly to the interpretation of the data of the present experiment.

The administration of ISO by IP or SC injection resulted in increases in mean serum CK levels (Table 3.6, 3.8). Using the IP route of administration, serum CK values were increased at all time points from 1 to 5 h post-dosing, with the peak occurring at 2 h post-dosing (a 138.1 % increase, P<0.05). At 5 h post-dosing, CK activity remained increased using the IP route of administration (a 103.2 % increase, NS). Serum CK levels were also elevated at all time points using the SC route except at 5 h post-dosing, and the peak in serum CK also occurred at 2 h (a 120.9 % increase, P<0.05). By the 4 h autopsy time point, the group mean CK level had returned to control levels. Therefore, the peak of the response of serum CK following the IP or SC injection of 5000 µg/kg ISO was similar regardless of the route of administration; however the pattern of the changes (in time) were not identical.

Using the IP route of ISO administration, the group mean serum LD activity was increased at all time points post-dosing, with statistical significance at 2 h (P<0.05), 3 h (P<0.001), 4 h (P<0.05) and 5 h (P<0.05) post-dosing (Table 3.6). The peak in serum levels appeared to occur at 3 h post-dosing, where the group mean LD was elevated to 2789.2 U/L which was 241.6 % above the control value of 816.6 U/L (P<0.001). When ISO was administered by SC injection, serum LD values were also raised at all time points; however no increases were statistically significant (Table 3.8). The peak for serum LD following SC injection occurred at 3 h post-dosing (a 192.6 % increase, NS). Therefore, the peak of the LD response appeared to be slightly larger following the IP administration of ISO, but the pattern of changes generally compared using both routes of administration.

Following the IP administration of ISO at 5000 µg/kg, the group mean aldolase levels appeared to be generally elevated above the mean control value at all time points, with the increases becoming statistically significant at 3 h (P<0.001) and 5 h (P<0.05) post-
dosing, increases of 58.0 % and 55.8 %, respectively over the mean control value of 46.2 U/L (Table 3.6). No clear alterations were observed in mean serum aldolase values when rats were treated with ISO by SC injection (Table 3.8).

The treatment of rats with 5000 µg/kg ISO by IP or SC injection caused elevations in serum concentrations of cTnI at all time points post-dosing; however the increases were NS at all time points for both routes of administration (Table 3.7, 3.9). For the IP route of administration (Table 3.7), the group mean fold increases for 1, 2, 3, 4 and 5 h were, respectively, 16.8, 52.8, 1.5, 3.7 and 15.0. The average of the cTnI mean fold increases at 1 to 5 h was 17.96. When ISO was administered by SC injection (Table 3.9), the group mean fold increases were 55.0, 88.9, 203.5, 44.0 and 39.5, for the 1, 2, 3, 4 and 5 h autopsy time points, respectively. The average of the cTnI mean fold increases at 1 to 5 h was 86.18. Therefore, the cTnI response to ISO was greater using the SC route of administration. The pattern of the changes, in time, showed some difference with the maximum level for the IP route occurring at approximately 2 h, but the peak for the SC route was at about 3 h.

Therefore, when the cTnI results following the 2 routes of dosing are compared, it appears that the SC route of administration results in a larger response to ISO administration. Prior to starting the experiment, it was known that the LD$_{50}$ for ISO in the rat was much lower by the SC route compared with IP dosing (SC, 600 µg/kg; IP, 128000 µg/kg). Upon administration in man, ISO may be initially conjugated with sulphate, or O-methylated by catechol-O-methyltransferase (COMT) and then conjugated with sulphate (Figure 3.3). ISO and 3-O-methyl ISO both are active, whereas ISO sulphate is inactive (Sweetman, 2006). In the rat, glucuronide rather than sulphate metabolites are formed. In man, the metabolism of ISO is dependent upon the route of administration employed, with a larger proportion of sulphate metabolite being formed by the oral and inhaled routes, in comparison with the IV route of metabolism. Therefore, in the present study, the route of administration of ISO may have affected the metabolism of the drug, with increased glucuronidation (and therefore, inactivation) via the IP route, resulting in a lower plasma concentration of active drug. Dollery et al. (1971) showed that when ISO was infused into the portal vein of a dog, the drug exerted 1 fifth of the effect of the same dose given IV via a systemic vein, although the same metabolic end products were observed. Therefore, the large differences between the 2 routes of administration in the present study are suggested to be due to conjugation in
the gut wall during absorption, due to absorption through the gut wall after IP administration.

When the various serum markers of cardiotoxicity are compared in the SC route of dosing study, it is seen that cTnI was the earliest marker (at 1 h) to be elevated following ISO administration; the peak was at 3 h following drug treatment, where the group mean result was increased 203.5-fold over the 0 h control value (a 20352.4 % elevation) (Table 3.9). For AST and LD, the peak activities also occurred at 3 h post-dosing, and these elevations were 62.3 % and 192.6 % of the control values, respectively. The peak in serum CK activity was at 2 h, where the mean result was 120.9 % of the control group mean. Therefore, of the serum markers investigated, it has been demonstrated that cTnI is the superior marker of cardiomyocyte injury in terms of both sensitivity and specificity.

From the 2 experiments described in this chapter, it has been shown that elevated serum levels of cTnI occur as early as 1 h post-dosing, and peak at approximately 2 to 3 h following drug administration. This highlights that careful attention must be paid to the timing of sample collections and this is also critical to data interpretation. Characteristic histopathological lesions of ISO-induced cardiac injury (myocardial degeneration/necrosis and myocyte loss) have also been shown to be present at 24 h post ISO administration (but not at 2 h post-dosing). However, the time course of cardiac injury induced by ISO administration has still to be investigated.
Figure 3.1. Experiment 1, dose ranging study, 1 – 25000 μg/kg; group mean relative heart weights in control (vehicle-treated, 0 μg/kg) female Hanover Wistar rats and rats treated with isoproterenol (ISO). (A) Relative heart weights at 2 h post-dosing; (B) relative heart weights at 24 h post-dosing. Values are means, SD as error bars; n=15 controls; n=5 for all ISO-treated groups. Treated groups were compared with pooled (n=15) control samples from Experiments A, B and C. ***Significantly different from control, P<0.001. Note the origin of the Y axis is 3.0 g/kg.
Figure 3.2. Experiment 1, dose ranging study, 1 – 25000 μg/kg; (A) myocardium from a control rat and (B) myocardium from a rat given a single intraperitoneal dose of isoproterenol at 25000 μg/kg and killed at 24 h post-dosing. There are multifocal areas of myocardial degeneration/necrosis, with mononuclear cells and fibroblasts. H&E, (A) x 400 original magnification (OM); (B) x 160 OM.
Figure 3.3. Metabolism of isoproterenol in man. From Dollery et al. (1971). COMT: catechol-O-methyltransferase.
Table 3.1. Experiment 1, dose ranging study, 1 – 25000 µg/kg; group mean body weights of control female Hanover Wistar rats and animals treated with isoproterenol (ISO) at the time of dosing (0 h) and at 24 h post-dosing\(^a\).

<table>
<thead>
<tr>
<th>Isoproterenol dose (µg/kg)</th>
<th>Group mean body weight at 0 h</th>
<th>Group mean body weight at 24 h</th>
<th>Body weight increase (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 (control)</td>
<td>150.6 (37.1)</td>
<td>153.5 (37.2)</td>
<td>2.9</td>
</tr>
<tr>
<td>1</td>
<td>190.6 (15.0)</td>
<td>190.6 (14.4)</td>
<td>0.0</td>
</tr>
<tr>
<td>5</td>
<td>187.8 (7.4)</td>
<td>191.4 (7.3)</td>
<td>3.6</td>
</tr>
<tr>
<td>10</td>
<td>179.0 (12.0)</td>
<td>183.6 (12.9)</td>
<td>4.6</td>
</tr>
<tr>
<td>50</td>
<td>171.2 (13.6)</td>
<td>172.2 (15.8)</td>
<td>1.0</td>
</tr>
<tr>
<td>100</td>
<td>173.0 (18.0)</td>
<td>177.2 (18.7)</td>
<td>4.2</td>
</tr>
<tr>
<td>500</td>
<td>156.2 (8.1)</td>
<td>157.0 (6.2)</td>
<td>0.8</td>
</tr>
<tr>
<td>1000</td>
<td>130.0 (6.0)</td>
<td>131.2 (7.6)</td>
<td>1.2</td>
</tr>
<tr>
<td>5000</td>
<td>129.2 (15.6)</td>
<td>130.6 (14.8)</td>
<td>1.4</td>
</tr>
<tr>
<td>10000</td>
<td>102.2 (6.9)</td>
<td>105.0 (7.5)</td>
<td>2.8</td>
</tr>
<tr>
<td>25000</td>
<td>107.2 (7.1)</td>
<td>109.8 (8.9)</td>
<td>2.6</td>
</tr>
</tbody>
</table>

\(^a\) Results are means (SD). n=5/group, except for control (0 µg/kg ISO, vehicle-treated) where n=15. Data were not analysed statistically.
Table 3.2. Experiment 1, dose ranging study, 1 – 25000 µg/kg; serum clinical chemistry results in control (vehicle-treated, 0 µg/kg) female Hanover Wistar rats and rats treated with isoproterenol (ISO) at 2 h post-dosing<sup>a,b</sup>.

<table>
<thead>
<tr>
<th>Isoproterenol dose (µg/kg)</th>
<th>0</th>
<th>1</th>
<th>5</th>
<th>10</th>
<th>50</th>
<th>100</th>
<th>500</th>
<th>1000</th>
<th>5000</th>
<th>10000</th>
<th>25000</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lactate</td>
<td>5.168 (0.877)</td>
<td>4.993 (0.769)</td>
<td>5.482 (0.980)</td>
<td>5.612 (0.470)</td>
<td>5.180 (1.027)</td>
<td>4.856 (0.347)</td>
<td>4.842 (0.693)</td>
<td>6.330 (1.026)</td>
<td>6.820 (0.559)***</td>
<td>5.770 (0.444)</td>
<td>5.946 (0.623)</td>
</tr>
<tr>
<td>Glucose</td>
<td>8.99 (1.08)</td>
<td>8.85 (0.37)</td>
<td>9.54 (1.13)</td>
<td>8.10 (1.30)</td>
<td>8.52 (0.93)</td>
<td>8.64 (0.84)</td>
<td>8.80 (1.15)</td>
<td>7.46 (1.05)</td>
<td>8.10 (0.87)</td>
<td>7.88 (0.51)</td>
<td>7.66 (0.54)</td>
</tr>
<tr>
<td>NEFA</td>
<td>0.383 (0.121)</td>
<td>0.433 (0.054)</td>
<td>0.458 (0.055)</td>
<td>0.258 (0.059)</td>
<td>0.294 (0.036)</td>
<td>0.416 (0.088)</td>
<td>0.348 (0.106)</td>
<td>0.470 (0.173)</td>
<td>0.542 (0.122)</td>
<td>0.896 (0.200)***</td>
<td>1.074 (0.250)***</td>
</tr>
<tr>
<td>Potassium</td>
<td>4.38 (0.30)</td>
<td>4.28 (0.13)</td>
<td>4.48 (0.25)</td>
<td>4.60 (0.21)</td>
<td>4.54 (0.29)</td>
<td>4.56 (0.23)</td>
<td>4.64 (0.23)</td>
<td>4.28 (0.38)</td>
<td>4.38 (0.13)</td>
<td>4.60 (0.25)</td>
<td>4.50 (0.21)</td>
</tr>
<tr>
<td>ALT</td>
<td>47.9 (11.8)</td>
<td>48.0 (6.4)</td>
<td>47.4 (5.3)</td>
<td>49.4 (8.7)</td>
<td>42.8 (8.6)</td>
<td>43.6 (10.5)</td>
<td>39.0 (7.6)</td>
<td>47.2 (6.8)</td>
<td>41.2 (8.5)</td>
<td>47.0 (4.3)</td>
<td>54.8 (7.5)</td>
</tr>
<tr>
<td>AST</td>
<td>91.7 (18.2)</td>
<td>91.8 (7.5)</td>
<td>88.0 (5.4)</td>
<td>88.0 (14.1)</td>
<td>93.8 (19.6)</td>
<td>101.8 (25.3)</td>
<td>85.4 (13.1)</td>
<td>90.8 (7.0)</td>
<td>94.6 (7.8)</td>
<td>111.8 (12.7)</td>
<td>113.0 (11.2)</td>
</tr>
<tr>
<td>CK</td>
<td>878.2 (202.4)</td>
<td>822.0 (93.9)</td>
<td>956.4 (99.7)</td>
<td>876.0 (201.3)</td>
<td>964.4 (202.7)</td>
<td>1111.0 (533.7)</td>
<td>859.0 (160.3)</td>
<td>908.0 (117.1)</td>
<td>1020.2 (241.9)</td>
<td>1078.2 (229.7)</td>
<td>1126.0 (58.7)</td>
</tr>
<tr>
<td>LD</td>
<td>2145.3 (431.4)</td>
<td>2320.5 (287.7)</td>
<td>2555.4 (326.6)</td>
<td>2200.6 (551.1)</td>
<td>2182.0 (530.4)</td>
<td>2144.8 (312.8)</td>
<td>1995.6 (282.0)</td>
<td>2119.8 (418.7)</td>
<td>2468.6 (578.4)</td>
<td>2611.8 (641.0)</td>
<td>2882.0 (173.5)***</td>
</tr>
<tr>
<td>GLD</td>
<td>2.49 (1.47)</td>
<td>2.77 (1.80)</td>
<td>3.03 (0.68)</td>
<td>1.73 (2.14)</td>
<td>3.48 (0.54)</td>
<td>3.18 (1.33)</td>
<td>0.88 (1.19)</td>
<td>3.72 (2.21)</td>
<td>2.92 (2.28)</td>
<td>2.84 (1.48)</td>
<td>4.14 (1.17)</td>
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<tr>
<td>Aldolase</td>
<td>40.3 (7.6)</td>
<td>40.3 (2.6)</td>
<td>38.4 (3.0)</td>
<td>39.2 (5.8)</td>
<td>41.6 (8.3)</td>
<td>45.6 (19.9)</td>
<td>36.0 (4.5)</td>
<td>42.8 (4.8)</td>
<td>45.6 (7.9)</td>
<td>53.6 (8.9)***</td>
<td>55.6 (4.2)***</td>
</tr>
</tbody>
</table>

<sup>a</sup>Values are means, SD in parentheses; n=15 for control (0 µg/kg, vehicle-treated), except for GLD, where n=13, n=5 for ISO-treated rats. Animals were given a single intraperitoneal injection of ISO and autopsied at 2 h post-dosing. *Significantly different from n=15 control animals, P<0.05, ***P<0.001.

<sup>b</sup>Abbreviations and units: lactate, mmol/L; glucose, mmol/L; NEFA, non-esterified fatty acids, mmol/L; potassium, mmol/L; ALT, alanine aminotransferase, U/L; AST, aspartate aminotransferase, U/L; CK, total creatine kinase, U/L; LD, total lactate dehydrogenase, U/L; GLD, glutamate dehydrogenase, U/L; aldolase, U/L.
Table 3.3. Experiment 1, dose ranging study, 1 – 25000 μg/kg; serum cardiac troponin I (cTnI) results in individual control (vehicle-treated, 0 μg/kg) female Hanover Wistar rats and rats treated with isoproterenol (ISO) at 2 h post-dosing\textsuperscript{a,b}.

<table>
<thead>
<tr>
<th>Isoproterenol dose (pg/kg)</th>
<th>0</th>
<th>1</th>
<th>5</th>
<th>10</th>
<th>50</th>
<th>100</th>
<th>500</th>
<th>1000</th>
<th>5000</th>
<th>10000</th>
<th>25000</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.03</td>
<td>0.03</td>
<td>0.03</td>
<td>0.03</td>
<td>0.03</td>
<td>0.03</td>
<td>1.26</td>
<td>0.03</td>
<td>3.22</td>
<td>0.03</td>
<td>3.88</td>
<td>0.90</td>
</tr>
<tr>
<td>0.03</td>
<td>0.03</td>
<td>0.03</td>
<td>0.03</td>
<td>0.03</td>
<td>0.20</td>
<td>1.11</td>
<td>1.21</td>
<td>0.37</td>
<td>0.03</td>
<td>0.92</td>
<td>6.08</td>
</tr>
<tr>
<td>0.03</td>
<td>0.03</td>
<td>0.03</td>
<td>0.03</td>
<td>0.03</td>
<td>0.15</td>
<td>0.99</td>
<td>1.35</td>
<td>1.56</td>
<td>0.03</td>
<td>5.01</td>
<td>4.32</td>
</tr>
<tr>
<td>0.03</td>
<td>0.03</td>
<td>0.03</td>
<td>0.03</td>
<td>0.03</td>
<td>0.35</td>
<td>1.09</td>
<td>3.04</td>
<td>0.03</td>
<td>3.58</td>
<td>7.91</td>
<td>1.80</td>
</tr>
<tr>
<td>0.03</td>
<td>0.03</td>
<td>0.03</td>
<td>0.03</td>
<td>0.03</td>
<td>0.14</td>
<td>0.83</td>
<td>1.81</td>
<td>5.37</td>
<td>0.03</td>
<td>6.38</td>
<td>1.83</td>
</tr>
</tbody>
</table>

Mean: 0.030, 0.030, 0.030, 0.030, 0.086, 0.278, 1.252, 2.200 * 1.042, 0.740, 4.820 *** 2.986 ***
SD: 0.000, 0.000, 0.000, 0.080, 0.335, 0.327, 2.072, 1.370, 1.588, 2.651, 2.147

\textsuperscript{a}Animals were given a single intraperitoneal injection of ISO at dose levels of 0 (control, vehicle-treated), 1, 5, 10, 50, 100, 500, 1000, 5000, 10000, and 25000 μg/kg and autopsied at 2 h post-dosing.

\textsuperscript{b}Values are measured in μg/L. *Significantly different from n=15 control animals, P<0.05, ***P<0.001.
Table 3.4. Experiment 1, dose ranging study, 1 – 25000 µg/kg; serum clinical chemistry results in control (vehicle-treated, 0 µg/kg) female Hanover Wistar rats and animals treated with isoproterenol (ISO) at 24 h post-dosing a,b.*

<table>
<thead>
<tr>
<th>Isoproterenol dose (µg/kg)</th>
<th>0</th>
<th>1</th>
<th>5</th>
<th>10</th>
<th>50</th>
<th>100</th>
<th>500</th>
<th>1000</th>
<th>5000</th>
<th>10000</th>
<th>25000</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lactate</td>
<td>6.831 (1.722)</td>
<td>4.680 (0.465) ***</td>
<td>4.952 (1.201) *</td>
<td>4.942 (0.881)</td>
<td>5.690 (1.129)</td>
<td>5.674 (0.663)</td>
<td>5.302 (0.534)</td>
<td>6.214 (1.565)</td>
<td>6.402 (0.359)</td>
<td>7.184 (0.711)</td>
<td>7.044 (0.293)</td>
</tr>
<tr>
<td>Glucose</td>
<td>8.63 (1.12)</td>
<td>10.54 (0.74) ***</td>
<td>9.80 (1.00)</td>
<td>8.96 (0.69)</td>
<td>9.08 (0.76)</td>
<td>8.62 (1.13)</td>
<td>8.82 (1.03)</td>
<td>7.96 (0.63)</td>
<td>9.36 (0.69)</td>
<td>7.56 (0.62)</td>
<td>7.24 (0.32) *</td>
</tr>
<tr>
<td>NEFA</td>
<td>0.446 (0.101)</td>
<td>0.436 (0.088) ***</td>
<td>0.426 (0.113)</td>
<td>0.390 (0.146)</td>
<td>0.424 (0.119)</td>
<td>0.428 (0.154)</td>
<td>0.382 (0.077)</td>
<td>0.464 (0.116)</td>
<td>0.560 (0.199)</td>
<td>0.372 (0.089)</td>
<td>0.552 (0.130)</td>
</tr>
<tr>
<td>Potassium</td>
<td>4.53 (0.26)</td>
<td>4.74 (0.28)</td>
<td>4.60 (0.14)</td>
<td>4.38 (0.26)</td>
<td>4.32 (0.19)</td>
<td>4.32 (0.11)</td>
<td>4.60 (0.16)</td>
<td>4.16 (0.25)</td>
<td>4.80 (0.27)</td>
<td>4.70 (0.32)</td>
<td>5.38 (0.43) ***</td>
</tr>
<tr>
<td>ALT</td>
<td>51.1 (8.9)</td>
<td>51.2 (9.6)</td>
<td>52.4 (17.6)</td>
<td>44.4 (7.2)</td>
<td>40.6 (5.7)</td>
<td>39.6 (6.0)</td>
<td>42.8 (5.0)</td>
<td>44.0 (8.4)</td>
<td>48.0 (6.3)</td>
<td>56.8 (11.2)</td>
<td>62.4 (14.8)</td>
</tr>
<tr>
<td>AST</td>
<td>97.3 (15.4)</td>
<td>76.4 (10.2) ***</td>
<td>79.8 (21.9)</td>
<td>79.4 (10.6)</td>
<td>82.6 (5.6)</td>
<td>80.2 (10.8)</td>
<td>77.8 (9.9)</td>
<td>81.4 (6.6)</td>
<td>91.8 (8.6)</td>
<td>104.6 (24.0)</td>
<td>109.8 (11.3)</td>
</tr>
<tr>
<td>CK</td>
<td>982.8 (179.1)</td>
<td>599.6 (168.3) ***</td>
<td>651.6 (177.1) ***</td>
<td>889.2 (249.2)</td>
<td>932.0 (196.4)</td>
<td>937.0 (229.4)</td>
<td>737.6 (195.8)</td>
<td>839.2 (112.9)</td>
<td>944.2 (178.8)</td>
<td>965.4 (198.3)</td>
<td>1304.0 (154.0) *</td>
</tr>
<tr>
<td>LD</td>
<td>2422.9 (496.2)</td>
<td>1517.4 (497.4) *</td>
<td>1656.6 (460.3) *</td>
<td>2355.5 (674.0)</td>
<td>2369.4 (484.1)</td>
<td>2266.2 (565.1)</td>
<td>1684.0 (442.5)</td>
<td>2151.2 (418.3)</td>
<td>2421.8 (416.4)</td>
<td>2715.6 (667.7)</td>
<td>3482.8 (559.5) ***</td>
</tr>
<tr>
<td>GLD</td>
<td>3.30 (1.74)</td>
<td>3.24 (0.93)</td>
<td>3.24 (2.05)</td>
<td>2.75 (1.63)</td>
<td>0.86 (0.62) *</td>
<td>1.68 (1.28)</td>
<td>4.04 (0.85)</td>
<td>4.94 (0.70)</td>
<td>3.46 (1.43)</td>
<td>4.56 (1.32)</td>
<td>4.62 (1.61)</td>
</tr>
<tr>
<td>Aldolase</td>
<td>43.3 (7.4)</td>
<td>30.6 (5.5) ***</td>
<td>31.0 (7.0) *</td>
<td>39.2 (7.5)</td>
<td>42.4 (4.6)</td>
<td>37.8 (4.8)</td>
<td>32.0 (8.2) *</td>
<td>37.2 (5.2)</td>
<td>41.8 (6.5)</td>
<td>52.8 (10.1)</td>
<td>57.8 (7.0) ***</td>
</tr>
<tr>
<td>cTnl</td>
<td>0.030 (0.000)</td>
<td>0.030 (0.000)</td>
<td>0.030 (0.000)</td>
<td>0.030 (0.000)</td>
<td>0.030 (0.000)</td>
<td>0.030 (0.000)</td>
<td>0.052 (0.044)</td>
<td>0.030 (0.000)</td>
<td>0.030 (0.000)</td>
<td>0.088 (0.086)</td>
<td>0.114 (0.144)</td>
</tr>
</tbody>
</table>

aValues are means, SD in parentheses; n=15 for the control group (0 µg/kg, vehicle-treated), except for GLD where n=14, n=5 for ISO-treated rats, except for GLD at 50 µg/kg, where n=4. Animals were given a single intraperitoneal injection of ISO and autopsied at 24 h post-dosing. *Significantly different from n=15 control animals, P<0.05, ***P<0.001.

bAbbreviations and units: as Table 3.2 and 3.3.
Table 3.5. Experiment 1, dose ranging study, 1 – 25000 μg/kg; incidence of microscopic findings in the hearts of control female Hanover Wistar rats and animals treated with isoproterenol (ISO) at 24 h post-dosing.

<table>
<thead>
<tr>
<th>Microscopic finding</th>
<th>Severity grade</th>
<th>Isoproterenol dose (μg/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>0</td>
</tr>
<tr>
<td>Chronic myocardial degeneration</td>
<td>NADb</td>
<td>15</td>
</tr>
<tr>
<td></td>
<td>Minimal</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Mild</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Moderate</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Marked</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Very marked</td>
<td>-</td>
</tr>
</tbody>
</table>

*Animals were given a single IP injection of ISO at dose levels of 0 (control, vehicle-treated), 1, 5, 10, 50, 100, 500, 1000, 5000, 10000 and 25000 μg/kg and autopsied at 24 h post-dosing.

bNAD = no abnormalities detected.
Table 3.6. Experiment 2, route of administration study; serum clinical chemistry results in control (vehicle-treated, 0 µg/kg) female Hanover Wistar rats and animals treated with 5000 µg/kg isoproterenol (ISO) by intraperitoneal (IP) injection and sampled at 0, 1, 2, 3, 4 and 5 h post-dosing\(^a, b\).

<table>
<thead>
<tr>
<th></th>
<th>Time post-dosing (h)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
</tr>
<tr>
<td>Lactate</td>
<td>4.934 (1.387)</td>
</tr>
<tr>
<td>Glucose</td>
<td>10.36 (2.05)</td>
</tr>
<tr>
<td>NEFA</td>
<td>0.342 (0.073)</td>
</tr>
<tr>
<td>Potassium</td>
<td>5.42 (1.28)</td>
</tr>
<tr>
<td>ALT</td>
<td>55.0 (5.1)</td>
</tr>
<tr>
<td>AST</td>
<td>76.4 (7.5)</td>
</tr>
<tr>
<td>CK</td>
<td>442.4 (211.5)</td>
</tr>
<tr>
<td>LD</td>
<td>816.6 (527.9)</td>
</tr>
<tr>
<td>GLD</td>
<td>5.48 (0.70)</td>
</tr>
<tr>
<td>Aldolase</td>
<td>48.2 (7.9)</td>
</tr>
</tbody>
</table>

\(^a\)Values are means, SD in parentheses; n=5/group, except for lactate and CK at 3 h, where n=4. Animals were given a single IP injection of ISO at 5000 µg/kg and autopsied at 1, 2, 3, 4 and 5 h post-dosing. A further group of (n=5) control animals was autopsied at 0 h. *Significantly different from control, \(P<0.05\), ***\(P<0.001\).

\(^b\)Abbreviations and units: as Table 3.2.
Table 3.7. Experiment 2, route of administration study; serum cardiac troponin I (cTnI) results in individual control (vehicle-treated, 0 μg/kg) female Hanover Wistar rats and rats treated with 5000 μg/kg isoproterenol (ISO) by intraperitoneal (IP) injection and sampled at 0, 1, 2, 3, 4 and 5 h post-dosing$^{a,b}$.

<table>
<thead>
<tr>
<th>Time post-dosing (h)</th>
<th>0</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt;0.03</td>
<td>0.04 (17.2)</td>
<td>4.00 (89.9)</td>
<td>&lt;0.03 (1)</td>
<td>0.04 (1)</td>
<td>0.23 (4.2)</td>
<td></td>
</tr>
<tr>
<td>&lt;0.03</td>
<td>0.80 (17.2)</td>
<td>4.80 (108.1)</td>
<td>NS</td>
<td>0.05 (0.1)</td>
<td>0.23 (4.2)</td>
<td></td>
</tr>
<tr>
<td>&lt;0.03</td>
<td>1.91 (42.4)</td>
<td>1.27 (27.9)</td>
<td>&lt;0.03 (1)</td>
<td>0.07 (0.6)</td>
<td>0.03 (1)</td>
<td></td>
</tr>
<tr>
<td>&lt;0.03</td>
<td>1.10 (24.0)</td>
<td>0.10 (1.3)</td>
<td>&lt;0.03 (1)</td>
<td>0.84 (18.1)</td>
<td>2.82 (63.1)</td>
<td></td>
</tr>
<tr>
<td>0.10</td>
<td>0.07 (0.6)</td>
<td>1.66 (36.7)</td>
<td>0.35 (7.0)</td>
<td>&lt;0.03 (1)</td>
<td>0.21 (3.8)</td>
<td></td>
</tr>
</tbody>
</table>

Mean 0.044 0.784 [16.8]$^d$ 2.366 [52.8] 0.110 [1.5] 0.206 [3.7] 0.704 [15.0]

SD 0.031 0.780 1.964 0.160 0.355 1.186

$^{a}$Animals were given a single IP injection of ISO at 5000 μg/kg and autopsied (n=5) at 1, 2, 3, 4 and 5 h post-dosing. A further group of (n=5) control animals was autopsied at 0 h. *Significantly different from control, P<0.05, **P<0.001.

$^{b}$Values for cTnI are measured in μg/L. NS indicates no sample for analysis.

$^{c}$ indicates the “fold increase” of an individual value over the mean control group value.

$^{d}$ indicates the “fold increase” of a group mean value over the mean control value.
Table 3.8. Experiment 2, route of administration study; serum clinical chemistry results in control (vehicle-treated, 0 µg/kg) female Hanover Wistar rats and animals treated with 5000 µg/kg isoproterenol (ISO) by subcutaneous (SC) injection and sampled at 0, 1, 2, 3, 4 and 5 h post-dosing\(^a,b\).

<table>
<thead>
<tr>
<th>Time post-dosing (h)</th>
<th>0</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lactate</td>
<td>7.606 (3.033)</td>
<td>5.910 (1.109)</td>
<td>4.902 (1.418)</td>
<td>5.666 (1.638)</td>
<td>5.618 (1.481)</td>
<td>6.170 (0.750)</td>
</tr>
<tr>
<td>Glucose</td>
<td>10.38 (1.38)</td>
<td>7.32 (0.97) ***</td>
<td>7.48 (1.36) ***</td>
<td>8.38 (1.13) *</td>
<td>8.42 (0.98)</td>
<td>8.00 (1.08) *</td>
</tr>
<tr>
<td>NEFA</td>
<td>0.314 (0.087)</td>
<td>1.292 (0.286) ***</td>
<td>0.866 (0.168) ***</td>
<td>0.678 (0.154) *</td>
<td>0.506 (0.037)</td>
<td>0.512 (0.179)</td>
</tr>
<tr>
<td>Potassium</td>
<td>5.50 (0.62)</td>
<td>4.52 (0.89)</td>
<td>5.28 (0.59)</td>
<td>5.54 (0.32)</td>
<td>5.46 (1.37)</td>
<td>4.84 (0.85)</td>
</tr>
<tr>
<td>ALT</td>
<td>58.8 (4.2)</td>
<td>56.4 (7.7)</td>
<td>61.4 (5.4)</td>
<td>57.6 (7.5)</td>
<td>57.0 (2.9)</td>
<td>58.4 (5.0)</td>
</tr>
<tr>
<td>AST</td>
<td>78.6 (11.7)</td>
<td>95.8 (16.5)</td>
<td>125.6 (46.8)</td>
<td>127.6 (47.8)</td>
<td>114.2 (19.1)</td>
<td>124.2 (29.3)</td>
</tr>
<tr>
<td>CK</td>
<td>522.8 (105.3)</td>
<td>903.8 (425.1)</td>
<td>1154.8 (661.6)*</td>
<td>800.0 (393.1)</td>
<td>526.2 (105.8)</td>
<td>428.3 (61.8)</td>
</tr>
<tr>
<td>LD</td>
<td>632.4 (177.2)</td>
<td>1451.8 (400.5)</td>
<td>1664.8 (849.3)</td>
<td>1850.6 (1373.8)</td>
<td>1050.0 (297.4)</td>
<td>1455.2 (1373.2)</td>
</tr>
<tr>
<td>GLD</td>
<td>5.44 (1.27)</td>
<td>3.86 (1.27)</td>
<td>3.78 (1.01)</td>
<td>4.64 (1.84)</td>
<td>3.74 (0.97)</td>
<td>4.94 (1.44)</td>
</tr>
<tr>
<td>Aldolase</td>
<td>42.8 (2.3)</td>
<td>50.4 (7.5)</td>
<td>59.6 (13.3)</td>
<td>60.2 (18.2)</td>
<td>54.2 (8.0)</td>
<td>58.6 (16.2)</td>
</tr>
</tbody>
</table>

\(^a\)Values are means, SD in parentheses; n=5/group, except for lactate and CK at 5 h, where n=4. Animals were given a single SC injection of ISO at 5000 µg/kg and autopsied at 1, 2, 3, 4 and 5 h post-dosing. A further group of (n=5) control animals was autopsied at 0 h. *Significantly different from control, P<0.05, ***P<0.001.

\(^b\)Abbreviations and units: as Table 3.2.
Table 3.9. Experiment 2, route of administration study; serum cardiac troponin I (cTnI) results in individual control (vehicle-treated, 0 µg/kg) female Hanover Wistar rats and rats treated with 5000 µg/kg isoproterenol (ISO) by subcutaneous (SC) injection and sampled at 0, 1, 2, 3, 4 and 5 h post-dosing.\(^a,b\).

<table>
<thead>
<tr>
<th>Time post-dosing (h)</th>
<th>0</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.07</td>
<td>1.69 (39.2)(^c)</td>
<td>1.99 (46.4)</td>
<td>21.51 (511.1)</td>
<td>2.57 (60.2)</td>
<td>NS</td>
<td></td>
</tr>
<tr>
<td>0.05</td>
<td>3.17 (74.5)</td>
<td>5.27 (124.5)</td>
<td>6.35 (150.2)</td>
<td>4.50 (106.1)</td>
<td>0.99 (22.6)</td>
<td></td>
</tr>
<tr>
<td>&lt;0.03</td>
<td>3.32 (78.0)</td>
<td>4.39 (103.5)</td>
<td>1.49 (34.5)</td>
<td>1.44 (33.3)</td>
<td>1.27 (29.2)</td>
<td></td>
</tr>
<tr>
<td>0.03</td>
<td>1.96 (45.7)</td>
<td>4.37 (103.0)</td>
<td>11.46 (271.9)</td>
<td>0.36 (7.6)</td>
<td>1.44 (33.3)</td>
<td></td>
</tr>
<tr>
<td>&lt;0.03</td>
<td>1.62 (37.6)</td>
<td>2.85 (70.0)</td>
<td>2.14 (50.0)</td>
<td>0.57 (12.6)</td>
<td>3.10 (72.8)</td>
<td></td>
</tr>
</tbody>
</table>

Mean: 0.042, 2.352 [55.0]\(^d\), 3.774 [88.9], 8.590 [203.5], 1.888 [44.0], 1.700 [39.5]

SD: 0.018, 0.827, 1.324, 0.249, 1.700, 0.952

\(^a\)Animals were given a single SC injection of ISO at 5000 µg/kg and autopsied (n=5) at 1, 2, 3, 4 and 5 h post-dosing. A further group of (n=5) control animals was autopsied at 0 h. *Significantly different from control, P<0.05. ***P<0.001.

\(^b\)Values for cTnI are measured in µg/L. NS indicates no sample for analysis.

\(^c\)\{\} indicates the "fold increase" of an individual value over the mean control group value.

\(^d\)[\] indicates the "fold increase" of a group mean value over the mean control value.
Table 3.10. Experiment 1, dose ranging study, 1 – 25000 μg/kg; comparison of group mean cardiac troponin I (cTnI) levels at 2 h post-dosing with the group mean pathological lesion scores (severity grading) at 24 h post-dosing in the hearts of control female Hanover Wistar rats and animals treated with isoproterenol (ISO)\textsuperscript{a,b}.

<table>
<thead>
<tr>
<th>Isoproterenol dose (μg/kg)</th>
<th>2 h serum cTnI mean (SD)</th>
<th>24 h chronic myodegeneration score mean (SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0.030 (0.000)</td>
<td>0.0 (0.0)</td>
</tr>
<tr>
<td>1</td>
<td>0.030 (0.000)</td>
<td>0.0 (0.0)</td>
</tr>
<tr>
<td>5</td>
<td>0.030 (0.000)</td>
<td>0.0 (0.0)</td>
</tr>
<tr>
<td>10</td>
<td>0.086 (0.080)</td>
<td>0.4 (0.5)</td>
</tr>
<tr>
<td>50</td>
<td>0.278 (0.335)</td>
<td>0.4 (0.5)</td>
</tr>
<tr>
<td>100</td>
<td>1.252 (0.327)</td>
<td>0.6 (0.5)</td>
</tr>
<tr>
<td>500</td>
<td>2.200 (2.072) *</td>
<td>2.0 (0.7) ***</td>
</tr>
<tr>
<td>1000</td>
<td>1.042 (1.370)</td>
<td>1.4 (1.1) ***</td>
</tr>
<tr>
<td>5000</td>
<td>0.740 (1.588)</td>
<td>1.8 (0.8) ***</td>
</tr>
<tr>
<td>10000</td>
<td>4.820 (2.651) ***</td>
<td>2.0 (1.2) ***</td>
</tr>
<tr>
<td>25000</td>
<td>2.986 (2.147) ***</td>
<td>2.2 (0.4) ***</td>
</tr>
</tbody>
</table>

\textsuperscript{a}Animals were given a single IP injection of ISO at 0 μg/kg (vehicle control) and levels from 1 to 25000 μg/kg, and autopsied at 2 h and 24 h post-dosing. *Significantly different from control, \( P<0.05 \), ***\( P<0.001 \). There were 5 rats at each ISO dose level and 15 control (0 μg/kg) animals at 2 h and 24 h post-dosing.

\textsuperscript{b}Values for cTnI are measured in μg/L. Chronic myocardial degeneration scores: 0 = no abnormalities detected; 1 = minimal; 2 = mild; 3 = moderate.
CHAPTER 4: TIME COURSE STUDY ON THE CARDIOTOXICITY OF ISOPROTERENOL

4.1 INTRODUCTION

4.1.1 Background

Isoproterenol (ISO) is a synthetic catecholamine and a potent non-selective β-adrenoceptor agonist. Administration of ISO at high doses in the rat induces a severe myocardial necrosis, with the severity of the lesion being directly proportional to the dose administered (Rona et al., 1959a). Ferrans et al. (1969) showed that ISO-induced cardiac lesions in the rat were characterised by large, infarct-like areas of necrosis with hyalinisation and/or vacuolisation of the myofibres, interstitial and intracellular oedema and extensive inflammation, with inflammatory changes appearing as early as 6 to 10 h following a single subcutaneous (SC) injection of 50000 µg/kg. However, Rona et al. (1961) found that following a single SC injection of 85000 µg/kg ISO, increased eosinophilia was evident at 2 h post-dosing, neutrophil infiltration and interstitial oedema appeared by 8 h post drug administration, and focal myocardial necrosis was present at 24 h post-dosing, peaking in severity at 48 h. At 72 h post ISO treatment, the interstitial oedema regressed and there was a proliferation of fibroblasts.

ISO administration is known to induce an increase in a number of established cardiac serum markers, such as aspartate aminotransferase (AST), lactate dehydrogenase (LD) and creatine kinase (CK) (Wexler and Kittinger, 1963; Wexler, 1970; Judd and Wexler, 1974). In Chapter 3 (Preliminary studies on the cardiotoxicity of isoproterenol), it was demonstrated that cardiac troponin I (cTnl) levels are increased as early as 1 h post-dosing following the single SC administration of ISO at 5000 µg/kg; however in that study the time course of histopathological changes were not assessed.

Little work has been performed in the assessment of cardiac troponin (cTn) levels following ISO-induced toxic insult, but it is known that serum cTnT and cTnl concentrations may be raised as early as 2 h post-dosing following a single SC injection of ISO at 4000 µg/kg to Wistar rats (Bertinchant et al., 2003). O’Brien et al. (2006) reported that when male Sprague Dawley rats were treated with a single SC dose of ISO at 100000 µg/kg and serum collected at 2, 4, 6 and 24 h post-dosing, increased levels of
cTnI were first detectable at 2 h. Levels of cTnI peaked at 4 h post-dosing, when the group mean cTnI value was approximately 35.00 µg/L; the pre-dosing control value in this experiment was 0.07 µg/L. At 24 h, the mean cTnI value in the ISO-treated animals was approximately 7.00 µg/L. Minimal, multifocal necrosis of single myofibrillar cells was detected at 2 h post ISO administration, with mineralisation evident at 4 to 6 h, and inflammation and oedema at 24 h. Therefore, it was concluded that serum cTnI levels correlated well with the time of lesion development following the onset of cardiac injury.

Herman et al. (2006) demonstrated that in male Sprague Dawley rats treated with a single dose of ISO at 8 to 500 µg/kg, cTnT concentrations were elevated at all dose levels at 3 h post-dosing; however, no histopathological evidence of cardiac injury was present until 6 h following drug administration, and lesions were only present at dose levels of 32 µg/kg and above (i.e. not at 8 or 16 µg/kg). Therefore, this suggested that cTn measurement may detect damage not detectable by light microscopy (i.e. ultrastructural changes).

York et al. (2007) investigated female Hanover Wistar rats given a single intraperitoneal (IP) dose of ISO at 50000 µg/kg with animals sampled at 0 (vehicle-treated), 1, 2, 4, 6, 12, 24 and 48 h post-dosing. At 1 h, all rats treated with 50000 µg/kg ISO (n=4) showed positive cTnI and cTnT responses (a mean cTnI level of 4.265 µg/L compared with a control value of <0.030 µg/L; a mean cTnT value of 1.535 µg/L compared with <0.010 µg/L control); however the earliest microscopic changes were observed at 4 h post-dosing, where 1 animal showed a minimal myofibre lesion characterised by eosinophilia and swelling of cardiac myofibres. Levels of cTnI and cTnT peaked at 2 h following ISO administration. No cTnI signal was detected at 48 h post-dosing; however there was still a small cTnT signal at this time point. Histopathological lesions increased in incidence and severity from 1 h post-dosing, and at 24 h and 48 h, all animals showed mild to moderate chronic myodegeneration, which was characterised by a loss of cardiac myofibres and a mononuclear cell infiltration. This ISO-treated rat model therefore showed that there was a close association between serum cTn levels and the histopathological assessment of cardiac injury; however York et al. (2007) considered that there was a temporal disconnect, with maximal cTn responses occurring before the maximal severity of histopathological lesions.
4.1.2 Aims of the present study

The aims of the present study were to establish the timing of peak serum cTn (cTnI and cTnT) levels in the rat induced by a single SC exposure to ISO, and investigate correlations between the timing and the magnitude of cTn increases in relation to the histopathology of cardiac lesion development. Other biomarkers of cardiac injury in serum or plasma were also to be studied and evaluated, and related to changing cTn levels.
4.2 MATERIALS AND METHODS

4.2.1 Experimental protocol

OBJECTIVE: To establish the timing of peak troponin levels in the rat following a single dose of isoproterenol and to investigate correlations between the timing and magnitude of troponin increases with the histopathology of cardiac lesion development

4.2.1.1 Animal handling

ISO dosing solutions were prepared at concentrations of 20 and 800 µg/ml, as described in Chapter 2.4.1. The vehicle was phosphate buffered saline (PBS). Male Hanover Wistar rats (n=170; mean weight 228.7 g) were randomly divided into 34 groups (n=5/group) (Table 4.1). Each group of 5 rats occupied 1 cage. Animals were treated with a single SC dose of ISO at dose levels of 0 (PBS, vehicle), 100 or 4000 µg/kg. Body weights were recorded immediately before PBS/ISO administration, and then daily up to 72 h post-dosing, and before autopsy (Chapter 2.1). Animals from each of the 3 ISO dose level groups (n=5/group) were autopsied at 0.5, 1.0, 2.0, 3.0, 4.0, 6.0, 8.0, 12.0, 24.0, 48.0 and 72.0 h post-dosing for clinical chemistry and histopathological investigations; a further group of (n=5) control (vehicle-treated) rats were autopsied at 0 h.

4.2.1.2 Sample handling

At autopsy, animals were killed by exsanguination under deep isoflurane anaesthesia (as described in Chapter 2.6) for the preparation of serum and plasma. Hearts were removed, weighed and placed in fixative (Chapter 2.10). Serum and plasma clinical chemistry analysis was performed on all animals in all groups (Chapter 2.8.1 and 2.8.2). Serum parameters measured were: heart fatty acid-binding protein (H-FABP), cTnI (measured on both the ACS: 180S and the Beckman Access systems), cTnT (determined on the Roche Elecsys 2010) and AST. Plasma parameters measured were: total CK, and CK-MB, CK-BB and CK-MM activities and total LD, and LD1, LD2, LD3, LD4 and LD5 activities. The relative percentages of the CK and LD isoenzymes were determined by plasma electrophoresis.
Sections of hearts (H&E) were examined microscopically from all animals in all groups. Hearts were scored on a lesion severity scale of 0 to 4: 0 = no abnormalities detected (NAD), 1 = minimal, 2 = mild, 3 = moderate and 4 = marked; the definition of each lesion severity grading score is presented in Table 4.2. Staining with phosphotungstic acid haematoxylin (PTAH), Martius' Scarlet Blue (MSB) and immunostaining with an anti-cTnI antibody was performed on selected sections (Chapter 2.10).

4.2.2 Statistical analysis

Data were analysed using one-way analysis of variance (ANOVA) followed by Dunnett's test for post hoc multiple comparison. In cases of violation of the assumptions for parametric testing, the Kruskal-Wallis test was used in combination with Dunn's post-test. For correlation analyses, the 2-tail Pearson test was used. Statistical analysis was performed using GraphPad Prism version 3.00 for windows (GraphPad software, San Diego, California, USA).
4.3 RESULTS

OBJECTIVE: To establish the timing of peak troponin levels in the rat following a single dose of isoproterenol and to investigate correlations between the timing and magnitude of troponin increases with the histopathology of cardiac lesion development

4.3.1 Clinical signs and body weight changes

Male Hanover Wistar rats were given a single SC dose of PBS (vehicle control) or ISO at 100 or 4000 μg/kg. Following drug administration, animals in the high dose (4000 μg/kg ISO) group displayed reduced activity and lay in a prostrate position. The heart rate and respiratory rate of these animals appeared to be increased, and the animals drank large volumes of water. These signs of toxicity appeared approximately 20 min post-dosing but animals had recovered by 3.0 h post ISO administration. There were no intercurrent deaths (ICDs).

Body weights were determined daily over the 72 h treatment period and at autopsy. ISO administration caused no significant changes in body weight when the group mean body weights of the animals at the 3 dose levels (0, vehicle-treated; 100 and 4000 μg/kg) were compared at each autopsy time point.

4.3.2 Serum and plasma clinical chemistry

Administration of ISO at dose levels of 100 and 4000 μg/kg by SC injection induced changes in all the serum and plasma clinical chemistry parameters investigated (Figure 4.1 to 4.9).

4.3.2.1 Heart fatty acid-binding protein

The mean serum level of H-FABP in the control (0 μg/kg ISO) animals at 0 h was 1.726 ng/mL (Figure 4.1). In rats treated with ISO at the higher dose level of 4000 μg/kg, the mean serum H-FABP concentration at the 0.5 h time point post ISO dosing was significantly increased to 25.744 ng/mL (P<0.001), a 19.0-fold increase over the concurrent mean control value of 1.286 ng/mL. The mean serum H-FABP in animals
treated with ISO at 4000 μg/kg at 1.0 h post-dosing was 15.760 ng/mL, a 17.4-fold increase over the concurrent mean control value of 0.855 ng/mL (P<0.001). The mean level of serum H-FABP in the 4000 μg/kg ISO group peaked at 2.0 h post-dosing, where the mean value was 36.874 ng/mL, a 21.5-fold elevation over the concurrent control value of 1.640 ng/mL (P<0.001). After the 2.0 h time point, the mean values for H-FABP in the 4000 μg/kg ISO animals began to decrease, however levels remained above the concurrent control values at 3.0, 4.0, 6.0 and 8.0 h post-dosing, where the group mean H-FABP levels were 4.1-, 1.0-, 4.1- and 2.5-fold higher than in the concurrent controls (P<0.05, NS, P<0.05 and NS, respectively). At 12.0 post ISO dosing, the mean serum H-FABP result in the 4000 μg/kg ISO rats was 4.058 ng/mL, compared with the concurrent control value of 3.968 ng/mL (NS), suggesting a return to the base line control levels (Figure 4.1). At the 24.0, 48.0 and 72.0 h time points, the H-FABP levels in the 4000 μg/kg ISO groups were comparable with the relevant control values.

In rats treated with ISO at the lower dose level of 100 μg/kg, the mean serum H-FABP data showed a clear separation in relation to the results obtained from the 4000 μg/kg ISO animals (Figure 4.1). At 0.5 h, the mean serum level in the 100 μg/kg animals was 6.710 ng/mL, a 4.2-fold increase (NS) above the mean concurrent control figure of 1.286 ng/mL. At 1.0 h post ISO dosing, the mean H-FABP in the 100 μg/kg group appeared to peak, at 6.890 ng/mL, a 7.1-fold increase (NS) above the control level of 0.855 ng/mL. At the 2.0 h post-dosing time point, and at all times until 72.0 h, mean H-FABP values of the 100 μg/kg ISO group animals were directly comparable with the relevant control results, except at the 12.0 h time point, when the mean level in the ISO-treated rats was significantly reduced (P<0.001) below the concurrent control animals.

4.3.2.2 Cardiac troponin I

4.3.2.2.1 ACS: 180S

The mean level of serum cTnl, measured using the Bayer ACS: 180S system, in the vehicle-treated control rats (0 μg/kg ISO) at the beginning of the study (0 h) was 0.030 μg/L (Figure 4.2). In rats treated with ISO at 4000 μg/kg, the group mean cTnl level at the 0.5 h time point was significantly raised (P<0.001) to 0.718 μg/L, a 22.9-fold increase above the concurrent mean control value of 0.030 μg/L. At the 1.0 h post ISO
dosing time point, the mean serum level of cTnI in the 4000 μg/kg rats was 3.096 μg/L, a 102.2-fold increase above the mean concurrent control value of 0.030 μg/L (P<0.001). At 2.0 h, the mean cTnI concentration in the 4000 μg/kg group was 8.368 μg/L, the concurrent control value was 0.030 μg/L, giving a 277.9-fold elevation (P<0.001). The peak group mean (4000 μg/kg) serum cTnI level was at 3.0 h post ISO dosing, being 20.540 μg/L, a 683.7-fold statistically significant (P<0.001) increase above the value for the concurrent controls (0.030 μg/L). After peaking at 3.0 h post-dosing, the mean cTnI values of rats in the 4000 μg/kg ISO group gradually fell towards base line control values; the fold increases above the concomitant control values were: 4.0 h, 473.4-fold, P<0.001; 6.0 h, 351.7-fold, P<0.001; 8.0 h, 514.5-fold, P<0.001; 12.0 h, 328.8-fold, P<0.05; 24.0 h, 162.6-fold, P<0.001. At 48.0 h and 72.0 h, the group mean cTnI values were at base line control levels.

In rats treated with ISO at the lower dose level of 100 μg/kg, at 0.5 h post-dosing (Figure 4.2), the mean serum cTnI value was significantly increased (P<0.05) to 0.440 μg/L, a 13.7-fold elevation above the mean concurrent control value (0.030 μg/L); at 1.0 h the comparable result was a 78.6-fold increase (P<0.05). The mean serum cTnI value of the 100 μg/kg ISO animals peaked at 2.0 h post ISO administration; at this time the mean cTnI result was 5.878 μg/L, a 194.9-fold increase (P<0.05) above the mean concomitant control value. After the 2.0 h sampling time point, the group mean cTnI values gradually fell towards the base line control values, that is, at 3.0, 4.0, 6.0, 8.0, 12.0 and 24.0 h post-dosing, the levels being 2.905, 2.576, 1.746, 0.190, 0.614 and 0.136 μg/L; fold increases of 95.8, 84.9, 57.2, 5.3, 19.5 and 3.5, respectively, NS at all time points (Figure 4.2). At 48.0 h and 72.0 h post-dosing, mean cTnI levels of the 100 μg/kg ISO animals were directly comparable with the concurrent control values.

### 4.3.2.2.2 Beckman Access

Using the Beckman Access platform, the group mean cTnI concentration in the control (0 μg/kg ISO, vehicle-treated) animals at 0 h was 0.005 μg/L (Figure 4.3). In rats treated with the higher ISO dose of 4000 μg/kg at 0.5 h, the group mean cTnI value was raised significantly (P<0.001) to 0.936 μg/L, in comparison with the group concurrent control level of 0.004 μg/L, which represented a 233.0-fold elevation. At 1.0 h post ISO dosing, the mean cTnI level in the 4000 μg/kg rats was 3.448 μg/L and the mean cTnI level in the control animals was 0.018 μg/L; this was a 190.6-fold elevation (P<0.001).
The mean level of serum cTnl in the rats treated with ISO at 4000 μg/kg at 2.0 h post-dosing was 8.704 μg/L, a 2900.3-fold increase above the mean control level of 0.003 μg/L (P<0.001). The peak 4000 μg/kg group mean cTnl measured with the Beckman Access occurred at 3.0 h post ISO dosing, where the concurrent control value was 0.005 μg/L and the 4000 μg/kg ISO dose group mean was 18.195 μg/L; therefore, this represented a 3638.0-fold elevation above controls (P<0.001). After peaking at 3.0 h post-dosing, the group mean cTnl levels in the 4000 μg/kg group gradually returned towards control values; the fold increases above the concurrent control values were: 4.0 h, 4587.7-fold, P<0.001; 6.0 h, 2391.5-fold, NS; 8.0 h, 2581.0-fold, P<0.001; 12.0 h, 4659.0-fold, P<0.001; 24.0 h, not calculable (mean value for controls = 0.000 μg/L); 48.0 h, 54.7-fold, P<0.05 and 72.0 h, 14.7-fold, P<0.001 (Figure 4.3).

In rats treated with the lower ISO dose level of 100 μg/kg (Figure 4.3), the group mean cTnl concentration at 0.5 h was 0.624 μg/L, compared with a concurrent control value of 0.004 μg/L (a 155.0-fold increase, P<0.001). At 1.0 h post-dosing, the group (100 μg/kg ISO) mean cTnl value was 2.742 μg/L and the concurrent control level was 0.018 μg/L, giving a significant (P<0.001) elevation 151.3-fold above the control value. The mean cTnl in animals treated with ISO at 100 μg/kg peaked at 2.0 h post ISO administration, where the 100 μg/kg ISO-treated group mean was 6.330 μg/L, a 2109.0-fold increase over the concomitant mean control value of 0.003 μg/L (P<0.05). After the 2.0 h sampling point, the group mean cTnl values fell towards base line control values: at 3.0, 4.0, 6.0, 8.0, 12.0 and 24.0 h post-dosing, the cTnl levels were 4.250, 2.376, 1.922, 0.380, 0.528 and 0.195 μg/L; fold increases of 849.0, 791.0, 479.5, 75.0, 263.0 and not calculable (0 μg/kg group mean = 0.000 μg/l). At the 48.0 h and 72.0 h time points, the 100 μg/kg group mean cTnl levels determined on the Beckman Access platform were directly comparable with the concurrent control results (Figure 4.3).

4.3.2.3 Cardiac troponin T

At 0 h, the group mean serum cTnT level in the control (0 μg/kg ISO, vehicle-treated) animals was 0.010 μg/L (Figure 4.4). In rats treated with the higher dose level of ISO of 4000 μg/kg, the mean cTnT concentration at 0.5 h was increased significantly (P<0.001) to 0.240 μg/L, which represented a 23.0-fold increase above the mean concurrent control level of 0.010 μg/L. At 1.0 h post ISO administration, the mean cTnT in rats treated with 4000 μg/kg was 0.780 μg/L, a 77.0-fold elevation above the concurrent
control level of 0.010 μg/L (P<0.001). At the 2.0 h time point, the group mean cTnT level in the 4000 μg/kg group was significantly raised (P<0.001) above the mean concomitant control value of 0.010 μg/L to 1.744 μg/L, a 173.4-fold increase. The group mean cTnT concentration in the 4000 μg/kg ISO animals peaked at 3.0 h post-dosing, where the 4000 μg/kg ISO group mean was 3.374 μg/L, which represented a 336.4-fold increase above the concurrent control value of 0.010 μg/L (P<0.001). After peaking at 3.0 h post ISO administration, the group mean cTnT levels in the 4000 μg/kg rats began to return towards base line control levels; however levels remained above control values at all time points (including at 48.0 h post-dosing). The fold increases above the concurrent control values were: 4.0 h, 220.4-fold, P<0.001; 6.0 h, 280.0-fold, P<0.05; 8.0 h, 318.5-fold, P<0.001; 12.0 h, 165.6-fold, P<0.001; 24.0 h, 177.2-fold, P<0.001; 48.0 h, 28.6-fold, NS. Measurements of cTnT were not carried out at 72 h post-dosing.

In rats treated with the lower ISO dose level of 100 μg/kg, the mean serum cTnT level was significantly increased (P<0.001) at 0.5 h post-dosing, to 0.132 μg/L, a 12.2-fold elevation above the mean concurrent control value of 0.010 μg/L (Figure 4.4). At the 1.0 h sampling time point, the comparable result was a 60.0-fold increase (P<0.001). The group mean cTnT concentration at 2.0 h post ISO administration was raised significantly (P<0.001) to 1.034 μg/L, a 102.4-fold increase above the concurrent mean control level of 0.010 μg/L. At 3.0 h, the peak mean cTnT concentration in the 100 μg/kg group was 1.200 μg/L and the concurrent control value was 0.010 μg/L, giving a 119.0-fold elevation (P<0.05). After the 3.0 h sampling point, the group mean cTnT levels in the 100 μg/kg ISO dose group gradually fell to base line control values; at 4.0, 6.0, 8.0, 12.0 and 24.0 h post-dosing, the serum mean cTnT results were 0.785, 0.684, 0.078, 0.176 and 0.074 μg/L; fold increases of 77.5, 67.4, 6.8, 16.6 and 6.4, respectively, NS at all time points (Figure 4.4). At 48.0 h post-dosing, the mean cTnT concentration in rats treated with 100 μg/kg ISO was at the control value of 0.010 μg/L.

4.3.2.4 Aspartate aminotransferase

The group mean level of serum AST in the vehicle-treated control animals (0 μg/kg ISO) at 0 h was 69.2 U/L (Figure 4.5). In the higher dose ISO treatment group (4000 μg/kg), the group mean AST level at the 0.5 h and 1.0 h time points were 67.8 and 67.0 U/L, respectively; these activities were directly comparable with the base line
concurrent control activities. A significant increase (P<0.001) in the mean serum AST level in the 4000 μg/kg group was seen at 2.0 h post ISO dosing; the level at this time was 85.8 U/L, a 0.3-fold increase above the concurrent control figure of 66.0 U/L. At the 3.0, 4.0 and 6.0 h post-dosing time points, the group mean serum AST levels in the control group rats were 73.2, 64.2 and 58.8 U/L, and in the 4000 μg/kg animals the mean levels were 131.4, 131.6 and 147.2 U/L, giving 0.8- (P<0.001), 1.0- (P<0.001) and 1.5- (P<0.05) fold increases, respectively. The mean serum AST level peaked in the 4000 μg/kg ISO dose group at 8.0 h post-dosing; at this time, the mean level was 181.8 U/L, a 1.7-fold increase above the concurrent control value of 66.2 U/L (P<0.001). The mean serum activities of AST decreased gradually at the 12.0 h and 24.0 h post-dosing time points, the levels being 106.6 and 86.0 U/L, 0.6- and 0.3-fold elevations above the mean concurrent control levels of 67.0 and 65.4 U/L, P<0.05 and P<0.001, respectively. At the 48.0 h and 72.0 h autopsy time points, the mean serum AST activities in the 4000 μg/kg ISO-treated rats were directly comparable with the data from the concurrent control animals.

In rats treated with ISO at the lower dose level of 100 μg/kg, there was limited evidence that serum AST was altered by ISO administration (Figure 4.5).

4.3.2.5 Total creatine kinase and creatine kinase-MB

4.3.2.5.1 Total creatine kinase

The mean plasma total CK concentration in the vehicle-treated control animals (0 μg/kg ISO) at 0 h was 216.6 U/L (Figure 4.6). In the higher ISO dose level group (4000 μg/kg), the group mean CK activities were 195.6, 239.2 and 257.2 U/L at the 0.5, 1.0 and 2.0 h time points, respectively; these levels were directly comparable with the base line concurrent control values. At 3.0 h post-dosing, a significant elevation (P<0.001) in the 4000 μg/kg group mean plasma CK activity of 273.6 U/L was observed which was above the control value of 153.4 U/L, giving a 0.8-fold increase. At the 4.0 h sampling point, the mean plasma CK level in the 4000 μg/kg animals was similar to the concurrent control value; however, at 6.0 h, the group mean CK activity was raised significantly (P<0.001) in the 4000 μg/kg rats to 306.4 U/L, compared with a concurrent group mean of 177.4 U/L, a 0.7-fold change. At the remaining time points (8.0, 12.0, 24.0, 48.0 and 72.0 h post-dosing), the mean plasma total CK activities in the 4000
\( \mu g/kg \) ISO dose groups were similar to the concurrent control mean values. There was no clear peak observed in plasma total CK values at 4000 \( \mu g/kg \) ISO.

In animals treated with the lower ISO dose level of 100 \( \mu g/kg \), plasma group mean levels of CK were not altered clearly in comparison with the relevant concurrent control activities at any sampling point (Figure 4.6).

4.3.2.5.2 Creatine kinase-MB

At 0 h, the group mean plasma CK-MB level in the vehicle-treated control animals was 27.4 U/L (Figure 4.7A). At the 0.5 h and 1.0 h time points in the 4000 \( \mu g/kg \) ISO-treated animals, the mean plasma CK-MB activities were comparable with the concurrent control values; however, at 2.0 h post-dosing, the 4000 \( \mu g/kg \) ISO dose group mean was significantly elevated (\( P<0.001 \)) to 54.8 U/L, a 1.4-fold increase above the concurrent control level of 23.2 U/L. The group mean CK-MB value in the 4000 \( \mu g/kg \) ISO animals at 3.0 h post ISO administration was 63.4 U/L, the concurrent control level at this time point was 9.6 U/L, representing a 5.6-fold increase (\( P<0.001 \)). Levels of CK-MB in the 4000 \( \mu g/kg \) ISO animals peaked between 4.0 h and 8.0 h post ISO administration. At the 4.0 h time point, the mean concurrent control CK-MB activity was 15.8 U/L and the group mean in animals treated with ISO at 4000 \( \mu g/kg \) was 68.0 U/L, this increase was statistically significant (\( P<0.05 \)) and represented a 3.3-fold change. The mean plasma CK-MB activity at the 4000 \( \mu g/kg \) dose level was raised significantly (\( P<0.001 \)) to 52.8 U/L at 6.0 h post-dosing, compared with a mean concomitant control value of 11.3 U/L (a 3.7-fold increase). At 8.0 h, the mean CK-MB level in the 4000 \( \mu g/kg \) rats was 68.5 U/L, a 2.3-fold elevation above the mean concurrent control value of 20.6 U/L (\( P<0.05 \)). After the 8.0 h time point, levels of CK-MB in the 4000 \( \mu g/kg \) ISO animals appeared to have returned to base line control values.

In animals treated with the lower ISO dose level of 100 \( \mu g/kg \), CK-MB activities were not altered noticeably or significantly at any time in comparison with concurrent control values (Figure 4.7A).

Plasma electrophoresis also showed an increase in CK-MB activity in the animals given the higher dose of ISO (4000 \( \mu g/kg \)) and a lesser response in those given the lower dose.
of 100 μg/kg (Figure 4.7B). No alterations were observed in plasma levels of CK-MM or CK-BB in the present study.

4.3.2.6 Total lactate dehydrogenase and lactate dehydrogenase isoenzymes

4.3.2.6.1 Total lactate dehydrogenase

The group mean plasma total LD activity at 0 h in the vehicle-treated (0 μg/kg ISO) control animals was 184.6 U/L (Figure 4.8). At the 0.5 h autopsy time point, levels of LD in the 4000 μg/kg ISO animals were at similar levels to the concurrent control animals; however, at 1.0 h, the mean plasma total LD level in rats treated with ISO at 4000 μg/kg was significantly decreased (P<0.001), the activity in the ISO-treated animals was 239.8 U/L, compared with the control value of 390.0 U/L. At 2.0 h post-dosing, the 4000 μg/kg group mean LD activity was comparable to the mean concurrent control value. The group mean LD level in the 4000 μg/kg animals at 3.0 h post-dosing was increased significantly (P<0.001) to 485.0 U/L, compared with the concurrent control value of 137.0 U/L; this represented a 2.5-fold increase. At 4.0 h, total plasma LD activity in the high dose (4000 μg/kg ISO) rats was raised 1.5-fold above the control level, however this increase was not statistically significant. Levels of LD in the rats treated with 4000 μg/kg animals appeared to peak between 6.0 h and 8.0 h post-dosing: at 6.0 h, the group mean plasma LD level was 697.0 U/L (a 1.8-fold increase, P<0.05), and at 8.0 h, the mean LD level was 748.3 U/L (a 3.6-fold elevation, P<0.001). After 8.0 h, LD values in the 4000 μg/kg ISO group animals began to return towards baseline control levels. The fold increases above the relevant concurrent control values at 12.0 h and 24.0 h post-dosing were 1.1 and 0.6, respectively, P<0.05 at both time points. At 48.0 h and 72.0 h post ISO administration, mean LD activities in the 4000 μg/kg rats were directly comparable to the concurrent control values.

In the 100 μg/kg ISO-treated animals, no significant changes were seen in the mean total plasma LD levels at any of the sampling points, except at 1.0 h post-dosing, where the group mean LD activity was decreased significantly (P<0.001) below the concurrent control value (Figure 4.8).
At 0 h, the group mean plasma LD1 activity in the vehicle-treated (0 μg/kg ISO) animals was 12.2 U/L (Figure 4.9A). At 0.5 h and 1.0 h post-dosing, the group mean plasma LD1 levels in the animals treated at the higher ISO dose level of 4000 μg/kg ISO were similar to control values. At 2.0 h post ISO administration at 4000 μg/kg, the mean LD1 activity was raised 1.9-fold to 34.6 U/L above the concurrent control level of 11.8 U/L (P<0.001). The mean plasma LD1 value in the 4000 μg/kg animals at 3.0 h was increased to 66.2 U/L, compared with the concomitant control activity of 8.2 U/L (a 7.1-fold change, P<0.001). At the 4.0 h autopsy time point, the 4000 μg/kg ISO dose group mean was significantly increased (P<0.001) to 86.8 U/L, a 5.9-fold elevation above the concurrent control mean of 12.6 U/L. At 6.0 h, the mean plasma LD1 level in the 4000 μg/kg ISO group was 166.8 U/L, the concurrent control mean was 14.8 U/L, giving a 10.3-fold elevation (P<0.001). Levels of LD1 appeared to peak at 8.0 h post-dosing, where the group mean in animals treated with 4000 μg/kg ISO was 189.8 U/L while the concurrent control value was 6.4 U/L; a 28.7-fold increase, P<0.001. Levels of LD1 were returning towards base line control values at the 12.0 h post ISO administration time point; the fold increases above the concurrent control values were: 12.0 h, 6.0-fold, P<0.001; 24.0 h, 6.6-fold, P<0.001; 48.0 h, 0.9-fold, P<0.05. At 72.0 h post-dosing, the mean plasma LD1 activity in the 4000 μg/kg ISO animals compared with the concurrent control values.

In rats treated at the lower ISO dose level of 100 μg/kg animals, the mean plasma LD1 activities at 0.5 h and 1.0 h were directly comparable to the concurrent control values (Figure 4.9A). At the 2.0 h, 3.0 h, 4.0 h, 6.0 h, 8.0 h, 12.0 h, 24.0 h and 48.0 h autopsy time points, the group mean LD1 activities appeared to be raised above the relevant control levels, although no increases were statistically significant. The fold increases were 1.0-, 2.8-, 1.9-, 1.8-, 1.5-, 0.9-, 0.6- and 0.5-fold at these times, respectively. The peak in the LD1 levels for the 100 μg/kg dose level group occurred at 6.0 h post-dosing (a fold increase of 1.8). At the 72.0 h sampling point, the LD1 value in the animals treated with 100 μg/kg ISO was comparable with the group mean control value.
4.3.2.6.3 LD2 activity

The control (0 µg/kg, vehicle treated) plasma LD2 group mean at 0 h was 7.0 U/L (Figure 4.9B). At the 0.5 h autopsy sampling point, the mean LD2 level in the 4000 µg/kg animals was significantly increased (P<0.05) to 17.8 U/L, above the mean concurrent control value of 7.0 U/L, a 1.5-fold elevation. The group mean LD2 activity in animals treated with 4000 µg/kg ISO at 1.0 h post dosing was 28.6 U/L and the concomitant control level was 8.5 U/L, which represented a 3.4-fold increase, P<0.001. At 2.0 h, the mean LD2 value in the 4000 µg/kg rats was 64.0 U/L, a 7.9-fold increase above the mean concurrent control value of 7.2 U/L (P<0.001); at 3.0 h, the 4000 µg/kg ISO group LD2 mean was increased 25.7-fold above mean concurrent controls (P<0.001). The group mean (4000 µg/kg) plasma LD2 level at 4.0 h post-dosing was 154.0 U/L, a 17.3-fold increase above the value for the control animals (8.4 U/L; P<0.05). The group mean LD2 activity in the 4000 µg/kg ISO dose group peaked at 6.0 h to 8.0 h post ISO administration (Figure 4.9B). At 6.0 h, the group mean value was 241.0 U/L (a 24.4-fold increase, P<0.05) and at 8.0 h, the 4000 µg/kg group mean was 242.5 U/L (a 25.9-fold increase, P<0.001). After the 8.0 h time point, levels of LD2 gradually fell towards base line control values: at the 12.0 h, 24.0 h and 48.0 h autopsy time points, the group mean activities were increased 12.6-, 4.0- and 0.7-fold above concomitant control values, P<0.001, P<0.001 and P<0.05, respectively. At 72.0 h post-dosing, the mean plasma LD2 level of the 4000 µg/kg ISO animals was comparable to the concurrent control activity.

In animals treated with the lower ISO dose level of 100 µg/kg, the group mean plasma LD2 activity at 0.5 h post-dosing was raised significantly (P<0.05) to 15.4 U/L, a 1.2-fold increase over the concurrent mean control value of 7.0 U/L (Figure 4.9B). At the 1.0 h, 2.0 h, 3.0 h, 4.0 h and 6.0 h time points, the group mean LD2 activities of the 100 µg/kg animals were increased above the concurrent control levels: the fold increases were: 1.0 h, 1.0; 2.0 h, 3.1; 3.0 h, 8.2; 4.0 h, 5.1 and 6.0 h, 3.2; NS at all these time points. The 100 µg/kg LD2 activity peaked at 4.0 h post-dosing and from 8.0 h post-dosing, the mean plasma LD2 levels in the 100 µg/kg animals were very similar to the concurrent control values.
4.3.2.6.4 LD3 activity

At 0 h, the vehicle-treated control group mean was 6.8 U/L (Figure 4.9C). In rats treated with the higher dose level of ISO of 4000 µg/kg, the group mean plasma LD3 value at 0.5 h post ISO administration was comparable to the concurrent control level. At 1.0 h, 2.0 h and 3.0 h post ISO administration, the group mean LD3 activities were raised significantly (P<0.001 at these 3 time points) above the control activities, the increases were: 1.0-, 4.6- and 16.4-fold above the concurrent control values, respectively. The group mean level of LD3 in the 4000 µg/kg animals at 4.0 h post-dosing was 69.6 U/L compared with the control value of 5.2 U/L; a 12.4-fold increase (P<0.05). The mean plasma LD3 activity in rats treated with ISO at 4000 µg/kg peaked at 6.0 h post ISO administration; the mean result at this time point was 87.3 U/L, an 18.4-fold elevation over the concurrent control value of 4.5 U/L (P<0.001). At the 8.0 h, 12.0 h and 24.0 h time points, LD3 activities in the 4000 µg/kg ISO rats were increased significantly (P<0.001, P<0.05 and P<0.001, respectively); these increases were 5.0, 1.6- and 1.1-fold above the concurrent control levels. The 4000 µg/kg group mean LD3 levels at 48.0 h and 72.0 h post-dosing were similar to the values for the relevant controls.

In animals treated with 100 µg/kg ISO, the group mean plasma LD3 activities at 0.5 h and 1.0 h following ISO administration were comparable with control levels (Figure 4.9C). At the time points between 2.0 h and 6.0 h post-dosing, mean levels of LD3 in the 100 µg/kg animals were increased; elevations were 1.2-, 3.4-, 1.9- and 1.0-fold above control values (at 2.0 h, 3.0 h 4.0 h and 6.0 h, respectively), NS at any time point. At the sampling points from 8.0 h post ISO administration, the group mean LD3 levels in animals treated with 100 µg/kg were comparable with the concurrent control values.

4.3.2.6.5 Plasma electrophoresis

Plasma electrophoresis of the LD1, LD2, LD3, LD4 and LD5 isoenzymes further supported the evidence of a trend for a dose-related increase in plasma activities of LD1, LD2 and LD3 (Figure 4.9D), but this was not evident for the plasma activities of LD4 and LD5.
4.3.3 Gross pathology and histopathology

At autopsy, there was no macroscopic evidence of ISO-induced toxicity. The administration of the drug at the higher dose level of 4000 µg/kg had a significant effect on relative heart weights however (Figure 4.10). At the 0.5 h to 4.0 h post-dosing time points, the mean relative weights of the hearts from the 4000 µg/kg ISO-treated rats were directly comparable to those from the concurrent control animals. However, at the 6.0 h post-dosing time point, the group mean relative weight of the hearts from the 4000 µg/kg ISO animals was significantly greater than in the concurrent control animals (control, 3.16 g/kg; ISO-treated, 3.53 g/kg; an 11.7 % increase, P<0.05). At the 8.0 h, 12.0 h, 24.0 h, 48.0 h and 72.0 h post-dosing time points, the mean relative heart weights of the 4000 µg/kg ISO animals were also greater than in the concurrent control animals, the % increases being 6.8 % (NS), 23.2 % (P<0.001), 21.2 % (P<0.001), 18.0 % (P<0.001) and 15.9 % (P<0.001), greater than the concurrent controls, respectively (Figure 4.10).

It is of interest to note that in the rats treated at the higher ISO dose level of 4000 µg/kg, the mean relative heart weights were only greater than the concurrent controls at 9 of the 11 time points; at 2.0 h and 3.0 h, the mean relative heart weights in the ISO animals were lower (NS) than in the controls.

In rats treated with ISO at the lower dose level (100 µg/kg), the mean relative heart weights were increased above the concurrent control relative heart weights at each of the 11 sampling time points from 0.5 h to 72.0 h post-dosing (Figure 4.10). However, these increases were not statistically significant at any time point.

The severity and incidence of microscopic findings in the hearts of control animals, and in animals treated at 100 µg/kg and 4000 µg/kg ISO over the 12 time points from 0 h to 72.0 h, is summarised in Table 4.3A, 4.3B and 4.3C. 2 control animals showed minor background histological lesions, in 1 animal autopsied at 2.0 h post-dosing and in 1 animal autopsied at the 8.0 h time point (out of a total of 60 control animals studied in the experiment); in both cases the lesion consisted of minimal focal myocardial necrosis, in the mid-interventricular septum. The earliest histopathological change observed in the ISO-treated rats was myocardial degeneration, graded as minimal to moderate, seen in all 5 animals in both the 100 µg/kg and the 4000 µg/kg ISO dose level groups at the 0.5 h autopsy time point (Figure 4.11, 4.12). Myocardial degeneration was
characterised by contraction banding, loss of myofibre cross-striations, swelling of the myofibres and sarcoplasmic rarefaction, granular to fragmented sarcoplasm and a variable microvesicular or coarse vacuolation. Nuclear pyknosis was sometimes present. Myocardial degeneration was also observed in animals at the 1.0 h, 2.0 h, 3.0 h and 4.0 h time points (Figure 4.13, 4.14; Table 4.3A, 4.3B).

From the 6.0 h time point following ISO administration, myocardial degeneration was developing into necrosis (Figure 4.15, 4.16; Table 4.3B). Between 6.0 h and 24.0 h post-dosing, the hearts of affected animals displayed the histological changes associated with myocardial degeneration, but in addition a significant proportion of fibres showed hypereosinophilia and fragmentation, interstitial oedema and inflammatory cell infiltration (initially neutrophils, but later macrophages also appeared). Some myofibres showed macrophagic infiltration and digestion. Occasionally, karyorrhexis and karyolysis were present (Table 4.3B, 4.3C).

At 48.0 h after ISO administration, myocardial necrosis, graded as minimal to marked, was present, characterised by hypereosinophilic and necrotic myofibres, and phagocytic infiltration (Figure 4.17, 4.18; Table 4.3C). There was at this time a significant amount of sarcoplasm remaining in the cardiomyocytes, and there was little or no interstitial oedema present.

By 72.0 h post-dosing, myocardial degeneration/fibrosis, graded as minimal to marked, was the primary change (Figure 4.19, 4.20). This was characterised by early fibroplasia with a pronounced interstitial reaction, with large basophilic oval to spindle-shaped cells with prominent mitoses evident. There was little or no sarcoplasm remaining in the cardiomyocytes by this time point, and there was also a mononuclear cell infiltration.
The aims of the present study were to establish the timing of peak cTnl and cTnT levels in the rat following a single SC dose of ISO at 100 and 4000 μg/kg, and to correlate cTn increases with histopathological changes in the heart. Furthermore, the behaviour of other biomarkers of cardiac injury was to be investigated. ISO-induced myocardial injury, assessed by histopathological examination, was evident at all time points post-dosing in the majority of ISO-treated animals at both drug dose levels (Figure 4.11 to 4.20; Table 4.3A to 4.3C).

The time course of release of H-FABP into the serum following a single SC dose of ISO has been characterised in the present study (Figure 6.1). In animals treated with 4000 μg/kg ISO, serum H-FABP levels were increased significantly (P<0.001) at the first autopsy time point, at 0.5 h post-dosing. On histological examination, myocardial degeneration was present in all animals in the high dose group at this time point (Table 4.3A). Levels of H-FABP peaked at 2.0 h, and remained above concurrent control values until 8.0 h post-dosing; after this time point, H-FABP values were at control levels. In the 100 μg/kg ISO dose group, mean H-FABP results were above mean control values at 0.5 h and 1.0 h post-dosing (NS at both time points), however after this time, levels of serum H-FABP compared with control values. In addition, the data obtained for serum levels of H-FABP was quite variable at both dose levels, as evidenced by the large SDs at each time point.

It can be seen from the data in Figure 4.1 that H-FABP was a relatively sensitive marker in this experimental model of cardiac injury, as the marker was increased at 0.5 h following ISO administration, in parallel with the histopathological evidence of cardiac injury (Table 4.3A). For the 4000 μg/kg ISO group, the diagnostic time window for H-FABP was from 0.5 h to 8.0 h post-dosing that is, approximately 8 h in total. Therefore, as a serum marker, H-FABP is quite robust, as it persists in the circulation for several h after cardiac injury in this experimental model. However, in man, the peak in serum H-FABP levels (after the onset of symptoms of acute myocardial infarction, MI) occurs at about 5 to 10 h, and levels return towards baseline rapidly (Tanaka et al., 1991). In a mouse model of MI, serum H-FABP levels peaked at 4 h post infarction, and returned to control levels by 24 h (Aartsen et al., 2000). Therefore, in the present rat model, the
relatively short circulating half-life of H-FABP may, to a degree, limit the usefulness of the marker in the assessment of cardiac injury.

No studies have been reported that have assessed the utility of H-FABP in drug-induced myocardial injury, in man or in experimental animals. The present study is the first to map serum changes in H-FABP following toxic insult. Consequently, more work must to be carried out in the evaluation of this biomarker for the assessment of cardiotoxicity. Chan et al. (2004) showed that following MI in man, H-FABP peaked at 3 h after the onset of symptoms, which was 7 to 9 h before the peaks in cTnI and CK. In the present study, H-FABP levels in the 4000 µg/kg ISO dose level group were significantly (P<0.001) increased at 0.5 h post-dosing (Figure 4.1), as were the cTnI values measured on both the ACS: 180S system and Beckman Access system (Figure 4.2 and 4.3). However, in the lower ISO dose level (100 µg/kg) at 0.5 h post-dosing, H-FABP levels were elevated above the mean control value, but not significantly. Conversely, the mean cTnI concentrations in the 100 µg/kg ISO group at 0.5 h were raised significantly above the mean concurrent control values (Bayer ACS: 180S, P<0.05; Beckman Access, P<0.001). Therefore, it is considered necessary to evaluate further the early changes in serum levels of H-FABP and cTnI in the present model of ISO-induced cardiac injury.

Levels of cTnI, determined on the Bayer ACS: 180S system (Figure 4.2), were significantly elevated (P<0.05) in the high ISO dose group at 0.5 h post ISO administration, values then peaked at 3.0 h (P<0.001) post-dosing, and had returned to base line control values at the 48.0 h autopsy time point. When cTnI values were determined using the Beckman Access system, similar cTnI results were obtained to those measured on the Bayer ACS: 180S (Figure 4.3). Using the Beckman Access system, levels of cTnI in the 4000 µg/kg animals were also significantly elevated at 0.5 h (P<0.001), peaked at 3.0 h (P<0.001) and were still significantly increased at 72.0 h (P<0.001). Therefore, the diagnostic window for cTnI using both platforms in the present study was approximately 24 h at the high ISO dose level. For cTnT, concentrations were significantly elevated (P<0.001) at the first post-mortem time point, at 0.5 h for both ISO dose levels. The peak in cTnT response for 4000 µg/kg ISO occurred at 3.0 h following drug treatment, with levels still detectable at 48.0 h (4000 µg/kg) post-dosing. Following a single SC injection of ISO at 4000 µg/kg in Wistar rats, Bertinchant et al. (2000) showed that cTnI and cTnT were significantly increased at 2 h post drug treatment, values peaked at 4 h, and levels were still elevated at 24 h post-
dosing. However in the report by Bertinchant et al. (2000), no animals were sampled after the 24 h post-dosing time point. Therefore, the present study gives more detailed information regarding the behaviour of cTn following ISO-induced cardiomyocyte damage, compared with reports in the literature and highlights the cTn diagnostic time window in the ISO-induced model of cardiotoxicity in the rat.

When the cTnI and cTnT responses at 4000 µg/kg ISO are plotted and compared (Figure 4.21), it can be seen that the magnitude of cTnI release is greater than the cTnT release; however the pattern of release of both cTnI and cTnT are similar. For example, at 0.5 h post-dosing, there were significant cTnI and cTnT signals and the peak in both cTnI and cTnT values was at 3.0 h following drug treatment. Also, levels of cTnI and cTnT were still evident at 24.0 to 48.0 h post ISO administration. At 0.5 h post-dosing, mean serum cTnI levels were 0.718 (Bayer ACS: 180S) and 0.936 µg/L (Beckman Access), and 0.240 µg/L for cTnT. At 3.0 h, the appropriate results were 20.540, 18.195 and 3.374 µg/L, respectively, and at 24.0 h following a single SC injection of ISO at 4000 µg/kg, the results were 4.908, 2.980 and 1.782 µg/L, respectively. Therefore, it can be seen that although the degree of cTnI response is approximately 3 to 6 times higher than that for cTnT, both cTnI and cTnT are sensitive and specific markers of cardiomyocyte injury in this model of cardiotoxicity.

When cTnI results from individual animals measured on the Bayer ACS: 180S and the Beckman Access platforms are directly compared, it can be seen that there is a significant correlation (P<0.001, R²= 0.99) between the results (Figure 4.22). This highlights the usefulness of both methods in determination of cTnI in the laboratory rat. Furthermore, when the cTnI results using both cTnI platforms are compared with the results for cTnT obtained with the Elecsys 2010 (Roche) (Figure 4.23), it can be seen here also that there are significant correlations between cTnI and cTnT results (cTnI with the ACS: 180S compared with cTnT with the Elecsys 2010, P<0.001, R²= 0.87; cTnI with the Beckman Access compared with cTnT with the Elecsys 2010, P<0.001, R²= 0.86). These comparisons therefore demonstrate that these assays for either cTnI or cTnT are useful in the diagnosis of cardiomyocyte injury using clinical chemistry methods in this rat model of drug-induced cardiac injury.

The serum activities of AST were determined following ISO administration in the present study (Figure 4.5). It can be seen that at the 4000 µg/kg ISO dose level, values
for AST were first significantly elevated (P<0.001) at 2.0 h post-dosing. Maximal levels of AST were evident at 8.0 h, where the group mean was increased 1.7-fold above the concomitant control value. There remained a statistically significant elevation (P<0.001) of serum AST at 24.0 h post ISO administration; however at this time the mean levels was only 0.3-fold above the mean control value. Measurement of AST was not sensitive enough to detect cardiac damage at the lower ISO dose level of 100 µg/kg. Therefore, in the present study, AST provided no additional evidence of cardiac injury.

Plasma levels of total CK in the serum were significantly elevated (P<0.001) in the high dose (4000 µg/kg ISO) group at 3.0 h and 6.0 h post ISO administration (Figure 4.6); however these increases at 3.0 h and 6.0 h were the only 2 significant elevations observed for this parameter, and in general, CK measurement was not sensitive enough to detect cardiac injury in this animal model. However, the activities of the isoenzyme CK-MB were shown to be significantly increased at the 4000 µg/kg ISO dose level from 2.0 h to 8.0 h post-dosing (Figure 4.7A). Preus et al. (1988) also showed that CK-MB was significantly elevated between 2 and 8 h post ISO administration in male Wistar rats treated with a single SC injection at 5000 µg/kg. However, after the measurement of CK-MB at 8 h post-dosing, the next serum level was measured at 24 h (Preus et al., 1988), and therefore the present study gives a clearer representation of changes occurring in levels of plasma CK-MB following cardiomyocyte injury induced by ISO. CK-MB is the isoform of CK that is particularly associated with the heart in man and most laboratory species (Apple and Jaffe, 2006; Walker, 2006). Therefore, in the present study, the release of CK-MB into the plasma was indicative of myocardial damage; however the marker was not sensitive enough to detect cardiomyocyte injury at the lower ISO dose level of 100 µg/kg. In addition, no drug-induced increases in plasma CK-MM or CK-BB were observed in the present study (Figure 4.7B), suggesting that ISO administration did not cause injury to other tissues, such as the skeletal muscle (CK-MM) or the brain (CK-BB).

Measurement of total LD (Figure 4.8) showed that plasma activity was raised in the 4000 µg/kg ISO dose group between 2.0 h and 24.0 h post-dosing; however this marker was not sensitive enough to detect cardiac injury in the 100 µg/kg ISO animals. However, the quantification of LD isoenzyme activities in response to ISO administration at 4000 µg/kg resulted in the significant elevations of LD1, LD2 and LD3 plasma levels between approximately 2.0 h and 48.0 h (LD1), 0.5 h and 48.0 h.
(LD2) and 1.0 h and 24.0 h (LD3) post-dosing (Figure 4.9A to 4.9C). In addition, increases at 100 μg/kg ISO suggested there was some evidence of a dose-related increase in the isoenzyme results, although the elevations at the 100 μg/kg ISO dose level were not statistically significant. Nevertheless, LD isoenzyme determinations were relatively sensitive in the detection of cardiomyocyte injury in this animal model. Following the single SC administration of ISO at 5000 μg/kg to male Wistar rats, Preus et al. (1988) showed that serum LD1, LD2 and LD3 activities were raised significantly at 2 h, 2h and 4 h post-dosing, respectively. Furthermore, it was reported that levels of LD1 and LD2 remained significantly increased up to the final sampling point at 24 h post ISO administration; however serum LD3 levels fell to below the control values at 24 h post-dosing (Preus et al., 1988). In general, elevations in LD1 and LD2 activities are indicative of myocardial damage in both man and experimental animals (Wolf, 1986; Evans, 1991). Therefore, in the present study, the elevation of LD1 and LD2 gave further confirmation of the heart as the site of tissue injury. The increase in plasma LD3 levels in the present study was unexpected, as this isoenzyme is not generally associated with the heart (Crook, 2006). Nevertheless, Rotenberg et al. (1988) showed that LD3 levels were elevated in 35 % of patients with acute MI, and Preus et al. (1988) demonstrated significant increases in LD3 following ISO-induced cardiac injury in the rat. No increases in LD4 or LD5 levels were observed in the present study, suggesting that drug-induced injury did not occur in the liver or in skeletal muscle, as increases in circulating levels of these isoenzymes are particularly associated with injury to these tissues (Skillen 1984; Crook, 2006).

If the behaviours of the various serum and plasma biomarkers in the 4000 μg/kg ISO dose group are summarised and compared, it can be seen that there are differences in their responses following drug administration (Table 4.4). The earliest markers to be significantly elevated following ISO treatment at 4000 μg/kg were H-FABP, cTnI, cTnT and LD2; these parameters were significantly increased at 0.5 h post-dosing in the 4000 μg/kg ISO dose group. At the 0.5 h autopsy time point, histopathological examination demonstrated that all animals treated with 4000 μg/kg ISO showed minimal to moderate myocardial degeneration, indicating that cardiac tissue damage had already taken place at this time point (Table 4.3A). Therefore, H-FABP, cTnI, cTnT and LD2 can be viewed as sensitive markers of cardiac injury, as they are released in concurrence with histopathological evidence of myocardial damage. The biomarkers with the longest time windows for detection (0.5 to 2.0 h until 48.0 to 72.0 h post-
dosing) were cTnI, cTnT, LD1 and LD2. The robustness of a biomarker in the circulation (i.e. the length of the diagnostic window) is an important characteristic for an ideal marker of cardiac injury, as there must be a sufficient length of time to allow detection. This also highlights the importance of the timings of serum or plasma collection in preclinical animal studies, as an elevation in a serum or plasma parameter may be missed. Finally, if the fold increases of the various markers are compared (Table 4.4), it can be seen that the peak levels (as fold increases) of cTnI and cTnT are higher than those for other markers. For example, the peak cTnI concentration, measured by the ACS: 180S, occurred at 3.0 h post-dosing with ISO at 4000 μg/kg, where levels were increased 683.7-fold above the concurrent control value. In contrast, the peak AST result following ISO administration at 4000 μg/kg occurred at 8.0 h post-dosing, and the mean level was increased only 1.7-fold above the concomitant control result. Therefore, it is considered that the cTns have been shown in the present study to be the most sensitive indicators of cardiomyocyte damage induced by a single SC injection of ISO.

The administration of ISO induced dose-related increases in the relative heart weights of treated animals, with statistical significance (at the 4000 μg/kg dose level) at 6.0, 12.0, 24.0, 48.0 and 72.0 h post-dosing (Figure 4.10). At 6.0 to 48.0 h post-dosing, myocardial degeneration, degeneration/necrosis and necrosis was identified, and at 72.0 h following ISO administration myocardial degeneration/fibrosis was observed (Table 4.3B, 4.3C). The increase observed in relative heart weights (Figure 4.10) is postulated to be due to interstitial oedema, cellular influx of inflammatory cells and fibroplasia, rather than cardiac hypertrophy, which is generally considered to be a chronic response of the heart (Kizaki et al., 2005; Chapter 3, Preliminary studies on the cardiotoxicity of isoproterenol). This data (Figure 4.10) also highlights the importance of the timing of the assessment of cTn measurements in preclinical studies. For example, if a study has shown that a compound of interest causes fibroplasia in the heart, it is necessary to time blood collections for cTn determination prior to this histopathological change, as cTnI and cTnT are biomarkers of cardiac degeneration and necrosis, which will occur prior to fibroplasia.

The time course of histopathological changes in response to ISO administration was demonstrated in the present study. It was shown that myocardial degeneration may be observed as early as 0.5 h following the single SC administration of ISO at both 100 and 4000 μg/kg. This finding appears to be the earliest time point that light microscopy
changes have been observed following ISO administration. Rona et al. (1961) gave a single SC injection of ISO at 85000 μg/kg to male Wistar rats and reported that the normal structure of the myocardium was generally preserved at 30 min after dosing; however at 2 h, myofibres displayed increased eosinophilia, and there were areas of mast cells, histiocytes and lymphocytes in the epicardial portion of the heart. In addition in the present study, myocardial degeneration/necrosis was observed between 6.0 h and 24.0 h post-dosing, whereas Rona et al. (1961) observed these changes at 24 h post-dosing. Bertinchant et al. (2000) showed mild to moderate acute myofibrillar degeneration from 4 h post ISO administration at 4000 μg/kg SC to male and female Wistar rats. In the present study, myocardial degeneration/fibrosis was demonstrated at 72.0 h following treatment with ISO (Table 4.3C), and this change was characterised by early fibroplasia in conjunction with a pronounced interstitial reaction. These last observations are similar to those of Rona and Kahn (1969), who showed in the male Wistar rat, fibroblast proliferation and the removal of necrotic debris by histiocytes at 72 h following the SC administration of ISO as 2 doses of 85000 μg/kg 24 h apart.

In the present study, the histological evidence for the incidence and severity of myocardial changes over a 72 h post-dosing period has been investigated and related to the serum/plasma levels of a series of cardiac biomarkers. In general, cTnI and cTnT have been identified as biomarkers of choice. Using the rat model of ISO-induced cardiac injury, it became evident that there exists a dose association between the histological assessment of cardiac injury and serum levels of both cTnI and cTnT. However, there is a clear “temporal disconnect” with the maximal (i.e. peak) cTn responses preceding the maximal severity of the histopathological lesion observed. The earlier response of serum cTn values compared with microscopic changes has been observed in other studies (Bleuel et al., 1995; Bertinchant et al., 2000; O'Brien et al., 2006; York et al., 2007). The utility of the measurement of serum and plasma biomarkers to assess cardiac damage has been clearly demonstrated in the present study. This information therefore adds to the rather limited data in the literature. Kurata et al. (2007) found that plasma CK-MB, LD1 and LD2 activities and serum cTnI and cTnT values were raised significantly in male Crl: CD(SD) rats treated with a single SC injection of ISO at 40, 400 and 4000 μg/kg at 4 h post drug administration, and these biochemical changes occurred concurrently with histopathological evidence of minimal myofibrillar degeneration. However in the report of Kurata et al. (2007) only 1 time
point was chosen to evaluate the cardiac markers, and therefore, the present study presents a more detailed picture of the changes occurring in ISO-induced cardiotoxicity.

Nevertheless, ultrastructural studies with ISO have shown that morphological myocardial changes may be seen 4 to 8 min following drug treatment (Kutsuna, 1972). It would be of interest to complete a study investigating ultrastructural changes in the heart in response to ISO treatment in conjunction with cTn determinations.

In conclusion, in the present study it has been shown that ISO-induced cardiomyocyte injury may be detected by cTn measurements and histopathological evaluation as early as 0.5 h post drug administration. Levels of cTn peak at 3.0 h post dosing, during the early stages of cardiomyocyte myodegeneration. However the highest histological lesion scores occur during the phase of declining cTn values, from 24.0 h post dosing. Therefore, there is a temporal disconnect between maximal cTn values and the histopathological response in this experimental model. Concerning the validation of the cTns as novel serum markers of cardiomyocyte injury, this study has shown that cTnI and cTnT are the markers of choice in cardiac injury in comparison to the historically used enzyme markers (AST, CK and LD); however, the isoenzyme results for CK-MB, LD1, LD2 and LD3 are still considered useful in the confirmation of the site of tissue injury.
Figure 4.1. Group mean serum heart fatty acid-binding protein (H-FABP) levels in rats treated with a single subcutaneous dose of isoproterenol (ISO) at 0 (control), 100 and 4000 μg/kg at time points up to 72.0 h post-dosing. Values are means, SD as error bars; n=5 for control and ISO-treated rats, except at 1.0 h, 0 μg/kg (control) and at 8.0 h, 4000 μg/kg, where n=4. *Significantly different from the concurrent control, P<0.05; ***P<0.001.
Figure 4.2. Group mean serum cardiac troponin I (cTnI) levels in rats treated with a single subcutaneous dose of isoproterenol (ISO) at 0 (control), 100 and 4000 μg/kg at time points up to 72.0 h post-dosing, measured with the Bayer ACS: 180S kit. Values are means, SD as error bars; n=5 for control and ISO-treated rats, except at 1.0 h, 0 μg/kg (control); at 3.0 h, 0 (control) and 100 μg/kg, and at 8.0 h, 4000 μg/kg, where n=4. *Significantly different from the concurrent control, P<0.05; **P<0.001.
Figure 4.3. Group mean serum cardiac troponin I (cTnI) levels in rats treated with a single subcutaneous dose of isoproterenol (ISO) at 0 (control), 100 and 4000 µg/kg at time points up to 72.0 h post-dosing, using the Beckman Access system. Values are means, SD as error bars; n=5 for control and ISO-treated rats, except at 6.0 h, 4000 µg/kg and at 24.0 h, 4000 µg/kg, where n=1; at 8.0 h, 100 and 4000 µg/kg; at 12.0 h, 4000 µg/kg; at 48.0 h, 0 µg/kg (control) and at 72.0 h, 0 and 4000 µg/kg, where n=3; at 0 h, 0 µg/kg (control); at 1.0 h, 0 µg/kg (control); at 2.0 h, 0 µg/kg (control); at 3.0 h, 0 (control) and 4000 µg/kg; at 4.0 h, 0 µg/kg (control); at 8.0 h, 0 µg/kg (control); at 24.0 h, 100 µg/kg, and at 48.0 h, 4000 µg/kg, where n=4. *Significantly different from the concurrent control, P<0.05; ***P<0.001.
Figure 4.4. Group mean serum cardiac troponin T (cTnT) levels in rats treated with a single subcutaneous dose of isoproterenol (ISO) at 0 (control), 100 and 4000 μg/kg at time points up to 48.0 h post-dosing, measured with the Roche Elecsys 2010. Values are means, SD as error bars; n=5 for control and ISO-treated rats, except at 0 h, 0 μg/kg (control), where n=3; at 0 h, 100 μg/kg; at 1.0 h, 0 μg/kg (control); at 3.0 h, 0 (control) and 100 μg/kg; at 4.0 h, 100 μg/kg, and at 8.0 h, 4000 μg/kg, where n=4. cTnT measurements were not performed on any animals at 72.0 h. *Significantly different from the concurrent control, P<0.05; **P<0.001.
Figure 4.5. Group mean serum aspartate aminotransferase (AST) levels in rats treated with a single subcutaneous dose of isoproterenol (ISO) at 0 (control), 100 and 4000 µg/kg at time points up to 72.0 h post-dosing. Values are means, SD as error bars; n=5 for control and ISO-treated rats, except at 1.0 h, 0 µg/kg (control), and at 8.0 h, 4000 µg/kg, where n=4. *Significantly different from the concurrent control, P<0.05; ***P<0.001.
Figure 4.6. Group mean plasma total creatine kinase (CK) levels in rats treated with a single subcutaneous dose of isoproterenol (ISO) at 0 (control), 100 and 4000 µg/kg at time points up to 72.0 h post-dosing. Values are means, SD as error bars; n=5 for control and ISO-treated rats, except at 1.0 h, 0 µg/kg (control), and at 8.0 h, 4000 µg/kg, where n=4. *Significantly different from the concurrent control, P<0.05; ***P<0.001.
Figure 4.7. (A) Group mean plasma CK-MB activity in rats treated with a single subcutaneous dose of isoproterenol (ISO) at 0 (control), 100 and 4000 µg/kg at time points up to 72.0 h post-dosing. Values are means, SD as error bars; n=5 for control and ISO-treated rats, except at 1.0 h, 0 µg/kg (control), and at 8.0 h, 4000 µg/kg, where n=4. *Significantly different from the concurrent control, P<0.05; ***P<0.001. (B) Plasma electrophoresis gel showing a dose-related increase in plasma CK-MB activity in individual animals at 3.0 h post-dosing. “M” represents marker, “C” represents a control (0 µg/kg) animal, “L” represents a low dose (100 µg/kg ISO) animal, and “H” a high dose (4000 µg/kg ISO) animal.
Figure 4.8. Group mean plasma total lactate dehydrogenase (LD) levels in rats treated with a single subcutaneous dose of isoproterenol (ISO) at 0 (control), 100 and 4000 µg/kg at time points up to 72.0 h post-dosing. Values are means, SD as error bars; n=5 for control and ISO-treated rats, except at 1.0 h, 0 µg/kg (control), and at 8.0 h, 4000 µg/kg, where n=4. *Significantly different from the concurrent control, P<0.05; ***P<0.001.
Figure 4.9. Group mean plasma lactate dehydrogenase isoenzyme LD1 (A) and LD2 (B) in rats treated with a single subcutaneous dose of isoproterenol (ISO) at 0 (control), 100 and 4000 μg/kg at time points up to 72.0 h post-dosing. Values are means, SD as error bars; n=5 for control and ISO-treated rats, except at 1.0 h, 0 μg/kg (control); at 6.0 h, 0 (control) and 4000 μg/kg, and at 8.0 h, 4000 μg/kg, where n=4. *Significantly different from the concurrent control, P<0.05; ***P<0.001.
Figure 4.9. (C) Group mean plasma lactate dehydrogenase isoenzyme LD3 activity in rats treated with a single subcutaneous dose of isoproterenol (ISO) at 0 (control), 100 and 4000 μg/kg at time points up to 72.0 h post-dosing. Values are means, SD as error bars; n=5 for control and ISO-treated rats, except at 1.0 h, 0 μg/kg (control); at 6.0 h, 0 (control) and 4000 μg/kg, and at 8.0 h, 4000 μg/kg, where n=4. *Significantly different from the concurrent control, P<0.05; ***P<0.001. (D) Plasma electrophoresis gel showing a dose-related increase in plasma LD1, LD2 and LD3 activities in individual animals at 3.0 h after the administration of ISO. “M” represents marker, “C” represents a control (0 μg/kg) animal, “L” represents a low dose (100 μg/kg ISO) animal, and “H” a high dose (4000 μg/kg ISO) animal.
Figure 4.10. Group mean relative heart weights in rats treated with a single subcutaneous dose of isoproterenol (ISO) at 0 (control), 100 and 4000 µg/kg at time points up to 72.0 h post-dosing. Values are means, SD as error bars; n=5 for all groups. *Significantly different from the concurrent control, P<0.05; ***P<0.001. Note the origin of the Y axis is 3.0 g/kg.
Figure 4.11. Myocardium from a rat administered 4000 μg/kg isoproterenol at 0.5 h post-dosing. Microvesicular vacuolation is evident. H&E; x 400 original magnification.

Figure 4.12. Myocardium from a rat administered 100 μg/kg isoproterenol at 0.5 h post-dosing. Contraction banding and loss of cross striations are evident. PTAH; x 200 original magnification.
Figure 4.13. Myocardium from a rat administered 4000 μg/kg isoproterenol at 2.0 h post-dosing. Contraction banding and swelling of the myofibres is illustrated. H&E, x 100 original magnification.

Figure 4.14. Myocardium from a rat administered 4000 μg/kg isoproterenol at 3.0 h post-dosing. Swelling of the myofibres is seen, with a variable microvesicular and coarse sarcoplasm. H&E; x 400 original magnification.
Figure 4.15. Myocardium from a rat administered 100 µg/kg isoproterenol at 8.0 h post-dosing. Inflammatory cell infiltration is illustrated. H&E; x 200 original magnification.

Figure 4.16. Myocardium from a rat administered 4000 µg/kg isoproterenol at 8.0 h post-dosing. There is sarcoplasmic rarefaction and swelling of the myofibres, with inflammatory and macrophagic cell infiltration. H&E; x 100 original magnification.
Figure 4.17. Myocardium from a rat administered 4000 μg/kg isoproterenol at 48.0 h post-dosing. Myocardial necrosis and macrophagic cell infiltration is evident. H&E; x 200 original magnification.

Figure 4.18. Myocardium from a rat administered 4000 μg/kg isoproterenol at 48.0 h post-dosing. This is the same animal as shown in Figure 4.17; the eosinophilic remains of the myofibre sarcoplasm in affected fibres no longer stains in the same way as normal cells. MSB; x 200 original magnification.
Figure 4.19. Myocardium from a rat administered 4000 μg/kg isoproterenol at 72.0 h post-dosing. There is a pronounced interstitial reaction with prominent mitoses. H&E; x 200 original magnification.
Figure 4.20. (A) Myocardium from a rat given 0 µg/kg isoproterenol (control). MSB; x 200 original magnification. (B) Myocardium from a rat administered 4000 µg/kg isoproterenol at 72.0 h post-dosing. Early fibroplasia and interstitial deposition of collagen is seen. MSB; x 200 original magnification.
Figure 4.21. Group mean serum concentrations of cardiac troponin I (cTnI) and cardiac troponin T (cTnT) in rats treated with a single subcutaneous dose of 4000 µg/kg ISO at time points up to 48.0 h post-dosing. cTnI concentrations were determined on the Bayer ACS: 180S and Beckman Access systems; cTnT on the Roche Elecsys 2010. Further information is presented in Figure 4.2, 4.3 and 4.4.
Figure 4.22. Comparison of techniques for the determination of serum cardiac troponin I (cTnl) concentrations. cTnl concentrations from individual animals were measured using the Bayer ACS: 180S system and the Beckman Access platform. n=140 XY pairs, Pearson correlation.
Figure 4.23. Comparison of techniques for the determination of serum cardiac troponin I (cTnI) and concentrations of cardiac troponin T (cTnT). cTnI concentrations were measured using the Bayer ACS: 180S and Beckman Access platforms and cTnT was measured using the Roche Elecsys 2010. (A) cTnI (ACS: 180S) compared with cTnT, n=148 XY pairs, Pearson correlation; (B) cTnI (Beckman Access) compared with cTnT, n=126 XY pairs, Pearson correlation.
Table 4.1. Experimental design, treatment groups, dose levels of isoproterenol (ISO) and autopsy time points post-dosing.

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<th>Time point (h)</th>
</tr>
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</tr>
<tr>
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</tr>
<tr>
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</tr>
<tr>
<td>4</td>
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<td>0.5</td>
</tr>
<tr>
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</tr>
<tr>
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</tr>
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<tr>
<td>34</td>
<td>4000</td>
<td>72.0</td>
</tr>
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</table>

*n=5 animals in all groups. Animals were treated with a single subcutaneous injection of ISO at 0 (PBS vehicle control), 100 or 4000 µg/kg, and autopsied at 0.5, 1.0, 2.0, 3.0, 4.0, 6.0, 8.0, 12.0, 24.0, 48.0 or 72.0 h post-dosing; a further control group was autopsied at 0 h, the time of dosing.
Table 4.2. Definitions of the cardiac histopathological lesion grading scores, with increasing severity.

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<tr>
<th>Histopathological lesion score</th>
<th>Definition</th>
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</thead>
<tbody>
<tr>
<td>NAD</td>
<td>No abnormalities detected.</td>
</tr>
<tr>
<td>1 (minimal)</td>
<td>Scattered lesions, involving small numbers of isolated fibres or small foci of several fibres.</td>
</tr>
<tr>
<td>2 (mild)</td>
<td>Increased numbers of isolated fibres involved, or small foci of several affected fibres, extending deeper from the subendocardial myocardium into the mid-ventricular myocardium.</td>
</tr>
<tr>
<td>3 (moderate)</td>
<td>Large foci of affected fibres involved and constituting 25-50% of the apical left ventricular subendocardium/myocardium. Clusters of affected fibres present in the mid-section of the left ventricle/interventricular septum. The heart may also show small foci of affected fibres at the base of the heart (interventricular septum or left ventricular wall), or in the left atrium.</td>
</tr>
<tr>
<td>4 (marked)</td>
<td>Large multifocal to coalescing affected fibres constituting over 50% of the apical left ventricular subendocardium/myocardium. Large clusters of affected fibres present in the mid-section of the left ventricle/interventricular septum. Generally showing large foci of affected fibres at the base of the heart (interventricular septum or left ventricular wall), or in the left atrium.</td>
</tr>
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Table 4.3A. Incidence of microscopic findings in the hearts of control male Hanover Wistar rats and animals treated with ISO at 0, 0.5, 1.0, 2.0 and 3.0 h post-dosing.

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<th>Microscopic finding</th>
<th>Severity grade</th>
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<th>1.0</th>
<th>2.0</th>
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<tr>
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<td>Number of rats in each group with histological changes</td>
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<tr>
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<tr>
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<td>3</td>
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n=5 for all groups. Animals were treated with a single subcutaneous dose of ISO at 0 (control, vehicle treated), 100 and 4000 µg/kg, and autopsied at 0.5, 1.0, 2.0, 3.0, 4.0, 6.0, 8.0, 12.0, 24.0, 48.0 and 72.0 h post-dosing. A further group of (n=5) control animals was autopsied at 0 h, the time of drug administration. *NAD* = no abnormalities detected.
Table 4.3B. Incidence of microscopic findings in the hearts of control male Hanover Wistar rats and animals treated with ISO at 4.0, 6.0, 8.0 and 12.0 h post-dosing^a.

<table>
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<th>Microscopic finding</th>
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<th>8.0</th>
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<tbody>
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<td>4000</td>
<td>0</td>
</tr>
<tr>
<td>Isoproterenol dose (µg/kg)</td>
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<td></td>
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<td></td>
</tr>
<tr>
<td>Myocardial degeneration</td>
<td>NAD^b</td>
<td>5</td>
<td>5</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
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^aAll other information as Table 4.3A. ^bNAD = no abnormalities detected.
Table 4.3C. Incidence of microscopic findings in the hearts of control male Hanover Wistar rats and animals treated with ISO at 24.0, 48.0 and 72.0 h post-dosing.

<table>
<thead>
<tr>
<th>Microscopic finding</th>
<th>Severity grade</th>
<th>Isoproterenol dose (µg/kg)</th>
<th>Time point (h)</th>
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<tr>
<td></td>
<td></td>
<td></td>
<td>24.0</td>
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<tr>
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<tr>
<td>Myocardial degeneration/necrosis</td>
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<tr>
<td>Myocardial necrosis</td>
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<tr>
<td>Myocardial degeneration/fibrosis</td>
<td>NAD</td>
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</tbody>
</table>

*aAll other information as Table 4.3A. *bNAD= no abnormalities detected.
Table 4.4. Comparison of the characteristics of serum and plasma biomarkers in rats treated at 4000 μg/kg ISO and autopsied at time points from 0 h to 72.0 h post-dosing.

<table>
<thead>
<tr>
<th>Biomarker</th>
<th>Mean value of control animals</th>
<th>First significant increase in biomarker</th>
<th>Peak level of biomarker</th>
<th>Time point of return of biomarker to control base line value (h)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Time post-dosing (h)</td>
<td>Level of biomarker</td>
<td>Level as fold increase over concurrent control</td>
</tr>
<tr>
<td>H-FABP</td>
<td>2.18</td>
<td>0.5</td>
<td>25.74</td>
<td>19.0</td>
</tr>
<tr>
<td>cTnI (ACS: 180S)</td>
<td>0.030</td>
<td>0.5</td>
<td>0.718</td>
<td>22.9</td>
</tr>
<tr>
<td>cTnI (Beckman Access)</td>
<td>0.004</td>
<td>0.5</td>
<td>0.936</td>
<td>233.0</td>
</tr>
<tr>
<td>cTnT</td>
<td>0.010</td>
<td>0.5</td>
<td>0.240</td>
<td>23.0</td>
</tr>
<tr>
<td>AST</td>
<td>65.0</td>
<td>2.0</td>
<td>85.8</td>
<td>0.3</td>
</tr>
<tr>
<td>CK</td>
<td>208.5</td>
<td>2.0</td>
<td>273.6</td>
<td>0.8</td>
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<tr>
<td>CK-MB</td>
<td>19.6</td>
<td>3.0</td>
<td>54.8</td>
<td>1.4</td>
</tr>
<tr>
<td>LD</td>
<td>197.3</td>
<td>3.0</td>
<td>485.0</td>
<td>2.5</td>
</tr>
<tr>
<td>LD1</td>
<td>11.4</td>
<td>2.0</td>
<td>34.6</td>
<td>1.9</td>
</tr>
<tr>
<td>LD2</td>
<td>7.3</td>
<td>0.5</td>
<td>17.8</td>
<td>1.5</td>
</tr>
<tr>
<td>LD3</td>
<td>6.5</td>
<td>1.0</td>
<td>19.2</td>
<td>1.0</td>
</tr>
</tbody>
</table>

^Abbreviations and units: H-FABP, heart fatty acid-binding protein, ng/mL; cTnI, cardiac troponin I, μg/L; cTnT, cardiac troponin T, μg/L; AST, aspartate aminotransferase, U/L; CK, total creatine kinase, U/L; CK-MB, creatine kinase MB isoform, U/L; LD, total lactate dehydrogenase, U/L; LD1 to LD3, lactate dehydrogenase isoenzymes 1 to 3, U/L.
^Control (PBS-dosed) animals, H-FABP, n=59; cTnI (ACS: 180S), n=58; cTnI (Beckman Access), n=50; cTnT, n=56; AST, n=59; CK, n=59; CK-MB, n=58; LD, n=59; LD1, n=58; LD2, n=58; LD3, n=58.
^This time point is considered to be the first insignificant value after the peak level; in the case of H-FABP and CK, this is an interpretative value.
^"NP" indicates that the biomarker was still increased above concurrent control levels at the final time point measured. For cTnI (Beckman Access) the final time point was at 72.0 h post-dosing and for cTnT, the final time point was 48.0 h post-dosing.
^"NR" indicates that the biomarker of interest did not return to base line levels (due to lack of sensitivity).
^Values presented are from the last of the 2 or 3 time points where the biomarker peak occurred.
5.1 INTRODUCTION

5.1.1 Background

The previous study on isoproterenol (Chapter 4; Time course study on the cardiotoxicity of isoproterenol) highlighted the possible utility of heart fatty acid-binding protein (H-FABP) as a very early serum marker of cardiac injury in the rat. The study demonstrated that following a single subcutaneous (SC) dose of isoproterenol (ISO) at 100 and 4000 μg/kg in the male Hanover Wistar rat, increased serum levels of H-FABP were detectable at 30 min post-dosing (the first autopsy time point) and levels peaked at 2.0 h following ISO administration (4000 μg/kg). The present investigation was therefore designed to investigate further the utility of the protein in the diagnosis of cardiac damage following a single SC dose of ISO at 4000 μg/kg.

5.1.2 Aims of the present study

The aims of the present study were to assess whether H-FABP is a useful serum marker of myocardial injury in the rat as induced by exposure to a single dose of ISO, to establish the very early time course of the H-FABP increases, and to correlate the elevations with the histopathological evidence of myocardial injury.
5.2 MATERIALS AND METHODS

5.2.1 Experimental protocol

OBJECTIVE: To investigate the very early time course and changes in serum H-FABP levels following a single dose of isoproterenol and to correlate elevations with the histopathology of cardiac injury

5.2.1.1 Animal handling

An ISO dosing solution was prepared at a concentration of 800 µg/ml, as described in Chapter 2.4.1. The vehicle was phosphate buffered saline (PBS). Male Hanover Wistar rats (n=105; mean weight 192.3 g) were randomly divided into 21 groups (n=5/group). Each group of n=5 animals occupied 1 cage. Rats were treated with a single SC dose of ISO at dose levels of 0 (PBS, vehicle; n=50) or 4000 µg/kg (n=50). Animals from each of the 2 groups (n=5/group) were autopsied at 10, 20, 30, 40, 50, 60, 75, 90, 105 and 120 min post-dosing for clinical chemistry and histopathological investigations; a further group of (n=5) control (PBS vehicle-treated) rats were autopsied at 0 min, the time of ISO administration.

5.2.1.2 Sample handling

At autopsy, animals were killed by exsanguination under deep isoflurane anaesthesia (as described in Chapter 2.6) and hearts removed, weighed and placed in formalin fixative (Chapter 2.10). Serum was prepared and clinical chemistry analysis performed on all animals in all groups; parameters measured were: heart fatty acid-binding protein (H-FABP) and cardiac troponin I (cTnI; measured on the ACS: 180S and on the Centaur CP) (Chapter 2.8.2). The 0, 10, 20, 30, 75, 90 and 120 min post-dosing cTnI serum values were determined on the ACS: 180S, the 40, 50, 60 and 105 min samples on the Centaur CP.

Sections of hearts (H&E) were examined microscopically from all animals in all groups. Hearts were scored on a lesion severity scale of 0 to 4: 0 = no abnormalities detected (NAD), 1 = minimal, 2 = mild, 3 = moderate and 4 = marked.
5.2.2 Statistical analysis

Data were analysed using a 2-tail Student’s t test. For correlation analyses, the 2-tail Pearson test was used. Statistical analysis was performed using GraphPad Prism version 3.00 for windows (GraphPad software, San Diego, California, USA).
5.3 RESULTS

OBJECTIVE: To investigate the very early time course and changes in serum H-FABP levels following a single dose of isoproterenol and to correlate elevations with the histopathology of cardiac injury

5.3.1 Clinical signs and body weight changes

Male Hanover Wistar rats were given a single SC dose of PBS (vehicle control) or ISO at 4000 µg/kg. Following drug administration, animals given ISO at 4000 µg/kg displayed reduced activity and lay in a prostrate position. The heart rate and respiratory rate of these animals appeared to be increased, and they drank large volumes of water. These signs appeared approximately 20 min post-dosing and were still evident at 120 min post ISO administration. There were no deaths in the study.

5.3.2 Serum clinical chemistry

5.3.2.1 Heart fatty acid-binding protein

At 0 min (the time of ISO administration), the mean serum H-FABP level in the control (0 µg/kg ISO) animals was 1.610 ng/mL (Figure 5.1). The 4000 µg/kg ISO group mean serum H-FABP levels were higher than in the concurrent control animals at all time points but the results showed variability. In rats treated with 4000 µg/kg ISO, the group mean H-FABP value (13.846 ng/mL) at 10 min post-dosing was increased 2.4-fold over the relevant concurrent control result (4.130 ng/mL); however this elevation was not statistically significant. Likewise, at 20 min post-dosing, the 4000 µg/kg ISO group mean was 7.264 ng/mL, compared with the concurrent control result of 1.804 ng/mL (a 3.0-fold increase, NS). At the 30 min autopsy time point, the ISO-treated mean serum H-FABP level was significantly increased (P<0.001) above the control result; the ISO group mean value was 39.734 ng/mL and the concurrent control figure was 2.464 ng/mL (a 15.1-fold elevation). However, at 40 min post-dosing, the 4000 µg/kg ISO mean H-FABP level was raised over the control value, but the increase was NS. At the 50, 60, 75, 90, 105 and 120 min time points, the mean serum H-FABP results in the 4000 µg/kg ISO animals were significantly increased over the concurrent control values; the fold increases were: 50 min, 6.1, P<0.05; 60 min, 8.4, P<0.05; 75 min, 5.3,
respectively. It is interesting to note that there was no clear peak in the H-FABP increases, and the elevations in the ISO-treated animals at 30 min and 120 min post-dosing were similar.

5.3.2.2 Cardiac troponin I

Increases in the serum levels of cTnl in the ISO-treated animals were first apparent at the 20 min post-dosing time point (Figure 5.2). The mean serum cTnl result in the 0 µg/kg ISO (control, vehicle-treated) animals at 0 min was <0.030 µg/L. At 10 min, the control mean result was 0.030 µg/L, and in the ISO-treated rats the relevant result was 0.044 µg/L. At 20 min post-dosing, the mean cTnl level in the 4000 µg/kg ISO animals (0.434 µg/L) was increased over the concurrent control mean value of 0.030 µg/L, a 13.5-fold elevation (NS). From the 30 min time point, the group mean cTnl results in the ISO-treated animals were increased significantly over the concurrent control values; that is, at 30, 40, 50, 60, 75, 90, 105 and 120 min post-dosing, the mean levels in the ISO-treated animals being 1.724, 0.648, 0.754, 1.310, 8.362, 5.914, 5.370, 8.210 pg/L; fold increases of 56.5 (P<0.001), 63.8 (P<0.05); 74.4 (P<0.001), 108.2 (P<0.05), 277.7 (P<0.05), 196.1 (P<0.05), 91.6 (P<0.001) and 272.7 (P<0.001), respectively.

5.3.3 Gross pathology and histopathology

At autopsy, there was no macroscopic evidence of ISO-induced toxicity; however from the 30 min post-dosing time point, the stomachs of a proportion of the ISO-treated animals contained a large volume of water, e.g. 3 of 5 animals at 30 min, 5 of 5 animals at 120 min.

The administration of ISO had some effects on the relative heart weights of animals during the 120 min study period (Figure 5.3); however the data was inconsistent. At the 40 min post-dosing time point, the group mean relative heart weight of the ISO-treated rats was significantly greater than the concurrent control animals (control, 3.30 g/kg; ISO-treated, 3.63 g/kg, a 10.0 % increase, P<0.05). At the 50 min, 60 min and 120 min post-dosing time points, the mean relative heart weights of the 4000 µg/kg ISO animals were also significantly greater than in the concurrent control animals, the % increases
being 9.5 (P<0.05), 9.0 (P<0.001), and 7.8 (P<0.001), greater than the controls, respectively.

The incidence and severity of microscopic findings in the hearts of ISO-treated animals is summarised in Table 5.1A and 5.1B. ISO administration at a dose level of 4000 μg/kg induced myocardial degeneration, graded as minimal to moderate, at all time points from 10 min to 120 min post-dosing. Changes were characterised by contraction banding, loss of cross-striations, granular to fragmented sarcoplasm and a variable microvesicular or coarse vacuolation. A small proportion of the affected myocytes also showed nuclear pyknosis. Lesions increased in the grade of severity with time; at 10 min post-dosing, the mean lesion score was 1.0; at 60 min, the mean score was 2.2, and at 120 min post-dosing, the mean score was 2.8. Control animals (n=55 in total) showed no histological abnormalities.
5.4 DISCUSSION

The aims of the present study were to further investigate the utility of H-FABP as a serum marker of cardiac injury following a single SC injection of ISO at 4000 µg/kg, to establish the very early time course of the H-FABP increases and to correlate the elevations with the histopathological evidence of myocardial injury. The measurement of serum H-FABP in the assessment of drug-induced cardiotoxicity has not been described previously, and there are no reports in the literature employing the marker for this purpose.

Myocardial degeneration, graded as minimal to mild, was present in 4 of 5 ISO-treated animals at the first autopsy time point of 10 min post-dosing (Table 5.1A). After this time point, all rats treated with ISO showed drug-induced histopathological changes, which increased in the grade of severity over time (Table 5.1A, 5.1B). Myocardial degeneration was characterised by contraction banding, loss of cross-striations, granular to fragmented sarcoplasm and a variable microvesicular or coarse vacuolation. The myocardial alterations observed were similar to those seen previously (Chapter 4; Time course study on the cardiotoxicity of isoproterenol), where these changes were observed between 0.5 and 4.0 h following the single SC injection of ISO at 100 and 4000 µg/kg. However, the 10 min post-dosing time point appears to be the earliest that light microscopic evidence of cardiac injury induced by ISO has been reported. Although Csapó et al. (1972) demonstrated that 10 min following the single intraperitoneal (IP) injection of ISO to male Sprague Dawley rats, disorganisation of the myofibrils and contraction bands were evident, these changes were detected by electron microscopy.

A close examination of the H-FABP serum clinical chemistry data (Figure 5.1) in comparison with the pathological lesion scores shows that increased H-FABP levels were present at 10 min post-dosing, concurrently with the histopathological evidence of cardiac injury (Table 5.2A to 5.2D), although the H-FABP increases were NS at this time point. Serum cTnI levels were first increased at the 20 min autopsy time point, but again, this increase was NS (Figure 5.2). However, serum H-FABP and cTnI levels were both significantly raised (P<0.001) at the 30 min post-dosing time point, and both biomarkers remained elevated until the completion of the study at 120 min. In this early time course study, no clear peaks in serum H-FABP or cTnI were observed. However, the data for H-FABP was quite variable, but nevertheless the ISO-treated H-FABP
results were higher than the concurrent control values at all time points from 10 min post-dosing. The shape of the curve in Figure 5.2 suggests that the peak in serum cTnI values following a single SC injection of ISO at 4000 µg/kg occurs at or after 120 min post-dosing.

Individual animal H-FABP and cTnI values and myocardial degeneration scores (severity gradings) in all rats treated with ISO, concurrent control animals, and animals autopsied at 0 min (the time of administration), are presented in Table 5.2A to 5.2D; correlations of the H-FABP values, cTnI values and myocardial degeneration scores are shown in Figure 5.4, 5.5 and 5.6. Figure 5.4 demonstrates that there is a significant correlation (P<0.001) between individual H-FABP values and cTnI values ($R^2=0.3065$). In general, animals with higher H-FABP values tended to have higher cTnI values; however the $R^2$ was not very high. In Chapter 4 (Time course study on the cardiotoxicity of isoproterenol), it was demonstrated that the peak H-FABP value following a single SC dose of ISO at 4000 µg/kg occurred at 2.0 h post-dosing, whereas the maximal cTnI concentrations were seen at 3.0 h post-dosing. Therefore, in the present study, the temporal disconnect between peak H-FABP and cTnI levels may have been a factor contributing to the not very strong correlation between the 2 individual animal biomarker results.

Figure 5.5 presents the results of the correlation between individual animal H-FABP values and the myocardial degeneration scores; Figure 5.6 gives the correlation between individual animal cTnI values and the myocardial degeneration scores. It is seen in Figure 5.5 that there is a significant (P<0.001) correlation between H-FABP values and the severity grading of lesions ($R^2=0.4223$), with animals having higher serum concentrations of H-FABP tending to have more severe cardiac lesions. In a parallel fashion, there is a significant correlation (P<0.001) between individual animal cTnI values and the severity grading of the cardiac lesions ($R^2=0.3619$).

When the usefulness of H-FABP and cTnI measurements are compared, it has been shown in the present study that increased H-FABP levels appeared in the circulation earlier than the elevations of cTnI values (10 min post-dosing compared with 20 min post ISO administration) (Figure 5.1, 5.2). Histopathological evidence of cardiac injury was first seen at 10 min post-dosing (Table 5.1A). Therefore, H-FABP is considered to be an excellent very early marker of cardiac injury following ISO administration.
However, Figure 5.1 shows that the H-FABP data was quite variable compared with the values for cTnI. In addition, the fold increases for cTnI were much greater than for H-FABP. For example, the largest elevation in group mean H-FABP levels was at 120 min, where values were raised to 42.808 ng/mL, a 10.9-fold increase over the concurrent control value. The highest group mean cTnI value was also at 120 min post-dosing (17.756 μg/L); however this increase was 272.7-fold above control values. Therefore, cTnI is shown to be the more sensitive indicator of cardiac injury in this experimental model. When comparing H-FABP and cTnI, specificity is an issue. H-FABP is expressed in a number of tissues including skeletal muscle, myocardium and kidney (Pelsers et al., 2005); whereas cTnI is totally specific to the heart (Cummins and Perry, 1978). Therefore, although only cardiac injury was induced in this ISO experimental model, the usefulness of H-FABP as a cardiac marker may be reduced if other forms of tissue injury (such as skeletal muscle damage) are present.

In conclusion, the very early measurement of serum H-FABP or cTnI were both useful in the assessment of cardiac injury in this rat experimental model; however H-FABP appeared in the circulation earlier than cTnI, and in parallel with the histopathological evidence of myocardial injury. H-FABP is not specifically a cardiac marker, as it is expressed in tissues such as skeletal muscle and kidney, and further investigation of the release kinetics of H-FABP following toxic insult with cardiotoxic and myotoxic compounds is therefore required.
Figure 5.1. Group mean serum heart fatty acid-binding protein (H-FABP) levels in rats treated with a single subcutaneous dose of isoproterenol at 0 (control) and 4000 μg/kg at time points up to 120 min post-dosing. Values are means, SD as error bars; n=5 for control and ISO-treated rats, except at 90 min, 0 μg/kg (control), where n=4. *Significantly different from concurrent control animals, P<0.05; ***P<0.001. A further 5 control animals were autopsied at “Time 0”, the time of dosing.
Figure 5.2. Group mean serum cardiac troponin I (cTnl) levels in rats treated with a single subcutaneous dose of isoproterenol at 0 (control) and 4000 µg/kg at time points up to 120 min post-dosing. Values are means, SD as error bars; n=5 for control and ISO-treated rats, except at 60 min, 0 µg/kg (control) and at 90 min, 0 µg/kg (control), where n=4. *Significantly different from concurrent control animals, P<0.05; ***P<0.001. A further 5 control animals were autopsied at “Time 0”, the time of dosing. At 0, 10, 20, 30, 75, 90 and 120 min post-dosing, serum cTnl values were determined using the ACS: 180S system; the Centaur CP was used to measure serum cTnl levels at 40, 50, 60 and 105 min post-dosing.
Figure 5.3. Group mean relative heart weights in rats treated with a single subcutaneous dose of isoproterenol at 0 (control) and 4000 μg/kg at time points up to 120 min post-dosing. Values are means, SD as error bars; n=5 for all groups. *Significantly different from concurrent control animals, P<0.05; **P<0.001. A further 5 control animals were autopsied at “Time 0”, the time of dosing. Note the origin of the Y axis is 3.0 g/kg.
Figure 5.4. Individual animal serum cardiac troponin I (cTnI) values and individual animal serum heart fatty acid-binding protein (H-FABP) values in rats treated with a single subcutaneous dose of isoproterenol (ISO) at 0 (control) and 4000 µg/kg. Animals were autopsied at 10, 20, 30, 40, 50, 60, 75, 90, 105 and 120 min post-dosing; n=5 for each control and ISO-treated group at each time point. A further group of n=5 control animals were autopsied at the beginning of the study ("Time 0"). n=103 XY pairs; Pearson correlation.
Figure 5.5. Individual animal serum heart fatty acid-binding protein (H-FABP) values and myocardial degeneration scores (severity grading) of rats treated with a single subcutaneous dose of isoproterenol (ISO) at 0 (control) and 4000 μg/kg. Animals were autopsied at 10, 20, 30, 40, 50, 60, 75, 90, 105 and 120 min post-dosing; n=5 for each control and ISO-treated group at each time point. A further group of n=5 control animals were autopsied at the beginning of the study ("Time 0"). n=104 XY pairs; Pearson correlation.
Figure 5.6. Individual animal serum cardiac troponin I (cTnI) values and myocardial degeneration scores (severity grading) of rats treated with a single subcutaneous dose of isoproterenol (ISO) at 0 (control) and 4000 μg/kg. Animals were autopsied at 10, 20, 30, 40, 50, 60, 75, 90, 105 and 120 min post-dosing; n=5 for each control and ISO-treated group at each time point. A further group of n=5 control animals were autopsied at the beginning of the study ("Time 0"). n=103 XY pairs; Pearson correlation.
Table 5.1A. Incidence of microscopic findings in the hearts of control male Hanover Wistar rats and animals treated with isoproterenol (ISO) and autopsied at 0, 10, 20, 30, 40, 50 and 60 min post-dosing*.

<table>
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<tr>
<th>Microscopic finding</th>
<th>Severity grade</th>
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<th>10</th>
<th>20</th>
<th>30</th>
<th>40</th>
<th>50</th>
<th>60</th>
</tr>
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<tbody>
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<td>NAD</td>
<td>5</td>
<td>5</td>
<td>1</td>
<td>5</td>
<td>5</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>Minimal</td>
<td>-</td>
<td>-</td>
<td>3</td>
<td>-</td>
<td>1</td>
<td>-</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Mild</td>
<td>-</td>
<td>-</td>
<td>1</td>
<td>-</td>
<td>3</td>
<td>-</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>Moderate</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>1</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Marked</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
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</tr>
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<table>
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<th>0</th>
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<th>0</th>
<th>4000</th>
<th>0</th>
<th>4000</th>
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</thead>
<tbody>
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<td>Number of rats in each group with histological changes</td>
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*<sup>n=5 for all groups. Animals were treated with a single subcutaneous dose of ISO at 0 (control, vehicle-treated) and 4000 µg/kg, and autopsied at 10, 20, 30, 40, 50, 60, 75, 90, 105 and 120 min post-dosing. A further group of (n=5) control animals was autopsied at 0 min, the time of drug administration.

<sup>b</sup>NAD = no abnormalities detected.

<sup>c</sup>Lesion scoring: 0= NAD; 1= minimal; 2= mild; 3= moderate; 4= marked.
Table 5.1B. Incidence of microscopic findings in the hearts of control male Hanover Wistar rats and animals treated with isoproterenol and autopsied at 75, 90, 105 and 120 min post-dosing.

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*All other information as Table 5.1A.
Table 5.2A. Comparison of individual animal heart fatty acid-binding protein (H-FABP) levels, cardiac troponin I (cTnl) levels and the myocardial lesion degeneration scores (severity grading) in the hearts of male Hanover Wistar rats treated with isoproterenol (ISO) at 0 μg/kg (vehicle control) and autopsied at 10, 20, 30, 40, 50 and 60 min post-dosing.

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<th>Mean (SD)</th>
<th>cTnl level (μg/L)</th>
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</table>

*Values for H-FABP are measured in ng/mL, cTnl levels are measured in μg/L. Hearts were scored on a lesion severity grading scale of 0 to 4: 0 = no abnormalities detected (NAD), 1 = minimal, 2 = mild, 3 = moderate and 4 = marked. 5 control animals and 5 ISO-treated rats were autopsied at each time point.

*Significantly different from concurrent control animals, P<0.05; **P<0.001. NS indicates no sample for analysis.
Table 5.2B. Comparison of individual animal heart fatty acid-binding protein (H-FABP) levels, cardiac troponin I (cTnl) levels and the myocardial lesion degeneration scores (severity grading) in the hearts of male Hanover Wistar rats treated with isoproterenol (ISO) at 4000 μg/kg and autopsied at 10, 20, 30, 40, 50 and 60 min post-dosing.

<table>
<thead>
<tr>
<th>Time point (min)</th>
<th>H-FABP level (ng/mL)</th>
<th>Mean (SD) cTnl level (μg/L)</th>
<th>Mean (SD)</th>
<th>Myocardial degeneration score Mean (SD)</th>
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<td>1.15</td>
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*All other information as Table 5.2A.
Table 5.2C. Comparison of individual animal heart fatty acid-binding protein (H-FABP) levels, cardiac troponin I (cTnI) levels and the myocardial lesion degeneration scores (severity grading) in the hearts of male Hanover Wistar rats treated with isoproterenol (ISO) at 0 μg/kg (vehicle control) and autopsied at 75, 90, 105 and 120 min post-dosing.

<table>
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<tr>
<th>Time point (min)</th>
<th>H-FABP level (ng/mL)</th>
<th>Mean (SD)</th>
<th>cTnI level (μg/L)</th>
<th>Mean (SD)</th>
<th>Myocardial degeneration score Mean (SD)</th>
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“All other information as Table 5.2A.
Table 5.2D. Comparison of individual animal heart fatty acid-binding protein (H-FABP) levels, cardiac troponin I (cTnI) levels and the myocardial lesion degeneration scores (severity grading) in the hearts of male Hanover Wistar rats treated with isoproterenol (ISO) at 4000 µg/kg and autopsied at 75, 90, 105 and 120 min post-dosing.

<table>
<thead>
<tr>
<th>Time point (min)</th>
<th>H-FABP level (ng/mL)</th>
<th>Mean (SD)</th>
<th>cTnI level (µg/L)</th>
<th>Mean (SD)</th>
<th>Myocardial degeneration score</th>
<th>Mean (SD)</th>
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*All other information as Table 5.2A.
CHAPTER 6: STUDIES INVESTIGATING HEART FATTY ACID-BINDING PROTEIN AS A URINARY MARKER OF CARDIAC MUSCLE AND SKELETAL MUSCLE INJURY

6.1 INTRODUCTION

6.1.1 Background

2,3,5,6-tetramethyl-p-phenylenediamine (TMPD) belongs to an important group of industrial chemicals, the p-phenylenediamines (PPDs; Figure 6.1). The PPDs are used as antioxidants, antiozonants, photographic developers, dyestuff intermediates and as components of hair dye (Munday et al., 1990). PPDs are also formed during the metabolism of azo dyes in mammals (Munday et al., 1989). The PPDs are readily oxidised, forming their corresponding cation radicals, the semiquinonediamine Wurster salts or quinonediamines (Munday, 1992). In addition to the production of cation radicals, oxidation of PPDs also generates the superoxide radical, hydrogen peroxide and the hydroxyl radical (Munday, 1988).

6.1.2 Toxicity of the p-phenylenediamines

The unsubstituted PPD (Figure 6.1) is an industrial chemical, which causes skin irritation, keratoconjunctivitis, swelling of the conjunctiva and eczema of the eyelids on direct contact (Ioannou and Matthews, 1985).

However, more importantly from a toxicological viewpoint, studies have shown the potential of PPD to be mutagenic and/or carcinogenic. Ames et al. (1975) demonstrated that following oxidation by hydrogen peroxide, PPD was mutagenic to Salmonella typhimurium strain TA1538 (for the detection of frameshift mutations) in the presence of the S9 activating system; the mutagenic product formed was Bandrowski's base. PPD has also been shown to be mutagenic in recessive lethal assay studies on Drosophila melanogaster (Blijleven, 1977). However, Hossack and Richardson (1977) demonstrated that the compound showed no clear evidence of mutagenic potential, when investigated by the micronucleus test. The mutagenic and carcinogenic properties of PPD in the rat were investigated by Rojanapo et al. (1986), and it was demonstrated
that PPD was mutagenic to *Salmonella typhimurium* strain TA98 in the presence of rat liver S9 fraction following the oxidation of PPD with hydrogen peroxide. Daily topical application or subcutaneous (SC) injection of oxidised PPD for 18 months to female Wistar rats resulted in a statistically significant incidence of both benign and malignant mammary gland, uterine and soft tissue tumours. In males, no soft tissue or mammary gland tumours were observed; however tumours were present in some other organs such as the liver, kidney and urinary bladder. Ioannou and Matthews (1985) compared the absorption, distribution, metabolism and excretion of radioactive PPD in male and female F344 rats and B6C3F1 mice. After 24 h, only 10-15% of the total dose administered was present in the animal body, and it was demonstrated that clearance of PPD was primarily by the kidneys. It was shown that there were no real differences in metabolism between sexes or strains of animal, apart from a difference in urinary metabolites between rats and mice. PPD was also shown to bind to hepatic protein, but not hepatic DNA.

6.1.2.1 The myotoxicity of the p-phenylenediamines

A number of derivatives of PPD are known to cause cardiac and skeletal muscle toxicity *in vivo*. Studies have been performed investigating the myotoxic effects of the N- and ring-methylated derivatives of PPD. Jasmin and Gareau (1961) administered dimethyl-PPD (Figure 6.1) by SC injection to female Sprague Dawley rats at a dose of 2 mg twice daily for up to 20 days. Lesions were observed in the gastrocnemius muscle after 1 day of compound administration, where there was a diffuse swelling of the muscle fibres and an inflammatory cell (mainly neutrophils) infiltration. At days 2 and 3, there was interstitial oedema and the muscle fibres were granular with the loss of cross-striations; coagulation necrosis with fragmentation of the myofibres was present, with macrophage proliferation. From day 4 onwards, macrophage invasion continued, and rarely, there was connective tissue formation. After 10 days, the lesions became more discrete, suggesting some level of resistance was developing. Lesions were also observed in the myocardium after 3 days of compound administration. Foci of degeneration and mononuclear cell infiltration were found mainly in the subendocardial region, but also proximal to the epicardium. Over time, these foci became fibrotic. Therefore, the administration of dimethyl-PPD caused both skeletal muscle and myocardial lesions. It
should be noted that Jasmin and Gareau (1961) did not define which dimethyl-PPD was used.

The toxicity of the N-methylated PPDs was studied in more detail by Munday et al. (1989). PPD, monomethyl-PPD, N,N-dimethyl-PPD, N,N'-dimethyl-PPD, trimethyl-PPD and tetramethyl-PPD (Figure 6.1) were administered to female Sprague Dawley rats by SC injection at a dose level of 40 μmol/kg for 3 days, and animals were killed 24 h after receiving their final dose. No lesions were observed in the liver, kidney or spleen of the treated animals, however muscle necrosis was observed in the gastrocnemius, diaphragm, tongue and heart. Unsubstituted PPD caused no lesions; monomethyl-PPD and N,N-dimethyl-PPD caused lesions in the gastrocnemius, diaphragm and tongue; the remaining compounds affected the gastrocnemius, diaphragm, tongue and heart muscles. The earliest alteration observed in skeletal muscle was increased eosinophilia of the muscle fibres, followed by a loss of cross striations, histiocytic infiltration and degeneration. Lesions in the myocardium consisted of cytoplasmic vacuolation and loss of cross striations, degeneration and necrosis. The areas of necrosis were well defined infarct-like regions, while other areas of the heart remained normal. Plasma levels of aldolase and aspartate aminotransferase (AST) were raised in all groups treated with PPD; however in animals treated with N,N'-dimethyl-PPD, trimethyl-PPD and tetramethyl-PPD, plasma activities of creatine kinase (CK), lactate dehydrogenase (LD) and α-hydroxybutyric dehydrogenase (HBD) were also significantly elevated. The autoxidation rates of the test compounds were investigated in vitro, and it was found that those compounds which oxidised fastest were N,N'-dimethyl-PPD, trimethyl-PPD and tetramethyl-PPD (the most toxic compounds of the study), suggesting that free radical formation may be important in the pathogenesis of muscle damage caused by these compounds.

Following their studies on the N-methylated PPDs, Munday et al. (1990) investigated the myotoxic potential of the ring-methylated PPDs. 2-methyl-PPD, 2,5-dimethyl-PPD, 2,6-dimethyl-PPD and TMPD were administered to female Sprague Dawley rats by SC injection at dose levels of 50, 100 and 200 μmol/kg for 3 days, and animals were sacrificed 24 h after receiving their final dose. TMPD appeared to be the most myotoxic of the compounds studied, with the dose level of 50 μmol/kg/day inducing lesions in the gastrocnemius, diaphragm and tongue muscles. Similar changes were seen in rats given 100 μmol/kg/day of 2,5-dimethyl-PPD. Cardiac lesions were only observed in animals.
treated with 2-methyl-PPD, at 200 μmol/kg/day. No lesions were observed in the liver, kidney or spleen in any of the treated groups. Administration of TMPD at 50 μmol/kg/day resulted in significant elevations in plasma CK, ALD, LD, HBD, AST, alanine aminotransferase (ALT) and glutamate dehydrogenase (GLD). The toxicity of TMPD in the rat was investigated further by Draper et al. (1994). Male Sprague Dawley rats were given 4 doses of TMPD over 2 days at [total] dose levels of 25, 50 and 75 μmol/kg/day and were killed 18 h after the last TMPD dose. Gastrocnemius and soleus muscle necrosis was present in all treatment groups. At the highest TMPD dose level, small areas of inflammation were observed in the hearts of treated animals, and there was also some evidence of testicular damage at 75 μmol/kg/day. Serum levels of ALT, AST, CK, LD and HBD were significantly increased at the highest TMPD dose level only, indicating skeletal and cardiac muscle damage. Finally, Dare et al. (2002) treated female Sprague Dawley rats with a single dose of TMPD at 40.0 and 47.5 μmol/kg and male Sprague Dawley rats with a single dose of TMPD at 45.0 and 52.5 μmol/kg. Necrosis was present in the femoralis muscle of the treated animals and was characterised by necrotic single fibres. In the female animals, serum levels of ALT, AST, ALD and CK were significantly raised in both treatment groups. In the males, ALT and AST were increased significantly in both treatment groups. In addition, serum ALD and CK were raised in the 52.5 μmol/kg group only. Levels of ALT and AST were raised with no increases in GLD, suggesting muscle, and not hepatic damage. Urine analysis of the TMPD-treated animals indicated the retention of normal renal structure and function. Therefore, in the studies outlined above, TMPD has been shown to be a specific skeletal muscle toxicant.

6.1.3 The usefulness of heart fatty acid-binding protein as a marker of cardiac injury

The studies on isoproterenol (Chapter 4: Time course study on the cardiotoxicity of isoproterenol; Chapter 5: Time course study on the cardiotoxicity of isoproterenol and the utility of heart fatty acid-binding protein as an early serum marker of cardiac injury) have highlighted the possible utility of heart fatty acid-binding protein (H-FABP) as a serum marker of myocardial damage. However, H-FABP has also been shown to be a urinary marker of cardiac injury in man and in experimental animals. An elevation in urinary H-FABP has been shown in patients presenting with acute myocardial infarction.
(MI) as early as 1.5 h after symptom onset, with levels peaking at 5 to 10 h (Tanaka et al., 1991). In addition, Tsuji et al. (1993) showed that urinary H-FABP levels were elevated in 88.9 % (8 of 9 patients) of samples at 0 to 3 h following symptom presentation and in 87.5 % (7 of 8 patients) at 3 to 6 h. H-FABP proteinuria has also been observed at 3 h following aortic declamping after coronary artery bypass grafting in patients (Hayashida et al., 2000). In a dog model of myocardial injury (coronary occlusion and reperfusion), urinary H-FABP levels were increased in the urine around 45-75 minutes after reperfusion (Sohmiya et al., 1993). In addition, the total amount of H-FABP excreted into the urine correlated strongly with the estimated size of the cardiac infarct. Elevated urinary H-FABP levels have also been detected in rats with MI induced by coronary artery ligation (Volders et al., 1993); the mean amount of H-FABP detected in urine at 24 h post surgery was 79 µg in the MI rats compared with 23 µg in the sham-operated animals (P<0.001).

6.1.4 Aims of the present study

The aims of the present study were to assess whether H-FABP is a useful urinary marker of cardiac muscle and skeletal muscle injury in the rat as induced by exposure to a single dose of isoproterenol or TMPD, respectively; and if so, to establish the timing of urinary H-FABP elevations with both cardiac muscle and skeletal injury.
6.2 MATERIALS AND METHODS

6.2.1 Experimental protocols

6.2.1.1 Experiment 1, 24 h isoproterenol study

OBJECTIVE: To assess whether urinary H-FABP is a useful marker of cardiac muscle injury in the rat treated with a single dose of isoproterenol

6.2.1.1.1 Animal handling

An ISO dosing solution was prepared at a concentration of 800 µg/ml, as described in Chapter 2.4.1. The vehicle was phosphate buffered saline (PBS). Male Hanover Wistar rats (n=10; mean weight 195.3 g) were randomly divided into 2 groups (n=5/group). Animals were treated with a single SC dose of ISO at dose levels of 0 (vehicle control) or 4000 µg/kg at 15.00 h, and placed in metabolism cages (Chapter 2.1) for 24 h. Animals did not have access to diet, however mains drinking water was provided ad libitum. Urine samples were collected over ice every 8 h (i.e., 0–8 h, 15.00 to 23.00 h; 8–16 h, 23.00 to 07.00 h; 16–24 h, 07.00 to 15.00 h) and animals were autopsied at 24 h post-dosing for clinical chemistry and histopathological investigations.

6.2.1.1.2 Sample handling

The 8 h, 16 h and 24 h urine samples from all animals were analysed semi-quantitatively using dipsticks (Multistix). Parameters measured were: leukocytes, nitrite, urobilinogen, protein, pH, blood (haemoglobin), specific gravity (SG), ketones, bilirubin and glucose. Urine samples were also measured for heart fatty acid-binding protein (H-FABP), creatinine (CREAT) and corrected total protein (TPc) (Chapter 2.9). At autopsy, animals were killed by exsanguination under isoflurane anaesthesia (as described in Chapter 2.6). Serum was prepared and clinical chemistry analysis was performed on animals in both control and ISO-treated groups. Parameters measured were: cardiac troponin I (cTnI; measured on the Centaur CP), H-FABP, total creatine kinase (CK), total lactate dehydrogenase (LD) and aldolase (Chapter 2.8.2). At autopsy, hearts, kidneys and the right hind limb were removed, weighed (heart and kidneys) and placed in fixative (Chapter 2.10). Sections of tissues were examined from all animals in...
all groups. Samples from the gastrocnemius, quadriceps femoris and soleus muscles were examined as examples of skeletal muscle. Histopathological lesions were scored on a scale of 0 to 4: 0 = no abnormalities detected (NAD), 1 = minimal; 2 = mild, 3 = moderate and 4 = marked.

6.2.1.2 Experiment 2, 24 h 2,3,5,6-tetramethyl-p-phenylenediamine study

OBJECTIVE: To assess whether urinary H-FABP is a useful marker of skeletal muscle injury in the rat treated with a single dose of 2,3,5,6-tetramethyl-p-phenylenediamine

6.2.1.2.1 Animal handling

A TMPD dosing solution was prepared at a concentration of 2.268 mg/ml, as described in Chapter 2.4.2. The vehicle was vegetable oil. Male Hanover Wistar rats (n=10; mean weight 185.4 g) were randomly divided into 2 groups (n=5/group). Animals were treated with a single SC dose of TMPD at dose levels of 0 (vehicle control) or 40 μmol/kg (6.57 mg/kg) at 15.00 h, and placed in metabolism cages (Chapter 2.1) for 24 h. Animals did not have access to diet, however mains drinking water was provided ad libitum. Urine samples were collected over ice every 8 h (i.e., 0–8 h, 15.00 to 23.00 h; 8–16 h, 23.00 to 07.00 h; 16–24 h, 07.00 to 15.00 h) and animals were autopsied at 24 h post-dosing for clinical chemistry and histopathological investigations.

6.2.1.2.2 Sample handling

Sample handling was as described in Experiment 1 (24 h isoproterenol study).

6.2.2 Statistical analysis

Data from both studies were analysed using a 2-tail Student’s t test. Statistical analysis was performed using GraphPad Prism version 3.00 for windows (GraphPad software, San Diego, California, USA).
6.3 RESULTS

6.3.1 Experiment 1, 24 h isoproterenol study

OBJECTIVE: To assess whether urinary H-FABP is a useful marker of cardiac muscle injury in the rat treated with a single dose of isoproterenol

NB: The urine from 1 of the 5 rats from the control group (0 µg/kg ISO; PBS vehicle) was positive for nitrite in samples taken at 8, 16 and 24 h after vehicle treatment. At autopsy, macroscopic observations suggested an infection was present. The spleen was enlarged and the bladder was very full of urine, although urine production was normal. The testes were enlarged and there was a swelling/lump in the epididymis. In addition, there was a malodorous smell in the abdominal cavity and the abdominal organs appeared inflamed. This rat and data from this animal was excluded from the study, and therefore the control group consisted of n=4 animals.

6.3.1.1 Clinical signs and body weight changes

Male Hanover Wistar rats were treated with a single SC dose of ISO at 0 (PBS, vehicle-treated control) or 4000 µg/kg. All animals in both groups (control and ISO-treated) remained healthy and there were no clinical signs of toxicity. Body weights were established before compound administration, and at the autopsy at 24 h post-dosing. An examination of the data in Table 6.1 suggests that although maintaining animals in metabolism cages for 24 h caused a decrease in the body weight, there was no clear evidence of a difference between the control and ISO-treated animals.

6.3.1.2 Clinical chemistry

6.3.1.2.1 Urine clinical chemistry

Results from the analysis of the 0–8, 8–16 and 16–24 h urine samples are presented in Table 6.2A, 6.2B and 6.2C. Administration of ISO at a dose level of 4000 µg/kg by SC injection induced changes in the amount of urine produced, and levels of H-FABP, TPc and CREAT.
At 8 h post-dosing (Table 6.2A), the group mean volume of urine produced by the ISO-treated animals (n=5) was 11.18 ml, compared with 7.30 ml in the control rats (n=4). However, this increase in volume was not statistically significant. Between 8 and 16 h (Table 6.2B), the volume of urine produced by the ISO-treated rats was 6.58 ml compared with 9.75 ml (control). Again, this change (reduction) was not significant in the drug-treated animals. However, at 24 h post-dosing (Table 6.2C), administration of ISO caused a significant increase in the group mean volume of urine produced (3.24 ml compared with the mean control volume 1.98 ml; P<0.05). However, if the total volume of urine produced by individual animals in the 2 groups (control and ISO-treated) is compared (Table 6.2D), it can be seen that ISO administration did not alter the total urine output over the 24 h period; however the drug appeared to affect the pattern of urine output over time. In the 8 h period immediately after dosing, ISO treatment appeared to induce an increase in urine output (Table 6.2A), however this was followed by a period of reduced urine output, from 8–16 h post-dosing (Table 6.2B).

In the ISO-treated rats, the urinary mean level of H-FABP was significantly elevated at the 8 h sample point (Table 6.2A; P<0.001); the group mean H-FABP level for the ISO-treated rats was 18.738 ng/mL, compared with a control value of 1.680 ng/mL. At 16 h in the ISO-treated animals (Table 6.2B), the level of H-FABP remained elevated above the controls, with a group mean ISO-treated value of 8.628 ng/mL in comparison to 3.208 ng/mL for the control group (P<0.05). At 24 h, there were no differences between urinary H-FABP levels in the control and ISO-treated groups (Table 6.2C); however the mean control value was high at this time (15.560 ng/mL).

The group mean urinary TPc for the ISO-treated rats was significantly elevated above the mean control levels at all 3 sample points (8 h, P<0.05; 16 h, P<0.05; 24 h, P<0.05). In contrast, the administration of ISO appeared to decrease levels of urinary CREAT in comparison with the control. At 8 h post-dosing, the group mean CREAT concentration in the ISO-treated animals was 1.35 mmol/L, compared with 2.53 mmol/L for the control group, however this decrease was not significant. At the 16 h sample point (Table 6.2B), mean CREAT levels remained decreased in the ISO-treated rats, being 1.89 mmol/L in comparison with 2.30 mmol/L in the control animals; again this reduction was not significant. At 24 h post-dosing however (Table 6.2C), the group mean CREAT for the drug-treated animals was significantly reduced to 4.23 mmol/L.
compared with a mean control value of 7.22 mmol/L (P<0.05; a 41.4 % reduction); however the mean control CREAT level was high at this time point.

6.3.1.2.2 Serum clinical chemistry

At the 24 h autopsy time point, alterations were observed in the group mean serum levels of total CK, total LD, aldolase (Table 6.3) and cTnl (Table 6.4). In the ISO-treated animals, the mean level of serum H-FABP at the 24 h autopsy was at baseline control levels. The group mean total CK level for the ISO-treated animals was 652.4 U/L compared with a mean control value of 461.0 U/L; however this increase was not significant. The mean serum level of total LD however was significantly elevated in the ISO-treated animals (P<0.05; a 76.1 % increase). Likewise, the group mean serum aldolase for the ISO-treated group was 50.6 U/L compared with a mean of 37.5 U/L in the control group (P<0.05; a 34.9 % increase).

Individual serum cTnl concentrations of control (vehicle-treated) and ISO-treated animals are presented in Table 6.4. At 24 h post-dosing, the group mean cTnl value following ISO administration was 3.310 pg/L, compared with a mean control value of <0.010 pg/L (P<0.05; a 330.0-fold increase). A closer examination of the individual animal data shows that the lowest increase in serum cTnl in an ISO-treated animal (1.24 µg/L; rat number 6) was 123.0-fold above the mean control level; whereas the highest increase in cTnl (6.69 µg/L; rat number 7) was a 668.0-fold elevation above the group mean control level of <0.010 µg/L.

6.3.1.3 Gross pathology and histopathology

There was no macroscopic evidence of ISO-induced toxicity observed at the 24 h autopsy time point in the drug-treated animals.

Administration of ISO had a significant effect on the relative heart weights of the treated animals (Figure 6.2A) at 24 h post-dosing, but not on the relative kidney weights (Figure 6.2B). The group mean relative heart weight for the ISO-treated animals (Figure 6.2A) was significantly (P<0.001) increased to 4.17 g/kg compared to the control group mean, which was 3.35 g/kg (a 24.5 % change).
The severity and incidence of microscopic findings in the hearts of ISO-treated animals is summarised in Table 6.5. All animals treated with ISO showed myocardial degeneration/necrosis, graded as moderate. This lesion was characterised by contraction banding, loss of cross-striations, swelling of the myofibres and rarefaction, granular to fragmented sarcoplasm and a variable microvesicular or coarse vacuolation (Figure 6.3). A significant proportion of the myofibres exhibited hypereosinophilia/fragmentation, nuclear pyknosis with occasional karyorrhexis and karyolysis, interstitial oedema and inflammatory cell infiltration with some fibres showing mononuclear cell infiltration/digestion.

There was no histopathological evidence of renal or skeletal muscle injury induced by ISO treatment, as assessed by histopathological examination of the kidneys, and of the gastrocnemius, quadriceps femoris and soleus muscles.

6.3.2 Experiment 2, 24 h 2,3,5,6-tetramethyl-p-phenylenediamine study

OBJECTIVE: To assess whether urinary H-FABP is a useful marker of skeletal muscle injury in the rat treated with a single dose of 2,3,5,6-tetramethyl-p-phenylenediamine

6.3.2.1 Clinical signs and body weight changes

Male Hanover Wistar rats were given a single SC injection of vegetable oil (vehicle) or 40 µmol/kg TMPD. All animals remained healthy over the 24 h period following treatment, and there were no signs of toxicity relating to the administration of TMPD. Body weights of the animals were determined before compound administration, and at the 24 h autopsy point. Table 6.6 shows the individual animal weights before and after dosing with TMPD or vehicle. The data shows animals in both treatment groups lost approximately 20 g in the 24 h period of treatment; however there was no clear difference between the amount of weight lost by the control and TMPD-treated rats.
6.3.2.2  Clinical chemistry

6.3.2.2.1  Urine clinical chemistry

Urine clinical chemistry results from the 0–8, 8–16 and 16–24 h urine samples are presented in Tables 6.7A to 6.7F. TMPD administration at a dose level of 40 μmol/kg caused changes to the urinary pH, the presence of blood and H-FABP concentration. For urinary pH in the 16–24 h urine sample (Table 6.7E), the group mean urinary pH for the TMPD-treated animals was 6.0, compared with 5.0 in the controls (P<0.05). However, urinary pH was not altered in the urine samples at the 8 and 16 h collection time points. In the case of analysis for blood, there was evidence in the TMPD-treated animals of the presence of blood in the 0–8, the 8–16 and the 16–24 h urine samples. Blood positivity was recorded in all 5 animals in the TMPD-treated group at the 8, 16 and 24 h sampling points, respectively. Blood analysis was negative for the 5 control animals at each of the 3 time points.

Individual animal urinary H-FABP results at the 3 sampling time points are presented in Tables 6.7B, 6.7D and 6.7F. At the first urine sample time point (0–8 h; Table 6.7B), the group mean H-FABP level in the TMPD-treated animals was 612.73 ng/mL, compared with a mean control value of 6.06 ng/mL. This represented an TMPD-treated group mean fold increase of 100.1; however this was not statistically significant due to the large SD, caused by the variability of the data. At 16 h (Table 6.7D), the group mean urinary H-FABP concentration in animals treated with TMPD was 1259.90 ng/mL, compared with a control value of 4.49 ng/mL (a 279.7-fold increase, NS). Finally, in the 16–24 h urine samples, the group mean TMPD-treated H-FABP value was 1498.93 ng/mL (control value 15.24 ng/mL, a 97.3-fold increase); again, as at the 8 and 16 h time points, the increase at 24 h in the TMPD-treated rats was NS (which was again a reflection of the variability of the data).

6.3.2.2.2  Serum clinical chemistry

Results from the 24 h autopsy time point are summarised in Table 6.8. Treatment of male Hanover Wistar rats with a single SC dose of 40 μmol/kg TMPD caused elevations in serum levels of H-FABP and aldolase. At 24 h post-dosing, the group mean serum H-FABP concentration in the compound-treated animals was 11.228
ng/mL, in comparison to the control group mean of 1.192 ng/mL. This change represented an 8.4-fold increase and was statistically significant (P<0.05). Serum aldolase levels were also elevated in response to TMPD administration. The group mean serum aldolase activity in the TMPD-treated group was significantly (P<0.05) elevated to 882.0 U/L, compared with a control value of 61.8 U/L, representing a 13.3-fold increase. Levels of CK and LD in the TMPD-treated animals compared closely with the vehicle-dosed controls. Levels of serum cTnI were also directly comparable in the control and TMPD-treated animals (Table 6.8). The group mean cTnI results for both the TMPD-treated rats and control animals were <0.010 µg/L.

6.3.2.3 **Gross pathology and histopathology**

There was no macroscopic evidence of toxicity caused by administration of TMPD at the 24 h autopsy time point. The administration of TMPD had no effect on the group mean relative heart weights, or the group mean relative kidney weights (Figure 6.4).

The incidence and severity of pathological findings in the skeletal muscles (gastrocnemius, quadriceps femoris and soleus) of TMPD-treated rats are summarised in Table 6.9. TMPD administration at a dose level of 40 µmol/kg resulted in single myofibre necrosis, graded as minimal to mild, in all treated animals. Single fibre necrosis was characterised by the presence of scattered individual fibres or small groups of fibres with pale eosinophilic homogeneous sarcoplasm (Figure 6.5). Some of the affected fibres contained centrally placed nuclei, infiltrating mononuclear cells, or clumps of disorganised hypereosinophilic material (disorganised myofilaments). There were occasional foci of interstitial oedema and few infiltrating inflammatory cells. As well as single skeletal myofibre necrosis, myofibre degeneration, graded as minimal to moderate, was also present in all 5 animals treated with TMPD (Table 6.9). This change was typified by the presence of scattered individual fibres or groups of fibres showing pale eosinophilic sarcoplasm with a linear to granular pattern (disruption of the interfibrillar network) and microvesicular vacuolation (Figure 6.5). The severity of single myofibre necrosis and myofibre degeneration was most pronounced in the gastrocnemius muscle, with the quadriceps femoris slightly less affected (Table 6.9). The grading of the changes was least severe in the soleus muscle. No TMPD-related histopathological effects were observed in the heart or the kidneys.
6.4 DISCUSSION

The aims of the present studies were to assess whether H-FABP is a useful urinary marker of cardiac muscle and skeletal muscle injury in the rat as induced by exposure to a single dose of ISO or TMPD, respectively.

In Experiment 1 (24 h isoproterenol study), male Hanover Wistar rats were treated with a single dose of ISO at 4000 μg/kg, maintained in metabolism cages for the collection of urine. Animals were then autopsied at 24 h post ISO administration. The administration of ISO appeared to affect the pattern of urine production in the drug-treated rats (Tables 6.2A, 6.2B and 6.2C). Although the total amount of urine produced by the control and ISO-treated groups was approximately similar (Table 6.2D), urine production (volume) in the ISO-treated rats was slightly increased at the 0–8 h (NS) and 16–24 h (NS) urine sampling points, and slightly reduced compared with the controls at the 8–16 h (NS) sampling point.

During the storage phase of the micturition cycle, the bladder (the detrusor muscle, which is controlled by the parasympathetic nervous system) relaxes to accommodate the increasing volumes of urine produced. The internal urethral sphincter, which is controlled by the sympathetic nervous system, remains closed (contracted) during the storage phase, and is opened when the detrusor muscle is contracting during micturition (Widmaier et al., 2005). Detrusor muscle relaxation is brought about by β-adrenoceptor stimulation on the bladder itself. In the rat and human urinary bladder, the presence of β-adrenoceptors (β1, β2 and β3) have been confirmed at the mRNA level (using Northern blots, the polymerase chain reaction (PCR) and in situ hybridisation), with the β3 receptor being the most abundant subtype (Michel and Vrydag, 2006).

ISO, as a non-selective β-adrenoceptor agonist, will have an effect on the urinary bladder, and in particular on bladder relaxation. ISO has been demonstrated in the rat to relax the detrusor muscle in vitro (Uchida et al., 2005; Frazier et al., 2006) and to reduce bladder pressure in vivo (Takeda et al., 2000). Therefore, ISO should have an anti-diuretic effect. In the present study, a single SC injection of ISO at 4000 μg/kg reduced the group mean urine volume produced by the rats between 8 and 16 h only (Table 6.2B), during the period when the rats were the most active (23.00–07.00 h). The reduction in urine production was not statistically significant, but represented a 32.5 % decrease in comparison with the control mean volume. Zahajszky et al. (1982) infused
ISO (12 μg/kg/h) to anaesthetised dogs, and showed that urine flow decreased significantly in response to drug treatment. The ISO-induced antidiuresis was associated with reduced sodium and potassium excretion, but was independent of changes in renal haemodynamics: ISO infusion did not appear to alter glomerular filtration rate (GFR), total renal blood flow or distribution of cortical blood flow. Moosavi and Johns (2003) also observed reductions in urine flow and sodium excretion when ISO was infused to rats at 400 ng/kg/min (24 μg/kg/h) for 4 h. However, in this study, Moosavi and Johns (2003) also demonstrated that ISO initially reduced GFR and renal blood flow. In the present study, where ISO was administered as a single SC dose at 4000 μg/kg, there was no difference in total 24 h urine output between the control and ISO-treated groups (Table 6.2D), suggesting that although ISO had some antidiuretic effects during the 24 h post-dosing period, urine output (overall volume) was not affected by drug administration. Hertting (1964) showed that rats excrete 92 % of a single IV dose of ISO within 8 h of drug administration. Therefore, the significant increase in urine output observed in the present study between 16 and 24 h may have been due to the fact that the administered dose of ISO had been metabolised, and therefore the urinary bladder relaxation effects of the drug had disappeared, allowing micturition to occur.

The urinary clinical chemistry data collected during the 24 h period following treatment with ISO in the present study showed changes in urinary levels of TPc, CREAT, SG and H-FABP (Table 6.2A, 6.2B and 6.2C). Levels of TPc were significantly increased in the ISO-treated animals at 8, 16 and 24 h (P<0.05 for all time points), however dipstick analysis did not demonstrate the presence of protein in the ISO-treated animals at 8, 16 or 24 h. The maximum increase in urinary TPc in the ISO-treated animals was seen at the 8 h time point (Table 6.2A), where TPc was raised to 203.91 mg/mmol compared with a control value of 116.16 mg/mmol (a 75.5 % increase; P<0.05). In man, proteinuria may be a sign of renal functional impairment (Lamb et al., 2006), as protein is not normally (physiologically) present in the urine. High molecular weight (MW) proteins are retained in the circulation by the glomerular filter, whereas the lower MW proteins are reabsorbed by the tubular cells. However, rat urine has little high MW protein, but may be rich in low MW proteins, such as α 2μ-globulin (Olsen et al., 1990). Proteinuria may occur due to glomerular damage, increased glomerular vascular permeability (seen in inflammation) or be due to an increased circulating concentration of low MW proteins (Delaney et al., 2006). In the present study in the rat, there would have been an increase in serum H-FABP levels due to cardiac injury. In addition, the
low MW protein, myoglobin, is also released into the circulation following cardiac
damage in man (Roberts, 1977; Kemp et al., 2004) and animals (Spangenthal and Ellis,
1995; Walker, 2006). Furthermore, myoglobin has been shown to be present in the
Therefore, the presence of H-FABP and myoglobin in the circulation as a result of ISO-
induced myocardial damage may have contributed to the raised TPc observed in the
drug-treated animals (Table 6.2A, 6.2B and 6.2C).

In the present study, the group mean urinary CREAT concentrations in the ISO-treated
animals were decreased at all 3 urine sampling points (8, 16 and 24 h) and the decrease
was significant at 24 h (Table 6.2A, 6.2B and 6.2C). A common laboratory finding in
renal disease/injury is increased serum CREAT, often as a result of decreased GFR and
decreased tubular function (Delaney et al., 2006). As mentioned above, ISO may
decrease GFR (Moosavi and Johns, 2003). Serum CREAT was not measured in the
present study, however a decreased urinary CREAT does suggest that the serum
CREAT concentration may have been increased, indicating possible renal injury.

Therefore, the urine CREAT results possibly suggest that ISO administration may have
caused some injury to the kidneys. However, the group mean relative kidney weight
was not affected in the drug-treated animals (Figure 6.2B). In addition,
histopathological examination of the kidneys showed no evidence of ISO-induced renal
injury. The histopathological findings in the present study compare with findings in the
literature reporting that ISO treatment does not cause injury to the structure of the
kidney (Preus et al., 1988); however it is possible that a decreased GFR in conjunction
with a reduced renal blood flow induced by ISO may have caused a degree of hypoxia
in the kidney, with resultant changes in renal function.

The group mean urinary H-FABP concentration in ISO-treated rats was significantly
elevated at the 8 and 16 h urine sampling points (Table 6.2A and 6.2B), but not at the 24
h time point (Table 6.2C). H-FABP has been shown to be a urinary marker of cardiac
injury in man and in the rat (Tsuji et al., 1993; Volders et al., 1993; Aartsen et al.,
2000). Tanaka et al. (1991) showed that urinary H-FABP levels are elevated in patients
with acute MI. Sohmiya et al. (1993) showed in a dog model that the total amount of H-
FABP excreted strongly correlated with the estimated size of the cardiac infarct.
However, there have been no reports of studies that have used H-FABP as a marker of
drug-induced cardiotoxicity, either in serum or in urine. The present study therefore 
highlights the possible utility of H-FABP in this setting, especially if skeletal muscle 
toxicity can be ruled out (Table 6.7B, 6.7D, 6.7F, Table 6.8).

Cardiac injury induced by ISO was confirmed in the present investigation by an 
elevation in the mean serum cTnI level (Table 6.4; 3.310 μg/L compared with a control 
value of <0.010 μg/L; P<0.05) and also by the histopathological evidence of cardiac 
injury at 24 h post-dosing (Figure 6.3; Table 6.5). Serum levels of LD and aldolase were 
also significantly (P<0.05 for both parameters) elevated above the mean control values 
following ISO treatment (Table 6.3), also indicating the presence of cardiac injury 
(Wenzel and Lyon, 1967; Evans, 1996).

Histopathological examination of the gastrocnemius, quadriceps femoris and soleus 
muscles of the right hind limb of the ISO-treated rats showed no evidence of ISO- 
induced myocyte damage, therefore the serum LD and aldolase increases observed in 
Experiment 1 (24 h isoproterenol study) (Table 6.3) were likely to be due to myocardial 
injury, rather than skeletal muscle damage. Serum levels of H-FABP have been shown 
(Chapter 4: a time course study on the cardiotoxicity of isoproterenol) to be 
significantly elevated above control values in rats administered 100 and 4000 μg/kg ISO 
as early as 30 minutes post-dosing. In this investigation (Chapter 4), H-FABP levels 
appeared to peak at around 2 h post-dosing, and began returning towards control values 
from this time point. Also, in this experiment (Chapter 4), serum H-FABP values had 
returned to base line control values from 12 h post ISO administration. Therefore, in the 
present study (Table 6.3), the absence of an H-FABP signal in the 24 h serum sample 
would be expected, as serum levels would have peaked and returned to base line control 
values before the autopsy sampling point at 24 h.

In Experiment 1 (24 h isoproterenol study), the group mean relative heart weights of the 
ISO-treated animals were significantly (P<0.001) increased compared with control 
animals at the 24 h autopsy (Figure 6.2A). This is in agreement with previous work 
(Chapter 4: Time course study on the cardiotoxicity of isoproterenol), in which group 
mean relative heart weights were first increased at 24 h post-dosing. By 24 h after 
administration of ISO, histological changes within the heart include inflammatory cell 
infiltration and fibroplasia, and this is in line with the report of Kutsuna (1972). 
Therefore, the increase in relative heart weights observed with the administration of ISO
is suggested not to be related to the induction of cardiomyocyte hypertrophy, but rather the influx of extra-cardiac cells as part of the inflammatory response, increasing the absolute weight of the heart, in conjunction with tissue oedema.

In Experiment 1 (24 h isoproterenol study), ISO administration at a dose level of 4000 µg/kg induced characteristic cardiac changes, as seen previously in Chapter 3 (Preliminary studies on the cardiotoxicity of isoproterenol) and in Chapter 4 (Time course study on the cardiotoxicity of isoproterenol). Myocardial degeneration/necrosis, characterised by contraction banding, loss of cross-striations, swelling of the myofibres and rarefaction, granular to fragmented sarcoplasm and a variable microvesicular or coarse vacuolation was present in all animals treated with ISO (Figure 6.3). The histopathological alterations observed here have been reported by others (Rona et al., 1961; Bleuel et al., 1995; York et al., 2007).

There is some limited evidence in the literature that the administration of ISO may cause skeletal muscle myotoxicity. For example, Ng et al. (2002) investigated whether ISO induced skeletal muscle myocyte necrosis in the male Wistar rat. ISO was administered by a single SC injection at dose levels of 1 to 5000 µg/kg. Significant skeletal muscle myocyte necrosis was observed in the soleus muscle (a predominantly slow-twitch muscle) at dose levels of 1 µg/kg and above, and this occurred at dose levels below the dose level at which cardiomyocyte necrosis was seen (10 µg/kg). In a study by Goldspink et al. (2004), cell death in both types of tissue (skeletal muscle and myocardium) occurred by both apoptotic and necrotic pathways. The study also demonstrated, through selective β receptor blockade, that cardiomyocyte death occurred via the β1-adrenergic receptor, whereas skeletal muscle myocyte death was mediated by β2 receptor stimulation. In addition, Burniston et al. (2005) used the β2 receptor agonist, clenbuterol, to induce apoptosis in male Wistar rat heart and soleus muscle. The study by Burniston et al. (2005) also showed that cardiomyocyte death occurred via β1 receptor activation, whereas skeletal myocyte apoptosis was due to β2 receptor involvement. Therefore, in Experiment 1 (24 h isoproterenol study), the significant increases in serum LD and aldolase (Table 6.3) may have been due to skeletal muscle necrosis, rather than cardiomyocyte necrosis. However in this experiment, histological examination of the gastrocnemius, quadriceps femoris and soleus muscles showed no evidence of skeletal muscle injury. Furthermore, cTnI is a specific cardiac marker and therefore the appearance of this protein in the serum of the ISO-treated rats is a definite
indicator of cardiac damage (Table 6.4). However, the elevation of urinary H-FABP levels (Table 4.7 A and B) could possibly have been due to ISO-induced skeletal muscle damage.

H-FABP has been identified in human and rat kidney (Maatman et al., 1992). Therefore, it is possible that in Experiment 1 (24 h isoproterenol study), damage to the kidney would have resulted in increased levels of H-FABP in serum and urine. In addition, decreased renal function is known to result in higher serum H-FABP levels in coronary artery bypass grafting cases in patients with renal insufficiency, and this increased serum H-FABP is due to the reduced clearance of H-FABP. Therefore, if ISO had caused kidney damage in the present study, it is possible that H-FABP levels could have been elevated for a longer period due to decreased renal clearance. However, there was no histopathological evidence of ISO-induced kidney damage.

Renal disease (e.g. chronic renal impairment, renal dialysis) may cause false elevations in serum levels of the cardiac troponins in man (Buhaescu et al., 2005). However, it appears that it is the cTnT rather than the cTnI isoform which is elevated in renal disease (Bhayuna et al., 1995; Fahie-Wilson et al., 2006). Therefore, in Experiment 1 (24 h isoproterenol study), the elevation of cTnI following ISO administration was likely to be due to myocardial damage, rather than renal damage. cTnI is known to be a specific marker of cardiomyocyte injury (Apple, 1999; Wallace et al. 2004).

In Experiment 2 (24 h TMPD study), male Hanover Wistar rats were treated with a single dose of TMPD at 40 μmol/kg SC, maintained in metabolism cages for the collection of urine, and then autopsied at 24 h post compound administration. There was no macroscopic evidence of TMPD toxicity at autopsy, and neither the group mean relative heart weight, nor the group mean relative kidney weight was altered by TMPD administration (Figure 6.4).

The urinary clinical chemistry data from Experiment 2 (Table 6.7A to 6.7F) shows that TMPD administration was associated with the presence of blood in the urine and also an increase in urinary H-FABP concentration. At the 0–8, 8–16 and 16–24 h urine sampling points, blood was detected in the urine of the TMPD-treated animals by dipstick analysis (Table 6.7A, 6.7C and 6.7E). The presence of blood (haemoglobin) in the urine may be indicative of renal failure (Stonard, 1996); however no increases in TPc or histopathological changes to the kidney were observed in TMPD-treated
animals, so renal damage is considered to be unlikely. The dipstick test for the presence of haemoglobin in urine is equally sensitive to myoglobin (Bayer Multistix 10 SG data sheet; Lamb et al., 2006). Myoglobinuria is a common finding observed with skeletal muscle injury (e.g. trauma, rhabdomyolysis) in animals and man (Haller and Drachman, 1980; Huerta-Alardin et al., 2005). Therefore, the haemoglobin positivity observed in the present study with dipstick analysis may have actually been due to the presence of myoglobin as a consequence of the skeletal muscle damage.

The group mean urinary H-FABP for the animals treated with a single dose of TMPD was elevated above control levels at all three sampling time points (Table 6.7B, 6.7D and 6.7F). The TMPD group elevations were not statistically significant however, due to the variability of the data (i.e. large standard deviations). The fold increases for the H-FABP levels at 8, 16 and 24 h were large, being 100.1, 279.6 and 97.3, respectively. In comparison with the H-FABP values observed following ISO administration, where the largest increase in urinary H-FABP was a 10.2-fold increase at 8 h post-dosing (Table 6.2A), the elevations of urinary H-FABP in response to TMPD treatment were much larger. In addition, there was still a strong H-FABP signal at 24 h following TMPD administration (Table 6.7F). The lengthening of the diagnostic window in this way is probably due to the much greater amounts of H-FABP released into the circulation following the administration of TMPD rather than ISO. Although H-FABP has been proposed as a serum (Knowlton et al., 1989; Aartsen et al., 2000) and urinary (Volders et al., 1993; Hayashida et al., 2000) marker of cardiac injury, H-FABP is also present in to a lesser extent in skeletal muscle (Zschiesche et al., 1995; Pelsers et al., 2005). Therefore, the urinary H-FABP signal observed following TMPD administration may have been due to skeletal or cardiac muscle injury. Van Nieuwenhoven et al. (1995) however, showed that H-FABP is released into the circulation following myocardial damage much faster than following skeletal muscle injury. Therefore, this slower release from skeletal muscle may have contributed to the longer diagnostic window of urinary H-FABP observed following TMPD administration than following ISO treatment.

In Experiment 2 (24 h 2,3,5,6-tetramethyl-p-phenylenediamine study), the serum clinical chemistry results from the 24 h autopsy (Table 6.8) support the proposal that TMPD caused skeletal muscle injury, and that this occurs in the absence of cardiac damage. TMPD administration caused a significant increase in serum levels of aldolase
and H-FABP (P<0.05 for both parameters; Table 6.8). Increased serum levels of aldolase are indicative of skeletal muscle injury or disease (Panteghini et al., 2006) and this elevated response has been observed following TMPD administration (Munday et al., 1990; Dare et al., 2002). The group mean serum H-FABP in the TMPD-treated group was raised 8.4-fold above the controls, suggesting that skeletal muscle damage had occurred. In addition, there was no cTnI signal in the TMPD-treated animals (Table 6.8) or histopathological evidence of cardiac injury at 24 h post-dosing, providing further evidence that cardiac muscle damage had not been induced. TMPD is a PPD (Figure 6.1) and some compounds of the PPD group are known to cause myocardial damage, for example, dimethyl-PPD (Jasmin and Gareau, 1961) and trimethyl-PPD and tetramethyl-PPD, Munday et al., 1989). However, when TMPD was administered to female Sprague Dawley rats at 50 µmol/kg on 6 occasions, no cardiac damage was observed (Munday et al., 1990). Therefore in the present study (Experiment 2, 24 h 2,3,5,6-tetramethyl-p-phenylenediamine study), it is reasonable to conclude that only skeletal muscle damage was induced by TMPD.

Histopathological investigation of tissues in Experiment 2 (24 h 2,3,5,6-tetramethyl-p-phenylenediamine study) showed that TMPD administration induced single fibre necrosis, graded as minimal to mild, and myofibre degeneration, graded as minimal to moderate, in all compound-treated animals (Figure 6.5; Table 6.9). The gastrocnemius muscle was the most severely affected and the quadriceps femoris slightly less affected. The least severely affected muscle was the soleus muscle. These findings are consistent with the results of Blair (2001), who showed that in male rats given a single SC injection of TMPD at dose levels of 20, 30, 40, 50 and 60 µmol/kg, the soleus muscle was consistently less affected by TMPD than the gastrocnemius and quadriceps femoris muscles. In addition, the histopathological changes observed in the present study agree with the work by Munday et al. (1990), who showed that TMPD administration to female Sprague Dawley rats at 50, 100 and 200 µmol/kg/day for 3 days resulted in degenerative lesions in the gastrocnemius, diaphragm and tongue muscles, with no changes observed in the heart. Draper et al. (1994) however, did observe small areas of inflammation in the hearts of male Sprague Dawley rats administered TMPD at 75 µmol/kg/day for 2 days. No changes were seen in the hearts of animals treated at 25 and 50 µmol/kg/day for 2 days.
The clinical chemistry analysis in Experiment 1 (24 h isoproterenol study) revealed an elevation in serum LD activity in the drug-treated animals (Table 6.3). Serum LD was shown to be increased in male and female Wistar rats deprived of food for 24 h (Jenkins and Robinson, 1975). Therefore, it is possible that the LD elevation in the present study is a non drug treatment-related change; however the change was only seen with ISO administration, and not with TMPD treatment, suggesting that the LD elevation is indeed a drug-induced change.

Fasting may also alter H-FABP values in the rat (Turcotte et al., 1997). Female Sprague Dawley rats fasted for 12, 24 or 48 h showed an increase in FABP in the plasma membranes of red skeletal muscle, but not white skeletal muscle. Therefore, in both of the present experiments, the fasting of animals during the 24 h period of maintenance in the metabolism cages may have increased H-FABP values in both control and compound-treated animals.

In both Experiment 1 and Experiment 2, the control urinary H-FABP levels were highest in the 16–24 h urine samples, which were collected during the time period 07.00 to 15.00 h (Table 6.2C and 6.7F). During this period, the control urinary H-FABP was 15.56 and 15.24 ng/mL, for the ISO and TMPD study, respectively. At the other time points, mean control urinary H-FABP values ranged from 1.68 to 6.06 ng/mL (Tables 6.2A, 6.2B and 6.2C, and 6.7B, 6.7D and 6.7F). Therefore, it is possible that there may be some circadian variation in the physiological urinary excretion of H-FABP. Many hormones, enzymes and other body fluid constituents exhibit cyclical variations throughout the day (Young and Bermes, 2006). Pelsers et al. (1999) showed that plasma H-FABP concentrations in man show biological variation with time. Within day-to-day variation, levels of H-FABP in healthy male and female volunteers showed a significant (P<0.005) increase from daytime (09.30 to 17.00 h, 0.8 µg/L) to evening (17.00 to 01.00 h, 0.9 µg/L) to night (01.00 to 09.30 h, 1.1 µg/L). The authors linked these values to periods of activity, i.e. H-FABP levels were lowest during the highest period of activity, and highest during the least active hours. In the present study, serum levels of H-FABP in the control rats were highest during the daytime (07.00 to 15.00 h). Rats are nocturnal animals and therefore are least active during the daytime. Therefore, the results here in the rat agree with the work of Pelsers et al. (1999) in man. However, Glatz et al. (1984) assessed the fatty acid-binding capacity of the hearts of rats sacrificed at the mid-dark or mid-light phase of the light cycle and found a marked
variation in binding capacity, with increased binding (7-8 nmol/mg protein) in the mid-dark phase compared with the mid-light phase (3-4 nmol/mg protein).

In conclusion, H-FABP has been demonstrated to be a useful urinary marker of cardiotoxicity and skeletal muscle toxicity; however, the studies have also shown that the marker is not specific enough to differentiate between cardiac and skeletal muscle damage. To aid in a differential diagnosis, it may be possible to determine circulating levels of another specific cardiac or skeletal muscle injury marker. To differentiate cardiac muscle damage, cTnI is the most sensitive and specific marker in current use. To identify skeletal muscle damage, it could be proposed that a specific marker such as skeletal troponin I (sTnI) is employed. In man, sTnI has been investigated as a marker of exercise-induced muscle damage (Sorichter et al., 1997). Subjects (n=61) were placed on 4 exercise regimens, and elevations in CK, myosin heavy chain (MHC) fragments and sTnI were observed. sTnI was demonstrated to be released in parallel to CK and before MHC, and was the most sensitive marker of skeletal muscle injury. The usefulness of sTnI has also been investigated in the rat in a model of respiratory muscle injury (Simpson et al., 2004). Rats were made to breathe against an inspiratory resistive load to induce damage mainly to the diaphragm. sTnI appeared in the serum of respiratory-loaded rats after about 1.5 h. The source of the sTnI was the fast-twitch muscle of the diaphragm. Therefore, sTnI may prove to be useful in the differential diagnosis of skeletal muscle injury.
Figure 6.1. Structure of p-phenylenediamine (PPD) and its N-methylated and ring-methylated derivatives.
Figure 6.2. Experiment 1, 24 h isoproterenol study; group mean relative heart weights (A) and kidney weights (B) in rats treated with a single dose of isoproterenol (ISO) at 0 or 4000 µg/kg at 24 h post-dosing. Values are means, SD as error bars; n=4 for control animals, n=5 for ISO-treated rats. The kidney weight is a mean of the sum of the weight of the left and right kidneys. ***Significantly different from control, P<0.001. Note the origin of the Y axis for relative heart weights is 3.0 g/kg.
Figure 6.3. Experiment 1, 24 h isoproterenol (ISO) study; myocardium from a rat given a single subcutaneous dose of ISO at 4000 μg/kg and killed 24 h post-dosing. There is swelling of the myofibres and rarefaction, granular to fragmented sarcoplasm and a variable microvesicular or coarse vacuolation. H&E, x 400 original magnification.
Figure 6.4. Experiment 2, 24 h 2,3,5,6-tetramethyl-p-phenylenediamine study; group mean relative heart weights (A) and kidney weights (B) in rats treated with a single dose of 2,3,5,6-tetramethyl-p-phenylenediamine (TMPD) at 0 or 40 µmol/kg at 24 h post-dosing. Values are means, SD as error bars; n=5 for both groups. The kidney weight is a mean of the sum of the weight of the left and right kidneys. Note the origin of the Y axis for relative heart weights is 3.0 g/kg.
Figure 6.5. Experiment 2, 24 h 2,3,5,6-tetramethyl-p-phenylenediamine (TMPD) study, 1 – 25000 μg/kg; (A) quadriceps femoris from a control rat and (B) quadriceps femoris from a rat given a single subcutaneous dose of TMPD at 40 μmol/kg and killed at 24 h post-dosing. Single myofibre necrosis and myofibre degeneration. With necrosis, there are pale or hyper eosinophilic myofibres with loss of the normal cellular outline and sarcoplasm, mononuclear phagocytic cell infiltrate and the odd intercellular neutrophil. In degeneration, there is sarcoplasmic vacuolation, scattered pale myofibres with intermyofibrillar separation. H&E, x 200 original magnification.
Table 6.1. Experiment 1, 24 h isoproterenol study; body weights of individual control and isoproterenol- (ISO-) treated rats before dosing and at 24 h post-dosing.

<table>
<thead>
<tr>
<th>Isoproterenol dose (µg/kg)</th>
<th>Weight of rat (g) at 0 h</th>
<th>Weight of rat (g) at 24 h</th>
<th>Loss in weight (g)</th>
<th>Loss in weight as %&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>208</td>
<td>184</td>
<td>24</td>
<td>11.5</td>
</tr>
<tr>
<td></td>
<td>186</td>
<td>166</td>
<td>20</td>
<td>10.8</td>
</tr>
<tr>
<td></td>
<td>200</td>
<td>181</td>
<td>19</td>
<td>9.5</td>
</tr>
<tr>
<td></td>
<td>200</td>
<td>181</td>
<td>19</td>
<td>9.5</td>
</tr>
<tr>
<td>Mean</td>
<td>198.5</td>
<td>178.0</td>
<td>20.5</td>
<td>10.33</td>
</tr>
<tr>
<td>SD</td>
<td>9.1</td>
<td>8.1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4000</td>
<td>196</td>
<td>178</td>
<td>18</td>
<td>9.2</td>
</tr>
<tr>
<td></td>
<td>200</td>
<td>177</td>
<td>23</td>
<td>11.5</td>
</tr>
<tr>
<td></td>
<td>177</td>
<td>178</td>
<td>+1&lt;sup&gt;c&lt;/sup&gt;</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>190</td>
<td>170</td>
<td>20</td>
<td>10.5</td>
</tr>
<tr>
<td></td>
<td>202</td>
<td>180</td>
<td>22</td>
<td>10.9</td>
</tr>
<tr>
<td>Mean</td>
<td>193.0</td>
<td>176.6</td>
<td>16.4</td>
<td>8.50</td>
</tr>
<tr>
<td>SD</td>
<td>10.0</td>
<td>3.8</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup>Rats were treated with a single subcutaneous injection of ISO at 0 (vehicle control) or 4000 µg/kg at 0 h and maintained individually in metabolism cages for 24 h for the collection of urine. Animals were autopsied at 24 h post-dosing. During maintenance in metabolism cages, rats had access to water, but not to diet.

<sup>b</sup>Loss in weight as a percentage of weight at 0 h.

<sup>c</sup>Increase in body weight (1 g).
Table 6.2A. Experiment 1, 24 h isoproterenol study; urine clinical chemistry results in control male Hanover Wistar rats and animals treated with isoproterenol (ISO) at 8 h post-dosing\(^{a,b,c}\).

<table>
<thead>
<tr>
<th>Isoproterenol dose (µg/kg)</th>
<th>0</th>
<th>4000</th>
</tr>
</thead>
<tbody>
<tr>
<td>Volume produced (mL)</td>
<td>7.30 (2.80)</td>
<td>11.18 (2.86)</td>
</tr>
<tr>
<td>pH</td>
<td>7.3 (0.9)</td>
<td>6.8 (0.8)</td>
</tr>
<tr>
<td>SG</td>
<td>1.016 (0.003)</td>
<td>1.016 (0.005)</td>
</tr>
<tr>
<td>Leukocytes</td>
<td>NEG</td>
<td>NEG</td>
</tr>
<tr>
<td>Nitrite</td>
<td>NEG</td>
<td>NEG</td>
</tr>
<tr>
<td>Urobilinogen</td>
<td>3.2 (0.0)</td>
<td>3.2 (0.0)</td>
</tr>
<tr>
<td>Protein</td>
<td>NEG</td>
<td>NEG</td>
</tr>
<tr>
<td>Blood</td>
<td>NEG</td>
<td>NEG</td>
</tr>
<tr>
<td>Ketones</td>
<td>NEG</td>
<td>NEG</td>
</tr>
<tr>
<td>Bilirubin</td>
<td>NEG</td>
<td>NEG</td>
</tr>
<tr>
<td>Glucose</td>
<td>NEG</td>
<td>NEG</td>
</tr>
<tr>
<td>H-FABP</td>
<td>1.680 (0.494)</td>
<td>18.738 (4.162) ***</td>
</tr>
<tr>
<td>Tpc</td>
<td>116.16 (31.37)</td>
<td>203.91 (40.44) *</td>
</tr>
<tr>
<td>CREAT</td>
<td>2.53 (1.05)</td>
<td>1.35 (0.65)</td>
</tr>
</tbody>
</table>

\(^a\)Values are means, SD in parentheses; \(n=4\) for control and \(n=5\) for ISO-treated rats. Animals were treated with a single subcutaneous injection of ISO at 0 (vehicle control) or 4000 µg/kg and maintained individually in metabolism cages for 24 h for the collection of urine. \(^*\)Significantly different from control, \(P<0.05\); \(^{***}\)\(P<0.001\).

\(^b\)Urine was collected from 0 to 8 h post-dosing.

\(^c\)The following parameters were assayed using dipstick analysis (Multistix, Chapter 2.9): leukocytes, nitrite, urobilinogen, protein, pH, blood (haemoglobin), specific gravity, ketones, bilirubin and glucose. Abbreviations and units: SG, specific gravity; leukocytes, number of cells/µL; urobilinogen, µmol/L; protein, g/L; blood, number of erythrocytes/L; ketones, mmol/L; glucose, mmol/L; H-FABP, heart fatty acid-binding protein, ng/mL; Tpc, corrected total protein, mg/mmol; CREAT, creatinine, mmol/L; NEG, negative. Where a parameter is recorded as NEG, the urine samples for all animals in the group were negative.
Table 6.2B. Experiment 1, 24 h isoproterenol study; urine clinical chemistry results in control male Hanover Wistar rats and animals treated with isoproterenol (ISO) at 16 h post-dosing\textsuperscript{a,b}.

<table>
<thead>
<tr>
<th>Isoproterenol dose (µg/kg)</th>
<th>0</th>
<th>4000</th>
</tr>
</thead>
<tbody>
<tr>
<td>Volume produced (mL)</td>
<td>9.75 (6.34)</td>
<td>6.58 (2.05)</td>
</tr>
<tr>
<td>pH</td>
<td>7.1 (0.8)</td>
<td>7.0 (0.4)</td>
</tr>
<tr>
<td>SG</td>
<td>1.011 (0.006)</td>
<td>1.011 (0.004)</td>
</tr>
<tr>
<td>Leukocytes</td>
<td>NEG</td>
<td>NEG</td>
</tr>
<tr>
<td>Nitrite</td>
<td>NEG</td>
<td>NEG</td>
</tr>
<tr>
<td>Urobilinogen</td>
<td>3.2 (0.0)</td>
<td>3.2 (0.0)</td>
</tr>
<tr>
<td>Protein</td>
<td>NEG</td>
<td>NEG</td>
</tr>
<tr>
<td>Blood</td>
<td>NEG</td>
<td>NEG</td>
</tr>
<tr>
<td>Ketones</td>
<td>NEG</td>
<td>NEG</td>
</tr>
<tr>
<td>Bilirubin</td>
<td>NEG</td>
<td>NEG</td>
</tr>
<tr>
<td>Glucose</td>
<td>NEG</td>
<td>NEG</td>
</tr>
<tr>
<td>H-FABP</td>
<td>3.208 (4.012)</td>
<td>8.628 (2.587)*</td>
</tr>
<tr>
<td>TPC</td>
<td>127.15 (30.17)</td>
<td>193.31 (45.26)*</td>
</tr>
<tr>
<td>CREAT</td>
<td>2.30 (1.74)</td>
<td>1.89 (0.77)</td>
</tr>
</tbody>
</table>

\textsuperscript{a}Details of ISO administration, abbreviations and units, and other information are as Table 6.2A.

\textsuperscript{b}Significantly different from control, P<0.05.

\textsuperscript{b}Urine was collected from 8 to 16 h post-dosing.
Table 6.2C. Experiment 1, 24 h isoproterenol study; urine clinical chemistry results in control male Hanover Wistar rats and animals treated with isoproterenol (ISO) at 24 h post-dosing\textsuperscript{a,b}.

<table>
<thead>
<tr>
<th>Isoproterenol dose (µg/kg)</th>
<th>0</th>
<th>4000</th>
</tr>
</thead>
<tbody>
<tr>
<td>Volume produced (mL)</td>
<td>1.98 (0.49)</td>
<td>3.24 (0.43) *</td>
</tr>
<tr>
<td>pH</td>
<td>6.0 (0.0)</td>
<td>6.3 (0.4)</td>
</tr>
<tr>
<td>SG</td>
<td>1.030 (0.000)</td>
<td>1.020 (0.005) *</td>
</tr>
<tr>
<td>Leukocytes</td>
<td>NEG</td>
<td>NEG</td>
</tr>
<tr>
<td>Nitrite</td>
<td>NEG</td>
<td>NEG</td>
</tr>
<tr>
<td>Urobilinogen</td>
<td>3.2 (0.0)</td>
<td>3.2 (0.0)</td>
</tr>
<tr>
<td>Protein</td>
<td>NEG</td>
<td>NEG</td>
</tr>
<tr>
<td>Blood</td>
<td>NEG</td>
<td>NEG</td>
</tr>
<tr>
<td>Ketones</td>
<td>NEG</td>
<td>NEG</td>
</tr>
<tr>
<td>Bilirubin</td>
<td>NEG</td>
<td>NEG</td>
</tr>
<tr>
<td>Glucose</td>
<td>NEG</td>
<td>NEG</td>
</tr>
<tr>
<td>H-FABP</td>
<td>15.560 (4.571)</td>
<td>14.140 (4.955)</td>
</tr>
<tr>
<td>TPc</td>
<td>99.13 (16.61)</td>
<td>146.68 (36.27) *</td>
</tr>
<tr>
<td>CREAT</td>
<td>7.22 (2.53)</td>
<td>4.23 (0.91) *</td>
</tr>
</tbody>
</table>

\textsuperscript{a}Details of ISO administration, abbreviations and units, and other information are as Table 6.2A.
\textsuperscript{b}Significantly different from control, P<0.05.

\textsuperscript{b}Urine was collected from 16 to 24 h post-dosing.
Table 6.2D. Experiment 1, 24 h isoproterenol study; total urine output in individual control male Hanover Wistar rats and animals treated with isoproterenol (ISO) over the 24 h experimental period.

<table>
<thead>
<tr>
<th>Isoproterenol dose (µg/kg)</th>
<th>0</th>
<th>4000</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rat number</td>
<td>Total volume of urine produced (mL)</td>
<td>Rat number</td>
</tr>
<tr>
<td>1</td>
<td>29.1</td>
<td>6</td>
</tr>
<tr>
<td>2</td>
<td>13.7</td>
<td>7</td>
</tr>
<tr>
<td>3</td>
<td>23.9</td>
<td>8</td>
</tr>
<tr>
<td>4</td>
<td>9.4</td>
<td>9</td>
</tr>
<tr>
<td></td>
<td></td>
<td>10</td>
</tr>
<tr>
<td>Mean</td>
<td>19.0</td>
<td></td>
</tr>
<tr>
<td>SD</td>
<td>9.1</td>
<td></td>
</tr>
</tbody>
</table>

*aRats were treated with a single subcutaneous injection of ISO at 0 (vehicle control) or 4000 µg/kg at 0 h and maintained individually in metabolism cages for 24 h for the collection of urine. Animals were autopsied at 24 h post-dosing. During maintenance in metabolism cages, rats had access to water but not diet. Urine volumes are measured in mL; n=4 (vehicle controls), n=5 (ISO-treated).*
Table 6.3. Experiment 1, 24 h isoproterenol study; serum clinical chemistry results in control male Hanover Wistar rats and animals treated with isoproterenol (ISO) at 24 h post-dosing\textsuperscript{a,b}.

<table>
<thead>
<tr>
<th>Isoproterenol dose (µg/kg)</th>
<th>0</th>
<th>4000</th>
</tr>
</thead>
<tbody>
<tr>
<td>H-FABP</td>
<td>1.00 (0.00)</td>
<td>1.11 (0.25)</td>
</tr>
<tr>
<td>CK</td>
<td>461.0 (109.9)</td>
<td>652.4 (177.8)</td>
</tr>
<tr>
<td>LD</td>
<td>1146.3 (280.7)</td>
<td>2019.0 (496.5) *</td>
</tr>
<tr>
<td>Aldolase</td>
<td>37.5 (5.4)</td>
<td>50.6 (8.4) *</td>
</tr>
</tbody>
</table>

\textsuperscript{a}Values are means, SD in parentheses; n=4 for control animals, n=5 for ISO-treated rats. Rats were treated with a single subcutaneous injection of ISO at 0 (vehicle control) or 4000 µg/kg at 0 h and maintained individually in metabolism cages for 24 h for the collection of urine. Animals were autopsied at 24 h post-dosing. *Significantly different from control, P<0.05.

\textsuperscript{b}Abbreviations and units: H-FABP, heart fatty acid-binding protein, ng/mL; CK, creatine kinase, U/L; LD, lactate dehydrogenase, U/L; aldolase, U/L.
Table 6.4. Experiment 1, 24 h isoproterenol study; serum cardiac troponin I (cTnI) results in individual control male Hanover Wistar rats and animals treated with isoproterenol (ISO) at 24 h post-dosing\textsuperscript{a,b}.

\begin{table}[h]
\centering
\begin{tabular}{ccc}
\hline
Isoproterenol dose (\textmu g/kg) & & \\
\hline
0 & & 4000 \\
\hline
\multicolumn{3}{c}{\textbf{Rat number}}
\\
1 & <0.01 & 6 & 1.24 (123.0)\textsuperscript{c}
\\
2 & <0.01 & 7 & 6.69 (688.0)
\\
3 & <0.01 & 8 & 1.95 (194.0)
\\
4 & <0.01 & 9 & 5.09 (508.0)
\\
 & 10 & & 1.58 (157.0)
\\
\textbf{Mean} & <0.001 & & 3.310 (330.0)\textsuperscript{d}
\\
\textbf{SD} & 0.000 & & 2.435
\\
\hline
\end{tabular}
\end{table}

\textsuperscript{a}Rats were treated with a single subcutaneous injection of ISO at 0 (vehicle control) or 4000 \textmu g/kg at 0 h and maintained individually in metabolism cages for 24 h for the collection of urine. Animals were autopsied at 24 h post-dosing. *Significantly different from control, P<0.05.

\textsuperscript{b}Values for cTnI are measured in \textmu g/L.

\textsuperscript{c}{} indicates the “fold increase” of an individual value over the mean control group value.

\textsuperscript{d}[] indicates the “fold increase” of a group mean value over the mean control value.
Table 6.5. Experiment 1, 24 h ISO study; incidence of microscopic findings in the hearts of control (vehicle-treated) male Hanover Wistar rats and rats treated with isoproterenol (ISO)\textsuperscript{a}.

<table>
<thead>
<tr>
<th>Microscopic finding</th>
<th>Severity grade</th>
<th>Isoproterenol dose ((\mu)g/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>4000</td>
</tr>
<tr>
<td>Number of rats in each group with histological changes</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Myocardial degeneration/necrosis

- NAD\textsuperscript{b} 4
- Minimal -
- Mild -
- Moderate 5
- Marked -
- Very marked -

\textsuperscript{a}n=4 animals for 0 \(\mu\)g/kg; \(n=5\) for 4000 \(\mu\)g/kg. Rats were treated with a single subcutaneous injection of ISO at 0 (vehicle control) or 4000 \(\mu\)g/kg at 0 h and maintained individually in metabolism cages for 24 h for the collection of urine. Animals were autopsied at 24 h post-dosing.

\textsuperscript{b}NAD= no abnormalities detected.
Table 6.6. Experiment 2, 24 h 2,3,5,6-tetramethyl-p-phenylenediamine study; body weights of individual control and 2,3,5,6-tetramethyl-p-phenylenediamine-(TMPD-) treated rats before dosing and at 24 h post-dosing\(^a\).

<table>
<thead>
<tr>
<th>TMPD dose ((\mu)mol/kg)</th>
<th>Weight of rat (g) at 0 h</th>
<th>Weight of rat (g) at 24 h</th>
<th>Loss in weight (g)</th>
<th>Loss in weight as %(^b)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>186</td>
<td>168</td>
<td>18</td>
<td>9.7</td>
</tr>
<tr>
<td></td>
<td>174</td>
<td>151</td>
<td>23</td>
<td>13.2</td>
</tr>
<tr>
<td></td>
<td>186</td>
<td>163</td>
<td>23</td>
<td>12.4</td>
</tr>
<tr>
<td></td>
<td>196</td>
<td>175</td>
<td>21</td>
<td>10.7</td>
</tr>
<tr>
<td></td>
<td>172</td>
<td>152</td>
<td>20</td>
<td>11.6</td>
</tr>
<tr>
<td>Mean</td>
<td>182.8</td>
<td>161.8</td>
<td>21.0</td>
<td>11.49</td>
</tr>
<tr>
<td>SD</td>
<td>9.9</td>
<td>10.3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>40</td>
<td>187</td>
<td>168</td>
<td>19</td>
<td>10.2</td>
</tr>
<tr>
<td></td>
<td>197</td>
<td>177</td>
<td>20</td>
<td>10.2</td>
</tr>
<tr>
<td></td>
<td>188</td>
<td>170</td>
<td>18</td>
<td>9.6</td>
</tr>
<tr>
<td></td>
<td>191</td>
<td>172</td>
<td>19</td>
<td>9.9</td>
</tr>
<tr>
<td></td>
<td>177</td>
<td>160</td>
<td>17</td>
<td>9.6</td>
</tr>
<tr>
<td>Mean</td>
<td>188.0</td>
<td>169.4</td>
<td>18.6</td>
<td>9.9</td>
</tr>
<tr>
<td>SD</td>
<td>7.3</td>
<td>6.2</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

\(^a\)Animals were treated with a single injection of TMPD at 0 (vehicle control) or 40 \(\mu\)mol/kg and were maintained individually in metabolism cages for 24 h for the collection of urine samples, and were then autopsied at 24 h post-dosing.

\(^b\)Loss in weight as a percentage of weight at 0 h.
Table 6.7A. Experiment 2, 24 h 2,3,5,6-tetramethyl-p-phenylenediamine study; urine clinical chemistry results in control male Hanover Wistar rats and animals treated with 2,3,5,6-tetramethyl-p-phenylenediamine (TMPD) at 8 h post-dosing\textsuperscript{a,b,c}.

<table>
<thead>
<tr>
<th>TMPD dose (µmol/kg)</th>
<th>0</th>
<th>40</th>
</tr>
</thead>
<tbody>
<tr>
<td>Volume produced (mL)</td>
<td>5.28 (1.51)</td>
<td>4.16 (1.85)</td>
</tr>
<tr>
<td>pH</td>
<td>7.3 (0.8)</td>
<td>6.4 (0.9)</td>
</tr>
<tr>
<td>SG</td>
<td>1.015 (0.010)</td>
<td>1.023 (0.006)</td>
</tr>
<tr>
<td>Leukocytes</td>
<td>NEG</td>
<td>NEG</td>
</tr>
<tr>
<td>Nitrite</td>
<td>NEG</td>
<td>NEG</td>
</tr>
<tr>
<td>Urobilinogen</td>
<td>3.2 (0.0)</td>
<td>3.2 (0.0)</td>
</tr>
<tr>
<td>Protein</td>
<td>NEG</td>
<td>NEG</td>
</tr>
<tr>
<td>Blood</td>
<td>NEG</td>
<td>HAEM (130.0 [95.9])\textsuperscript{d}</td>
</tr>
<tr>
<td>Ketones</td>
<td>NEG</td>
<td>NEG</td>
</tr>
<tr>
<td>Bilirubin</td>
<td>NEG</td>
<td>NEG</td>
</tr>
<tr>
<td>Glucose</td>
<td>NEG</td>
<td>NEG</td>
</tr>
<tr>
<td>TPC</td>
<td>183.52 (56.41)</td>
<td>145.58 (62.94)</td>
</tr>
<tr>
<td>CREAT</td>
<td>3.49 (1.13)</td>
<td>4.62 (2.48)</td>
</tr>
</tbody>
</table>

\textsuperscript{a}Values are means, SD in parentheses; n=5 for control and TMPD-treated rats. Animals were treated with a single subcutaneous injection of TMPD at 0 (vehicle control) or 40 µmol/kg and maintained individually in metabolism cages for 24 h for the collection of urine.

\textsuperscript{b}Urine was collected from 0 to 8 h post-dosing.

\textsuperscript{c}The following parameters were assayed using dipstick analysis (Multistix, Chapter 2.9): leukocytes, nitrite, urobilinogen, protein, pH, blood (haemoglobin), specific gravity, ketones, bilirubin and glucose. Abbreviations and units: SG, specific gravity; leukocytes, number of cells/µL; urobilinogen, µmol/L; protein, g/L; blood, number of erythrocytes/L; ketones, mmol/L; glucose, mmol/L; TPC, corrected total protein, mg/mmol; CREAT, creatinine, mmol/L; NEG, negative. Where a parameter is recorded as NEG, the urine samples for all animals in the group were negative.

\textsuperscript{d}Haemolysis positivity (HAEM) is expressed as the group mean ( ) and SD [ ] of the number of erythrocytes/L; positivity was recorded in 5 of the 5 animals in the group.
Table 6.7B. Experiment 2, 24 h 2,3,5,6-tetramethyl-p-phenylenediamine study; urinary heart fatty acid-binding protein (H-FABP) results in individual control male Hanover Wistar rats and animals treated with 2,3,5,6-tetramethyl-p-phenylenediamine (TMPD) at 8 h post-dosing\textsuperscript{a,b,c}.

<table>
<thead>
<tr>
<th>Rat number</th>
<th>Dose of TMPD (μmol/kg)</th>
<th>Rat number</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>4000</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>7.31</td>
<td>6</td>
</tr>
<tr>
<td>2</td>
<td>2.80</td>
<td>7</td>
</tr>
<tr>
<td>3</td>
<td>1.98</td>
<td>8</td>
</tr>
<tr>
<td>4</td>
<td>5.30</td>
<td>9</td>
</tr>
<tr>
<td>5</td>
<td>12.89</td>
<td>10</td>
</tr>
</tbody>
</table>

Mean: 6.06
SD: 4.36

188.00 (30.0)\textsuperscript{c}
2334.60 (384.2)
42.95 (6.1)
487.06 (79.4)
11.06 (0.8)

612.73 [100.1]\textsuperscript{d}
980.78

\textsuperscript{a}Individual animal results and means and SD are presented; n=5 for both groups. Rats were treated with a single subcutaneous injection of TMPD at 0 (vehicle control) or 40 μmol/kg at 0 h and maintained individually in metabolism cages for 24 h for the collection of urine. Animals were autopsied at 24 h post-dosing.

\textsuperscript{b}Urine was collected from 0 to 8 h post-dosing.

\textsuperscript{c}Values of H-FABP are measured in ng/mL.

\textsuperscript{d}{} indicates the “fold increase” of an individual value over the mean control group value.

\textsuperscript{e}[] indicates the “fold increase” of a group mean value over the mean control value.
Table 6.7C. Experiment 2, 24 h 2,3,5,6-tetramethyl-p-phenylenediamine study; urine clinical chemistry results in control male Hanover Wistar rats and animals treated with 2,3,5,6-tetramethyl-p-phenylenediamine (TMPD) at 16 h post-dosing\(^a,b\).

<table>
<thead>
<tr>
<th>TMPD dose (μmol/kg)</th>
<th>0</th>
<th>40</th>
</tr>
</thead>
<tbody>
<tr>
<td>Volume produced (mL)</td>
<td>10.64 (3.04)</td>
<td>12.36 (8.28)</td>
</tr>
<tr>
<td>pH</td>
<td>6.6 (0.4)</td>
<td>6.3 (0.4)</td>
</tr>
<tr>
<td>SG</td>
<td>1.012 (0.006)</td>
<td>1.016 (0.010)</td>
</tr>
<tr>
<td>Leukocytes</td>
<td>NEG</td>
<td>NEG</td>
</tr>
<tr>
<td>Nitrite</td>
<td>NEG</td>
<td>NEG</td>
</tr>
<tr>
<td>Urobilinogen</td>
<td>3.2 (0.0)</td>
<td>3.2 (0.0)</td>
</tr>
<tr>
<td>Protein</td>
<td>NEG</td>
<td>NEG</td>
</tr>
<tr>
<td>Blood</td>
<td>NEG</td>
<td>HAEM (117.0 [79.0])(^c)</td>
</tr>
<tr>
<td>Ketones</td>
<td>NEG</td>
<td>NEG</td>
</tr>
<tr>
<td>Bilirubin</td>
<td>NEG</td>
<td>NEG</td>
</tr>
<tr>
<td>Glucose</td>
<td>NEG</td>
<td>NEG</td>
</tr>
<tr>
<td>TP:CREAT</td>
<td>233.84 (86.54)</td>
<td>247.37 (31.59)</td>
</tr>
<tr>
<td>CREAT</td>
<td>1.35 (0.77)</td>
<td>2.07 (2.09)</td>
</tr>
</tbody>
</table>

\(^a\)Details of TMPD administration, and abbreviations and units, and other details are presented in Table 6.7A.

\(^b\)Urine was collected from 8 to 16 h post-dosing.

\(^c\)Haemolysis positivity (HAEM) is expressed as the group mean ( ) and SD [ ] of the number of erythrocytes/L; positivity was recorded in 5 of the 5 animals in the group.
Table 6.7D. Experiment 2, 24 h 2,3,5,6-tetramethyl-p-phenylenediamine study; urinary heart fatty acid-binding protein (H-FABP) results in individual control male Hanover Wistar rats and animals treated with 2,3,5,6-tetramethyl-p-phenylenediamine (TMPD) at 16 h post-dosing$^{a,b,c}$. 

<table>
<thead>
<tr>
<th>Rat number</th>
<th>TMPD dose ($\mu$mol/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
</tr>
<tr>
<td>1</td>
<td>10.69</td>
</tr>
<tr>
<td>2</td>
<td>1.10</td>
</tr>
<tr>
<td>3</td>
<td>3.66</td>
</tr>
<tr>
<td>4</td>
<td>2.04</td>
</tr>
<tr>
<td>5</td>
<td>4.95</td>
</tr>
<tr>
<td>Mean</td>
<td>4.49</td>
</tr>
<tr>
<td>SD</td>
<td>3.77</td>
</tr>
</tbody>
</table>

$^a$Further details of TMPD administration are found in Table 6.7B.

$^b$Urine was collected from 8 to 16 h post-dosing.

$^c$Values of H-FABP are measured in ng/mL.

$^d${ } indicates the “fold increase” of an individual value over the mean control group value.

$^e$[ ] indicates the “fold increase” of a group mean value over the mean control value.
Table 6.7E. Experiment 2, 24 h 2,3,5,6-tetramethyl-p-phenylenediamine study; urine clinical chemistry results in control male Hanover Wistar rats and animals treated with 2,3,5,6-Tetramethyl-p-phenylenediamine (TMPD) at 24 h post-dosing\textsuperscript{a,b}.

<table>
<thead>
<tr>
<th>TMPD dose (µmol/kg)</th>
<th>0</th>
<th>40</th>
</tr>
</thead>
<tbody>
<tr>
<td>Volume produced (mL)</td>
<td>2.46 (0.67)</td>
<td>3.42 (2.92)</td>
</tr>
<tr>
<td>pH</td>
<td>5.0 (0.0)</td>
<td>6.0 (0.7) *</td>
</tr>
<tr>
<td>SG</td>
<td>1.026 (0.004)</td>
<td>1.027 (0.007)</td>
</tr>
<tr>
<td>Leukocytes</td>
<td>NEG</td>
<td>NEG</td>
</tr>
<tr>
<td>Nitrite</td>
<td>NEG</td>
<td>NEG</td>
</tr>
<tr>
<td>Urobinogen</td>
<td>3.2 (0.0)</td>
<td>3.2 (0.0)</td>
</tr>
<tr>
<td>Protein</td>
<td>NEG</td>
<td>NEG</td>
</tr>
<tr>
<td>Blood</td>
<td>NEG</td>
<td>HAEM (117.0 [79.0])\textsuperscript{c}</td>
</tr>
<tr>
<td>Ketones</td>
<td>NEG</td>
<td>NEG</td>
</tr>
<tr>
<td>Bilirubin</td>
<td>NEG</td>
<td>NEG</td>
</tr>
<tr>
<td>Glucose</td>
<td>NEG</td>
<td>NEG</td>
</tr>
<tr>
<td>TP:CREAT</td>
<td>166.68 (69.47)</td>
<td>226.57 (27.85)</td>
</tr>
<tr>
<td>CREAT</td>
<td>4.14 (0.81)</td>
<td>4.37 (1.75)</td>
</tr>
</tbody>
</table>

\textsuperscript{a}Details of TMPD administration, abbreviations and units, and other details are presented in Table 6.7A.

\textsuperscript{b}Significantly different from control, P<0.05.

\textsuperscript{c}Urine was collected from 16 to 24 h post-dosing.

\textsuperscript{c}Haemolysis positivity (HAEM) is expressed as the group mean ( ) and SD [ ] of the number of erythrocytes/L; positivity was recorded in 5 of the 5 animals in the group.
Table 6.7F. Experiment 2, 24 h 2,3,5,6-tetramethyl-p-phenylenediamine study; urinary heart fatty acid-binding protein (H-FABP) results in individual control male Hanover Wistar rats and animals treated with 2,3,5,6-tetramethyl-p-phenylenediamine (TMPD) at 24 h post-dosing\textsuperscript{a,h,c}.

<table>
<thead>
<tr>
<th>Rat number</th>
<th>TMPD dose (μmol/kg)</th>
<th>Rat number</th>
<th>TMPD dose (μmol/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>13.52</td>
<td>6</td>
<td>1068.30 (69.1)\textsuperscript{d}</td>
</tr>
<tr>
<td>2</td>
<td>9.47</td>
<td>7</td>
<td>5000.00 (327.1)</td>
</tr>
<tr>
<td>3</td>
<td>14.56</td>
<td>8</td>
<td>161.93 (9.6)</td>
</tr>
<tr>
<td>4</td>
<td>17.37</td>
<td>9</td>
<td>1159.05 (77.1)</td>
</tr>
<tr>
<td>5</td>
<td>21.29</td>
<td>10</td>
<td>105.38 (5.9)</td>
</tr>
<tr>
<td>Mean</td>
<td>15.24</td>
<td></td>
<td>1498.93 (97.3)\textsuperscript{e}</td>
</tr>
<tr>
<td>SD</td>
<td>4.41</td>
<td></td>
<td>2017.92</td>
</tr>
</tbody>
</table>

\textsuperscript{a}Further details of TMPD administration are found in Table 6.7B.

\textsuperscript{b}Urine was collected from 16 to 24 h post-dosing.

\textsuperscript{c}Values of H-FABP are measured in ng/mL.

\textsuperscript{d}{} indicates the “fold increase” of an individual value over the mean control group value.

\textsuperscript{e}[] indicates the “fold increase” of a group mean value over the mean control value.
Table 6.8. Experiment 2, 24 h 2,3,5,6-tetramethyl-p-phenylenediamine study; serum clinical chemistry results in control male Hanover Wistar rats and animals treated with 2,3,5,6-Tetramethyl-p-phenylenediamine (TMPD) at 24 h post-dosing\textsuperscript{a,b}.

<table>
<thead>
<tr>
<th>TMPD dose (μmol/kg)</th>
<th>0</th>
<th>40</th>
</tr>
</thead>
<tbody>
<tr>
<td>cTnl</td>
<td>&lt;0.010 (0.000)</td>
<td>&lt;0.010 (0.000)</td>
</tr>
<tr>
<td>H-FABP</td>
<td>1.192 (0.429)</td>
<td>11.228 (6.980) *</td>
</tr>
<tr>
<td>CK</td>
<td>1060.0 (474.9)</td>
<td>1203.4 (667.2)</td>
</tr>
<tr>
<td>LD</td>
<td>2900.8 (998.3)</td>
<td>2189.8 (736.5)</td>
</tr>
<tr>
<td>Aldolase</td>
<td>61.8 (15.8)</td>
<td>882.0 (438.2) *</td>
</tr>
</tbody>
</table>

\textsuperscript{a}Values are means, SD in parentheses; n=5 for all groups. Rats were treated with a single subcutaneous injection of TMPD at 0 (vehicle control) or 40 μmol/kg at 0 h and maintained individually in metabolism cages for 24 h for the collection of urine. Animals were autopsied at 24 h post-dosing. *Significantly different from control, P<0.05.

\textsuperscript{b}Abbreviations and units: cTnl, cardiac troponin I, μg/L; H-FABP, heart fatty acid-binding protein, ng/mL; CK, creatine kinase, U/L; LD, lactate dehydrogenase, U/L; aldolase, U/L.
Table 6.9. Experiment 2, 24 h 2,3,5,6-tetramethyl-p-phenylenediamine study; incidence of microscopic findings in the skeletal muscles of control (vehicle-treated) male Hanover Wistar rats and rats treated with 2,3,5,6-Tetramethyl-p-phenylenediamine (TMPD)*.

<table>
<thead>
<tr>
<th>Microscopic finding</th>
<th>Severity grade</th>
<th>TMPD dose (µmol/kg)</th>
<th>Number of rats in each group with histological changes</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>0</td>
<td>40</td>
</tr>
</tbody>
</table>

**Gastrocnemius**

- Single fibre necrosis
  - NAD\(^5\): 5
  - Minimal: -
  - Mild: -
  - Moderate: -
  - Marked: -

- Myofibre degeneration
  - NAD: 5
  - Minimal: -
  - Mild: -
  - Moderate: -
  - Marked: -

**Quadriceps femoris**

- Single fibre necrosis
  - NAD: 5
  - Minimal: -
  - Mild: -
  - Moderate: -
  - Marked: -

- Myofibre degeneration
  - NAD: 5
  - Minimal: -
  - Mild: -
  - Moderate: -
  - Marked: -

**Soleus**

- Single fibre necrosis
  - NAD: 5
  - Minimal: -
  - Mild: -
  - Moderate: -
  - Marked: -

- Myofibre degeneration
  - NAD: 5
  - Minimal: -
  - Mild: -
  - Moderate: -
  - Marked: -

\(^*\)n=5 for both groups. Rats were treated with a single subcutaneous injection of TMPD at 0 (vehicle control) or 40 µmol/kg at 0 h and placed individually in metabolism cages for 24 h for the collection of urine. Animals were autopsied at 24 h post-dosing. During maintenance in metabolism cages, rats had access to water but not diet. \(^5\)NAD= no abnormalities detected.
CHAPTER 7: STUDIES ON THE CARDIOTOXICITY OF
ALLYLAMINE

7.1 INTRODUCTION

7.1.1 Background

Allylamine (AA; 3-aminopropene) is a highly reactive primary amine used in the production of polymers and in the synthesis of pharmaceutical agents (Boor et al., 1980). A number of AA analogues are in development as therapeutic agents or are currently in use, e.g. Lamisil (active ingredient terbinafine, Novartis), an anti-fungal agent for human and veterinary use (Ramos et al., 2001).

In man, AA is a severe respiratory, eye and skin irritant (United States Environmental Protection Agency, 2000). Hine et al. (1960) demonstrated that exposure of male Long Evans rats for 7 h at concentrations from 5 to 40 ppm caused irritation to the mucous membranes of the eye and upper respiratory tract, a bloody nasal discharge, and in severe cases, alveolar haemorrhage and acute pulmonary oedema. Topical exposure of the compound to male New Zealand rabbits for several h resulted in local erythema and oedema, progressing to eschar formation lasting several weeks. Application of AA to the eyes of rabbits caused moderate eye irritation at a dose level of 2.5 ppm. Eppinger et al. (1934) were the first group to observe the ability of AA administered systemically to produce vascular lesions. Intravenous (IV) administration of the compound to dogs resulted in liver oedema, pancreatic degeneration and some vascular alterations. Administration of allyl formate, a chemically related compound, to dogs lowered blood pressure and produced irregular thickening of the mitral valves (Eppinger et al., 1934). However, AA is also reported to cause endocarditis, myocarditis and cardiac necrosis in addition to vascular alterations (Hine et al., 1960; Guzman et al., 1961; Boor et al., 1979). Therefore, the main histopathological alterations observed in the heart following AA administration can be grouped into 2 categories: myocardial and vascular changes.

Occupational exposure to AA is generally by inhalation (United States Environmental Protection Agency, 2000), but the compound shows cardiovascular toxicity in experimental animals when it is administered by various routes, such as the oral route (gavage, in the food and in the drinking water) and the IV route (Lalich, 1969; Boor and
Hysmith, 1987). An established method of inducing AA toxicity is by the oral route, either by gavage as a single dose or using repeat dosing in short term investigations, or in the drinking water for studies with longer time courses (Boor et al., 1979; Boor and Ferrans, 1985).

7.1.2 Myocardial changes in allylamine-induced cardiotoxicity

Studies looking at the development of myocardial changes with AA may be short term or long term. The length of the studies will determine the histopathological changes observed (Boor et al., 1979; Boor et al., 1980). Short term (i.e. acute, one or two doses) studies tend to employ the gavage route for compound administration, whereas the long term investigations use protocols where AA is added to the drinking water, e.g. where the compound has been administered for 1 to 7 days (Boor and Ferrans, 1982) and for 21 to 104 days (Boor et al., 1979).

7.1.2.1 Short term gavage studies

AA cardiovascular toxicity is characterised by an acute myocardial necrosis, which appears within 24 h of (gavage) compound administration (Conklin et al., 1999). Boor et al. (1979) administered a single dose of AA in aqueous solution to male Sprague Dawley rats by gavage at dose levels of 50, 100 and 150 mg/kg, and killed animals at 24 h post-dosing. Acute focal cardiac lesions were observed at the 2 higher dose levels, with myocardial necrosis present in 4 of 7 rats at the 150 mg/kg dose level and in 2 of 4 rats at 100 mg/kg. However, animals in the 150 mg/kg dose group did not survive to the 24 h time point, and died between 12 and 20 h post-dosing. The focal acute subendocardial necrosis was characterised by increased myofibre eosinophilia, myofibre vacuolation and lysis, and there were some areas of coagulation necrosis with interstitial haemorrhage. Boor and Ferrans (1982) administered AA in water to male Sprague Dawley rats by gavage at 100 mg/kg for 1 or 2 days (i.e. 1 or 2 doses). At 24 h (following 1 dose of AA), focal subendocardial interstitial oedema and increased numbers of interstitial cells were present. In rats given 2 doses of AA and killed 24 h later, acute subendocardial necrosis was present. The necrosis was characterised by focal myocardial degeneration, with the accumulation of lipid droplets and contraction band necrosis. There was also infiltration by macrophages and inflammatory cells. The myocardial interstitial cells exhibited frequent mitoses.
7.1.2.2 *Long term drinking water studies*

In a study by Boor *et al.* (1979), male Sprague Dawley rats were treated with AA in the drinking water at concentrations of 0.005, 0.05 or 0.1 % for 21 to 104 days. In a separate experiment, rats were treated with AA in the drinking water at a concentration of 0.1 % for 2 to 36 days. Myocardial necrosis, as described above, was seen after 4 days of AA consumption. Following longer periods of AA administration (e.g. for 21 days), the main lesion observed was subendocardial fibrosis, with associated neutrophil and macrophage infiltration. In another report, Boor *et al.* (1980) administered AA in the drinking water at 0.1 % for up to 3 months. Myocardial necrosis (as described above) was the earliest histological change, seen after 4 to 8 days of compound treatment. Up to 21 days, transmural subendocardial scarring was present, mostly affecting the apex and left ventricle of the heart. From 21 days to 3 months, extensive transmural fibrosis, often observed macroscopically as a ventricular aneurysm, was the predominant lesion seen. Occasionally, mural thrombi were also evident.

7.1.3 *Vascular changes in allylamine-induced cardiotoxicity*

Vascular changes in the heart following AA administration occur after the appearance of acute myocardial necrosis (Guzman *et al.*, 1961; Boor *et al.*, 1979; Boor and Ferrans, 1982). Boor *et al.* (1979) administered AA in the drinking water at a concentration of 0.1 %. Myocardial lesions were initially present after 4 days of AA treatment, whereas vascular alterations were first observed after 21 days. The vascular lesions were located in areas of extensive myocardial necrosis/fibrosis and were characterised by thickening of the blood vessel media, and hyalinisation affecting the small intra-myocardial vessels. Vascular lesions away from the fibrotic areas also occurred, including some located in the large coronary arteries at the base of the heart, but after a longer period of AA exposure (e.g. after 81 days of administration). Boor *et al.* (1980) also demonstrated the appearance of vascular lesions after 21 days of treatment with 0.1 % AA in the drinking water. Lesions were most common in the fibrotic areas of the myocardium, and included medial hypertrophy and intimal smooth muscle cell (SMC) proliferation. There was also some occlusion of the smaller arteries, but no evidence of vascular thrombosis.

Coronary artery hyalinosis was a histological alteration observed when AA was fed to Sprague Dawley rats in the diet for periods up to 210 days; the dose level was 20 to 40
ml of 5% AA in corn oil/kg diet (Lalich, 1969). Focal hyalinosis was located mainly in the proximal (rather than the distal) coronary arteries and there was also evidence of SMC hyperplasia. In severe cases, the coronary arteries also showed adventitial collagen spreading into the media of the vessels. Plaque formation was present in the intima of the aortic arch in some cases, with thickening of the media and the presence of SMC hyperplasia. SMC hyperplasia was also seen in Long Evans rats fed AA at a dose level of 2 g/kg diet for 84 to 281 days (Lalich et al., 1972).

In dogs administered 15 mg/kg AA IV as 1-3 daily doses, Waters (1948) observed lesions mainly in the small- and medium-sized coronary arteries. Lesions were composed of areas of vessel necrosis, with hyalinisation and proliferation of SMCs, and haemorrhage in the media and adventitia. Saito (1976) administered 15 mg/kg AA IV to male and female dogs in 10 injections at intervals of 3 or 4 days. No changes in coronary blood flow or coronary vascular resistance were observed; therefore AA did not cause haemodynamic alterations in this study.

7.1.4 Suggested mechanisms of allylamine toxicity

There are 2 main hypotheses on how AA causes myocardial necrosis: (1) directly through damage to the myocardium by AA or the metabolites of AA; or (2) indirectly via the induction of coronary artery vasospasm resulting in subendocardial ischaemia (Lalich et al., 1972; Boor et al., 1979; Conklin and Boor, 1998).

In vitro and in vivo metabolism studies suggest that AA is oxidatively deaminated by a semicarbazide-sensitive amine oxidase in the blood vessel wall to form the highly reactive metabolite, acrolein (Nelson and Boor, 1982; Boor et al., 1987). Acrolein is known to disrupt the thiol (-SH) balance of cells, denature proteins and interfere with nucleic acid synthesis (Ramos et al., 2001). In addition, oxidative deamination results in the production of hydrogen peroxide. Hydrogen peroxide may be converted to hydroxyl radicals (.OH) via the Fenton reaction (Timbrell, 2002). This in turn may lead to lipid peroxidation and oxidative stress. Awasthi and Boor (1994) administered AA as a single gavage dose at 150 mg/kg to male Sprague Dawley rats, and sampled animals at 1, 3 and 5 h post-dosing. In the aorta, epicardium and endocardium, there was a marked depletion of total and free -SH content. In addition, AA treatment resulted in an increased .OH production in the aorta, with lesser increases in the epicardium and
endocardium. These findings suggest a mechanism of AA-induced cardiotoxicity due to acrolein-induced lipid peroxidation. Conklin and Boor (1998) showed that in vivo administration of AA (100 mg/kg) for 10 days caused a subtle increase in heart rate (i.e. a positive chronotropic effect). In addition, acrolein is known to have some sympathomimetic actions (Egle and Hudgins, 1974). Therefore, the resultant positive chronotropic effects may contribute to the development of myocardial necrosis through hypoxic and ischaemic mechanisms. AA does not cause hypotension (Conklin and Boor, 1998), so hypotension is therefore not part of the mechanism of hypoxia and ischaemia. Biagini et al. (1990) demonstrated in vitro that both AA and acrolein inhibit electron transport in mitochondria isolated from rat hearts, with acrolein being the more potent inhibitory compound for the oxidation of glutamate, malate and malonate, however there was no difference in the level of inhibition of succinate oxidation by the 2 compounds. The addition of an uncoupling agent did not reverse the inhibition of mitochondrial electron transport, indicating that AA and acrolein inhibit electron transport with little direct effect on the coupling mechanism.

Conklin and Boor (1998) investigated the effects of AA on rat aortic rings in vitro. AA at 1 μM to 1 mM induced concentration-dependent, long-lasting contractions and phasic tension oscillations in the aortic rings. Therefore, these results suggest a direct vasospasm effect of AA on the vasculature. Vasospasm in vivo may, in turn, cause an ischaemic event, resulting in myocardial necrosis.

However, although AA toxicity is associated with vasculotoxicity (Lalich et al., 1972), the acute myocardial necrosis induced by AA appears before the development of morphologically evident vascular alterations (Guzman et al., 1961; Boor et al., 1979; Boor and Ferrans, 1982). Therefore, the myocardial necrosis caused by direct cardiomyocyte damage by AA or the metabolites of AA may be the major cause of AA-induced cardiotoxicity.

7.1.5 Effects on clinical chemistry parameters

Only one study has been identified in the literature that investigated clinical chemistry parameters in response to AA administration. Boor et al. (1979) administered AA as a single gavage dose at 50 and 100 mg/kg to male Sprague Dawley rats and killed animals at 24 h post-dosing. The serum CK activity was shown to be raised to 260 % above
control values for the 50 mg/kg group (P<0.002) and to 180 % above controls for the 100 mg/kg group (not significant).

7.1.6 Allylamine toxicity to other target organs

The first histopathological study on AA, which was carried out by Eppinger et al. (1934), showed that IV administration of the compound in the dog resulted in liver oedema, gastric submucosal oedema and degeneration of the pancreas, in addition to cardiovascular changes. Gavage administration of AA at 20, 40, 80 and 100 mg/kg to mice resulted in gross changes, namely gastrointestinal tract congestion, discoloured livers and pale spleens, but no histological evidence of tissue injury (Hine et al., 1960). AA exposure has also been shown to induce diuresis with a concurrent increase in water intake in rats treated with a single gavage dose of AA at 100 mg/kg (Conklin et al., 1999); a significant increase in sodium ion excretion was also observed in AA-treated rats in this study. Boor (1985) demonstrated that the kidney was the primary excretory organ in rats treated with a single gavage dose of AA at 150 mg/kg. It was also shown in this study that no histopathological abnormalities were present in the kidneys of AA-treated rats. Lalich (1969) demonstrated that arterial nephrosclerosis was evident in male and female Sprague Dawley rats fed AA at a dose level of 20 to 40 ml of 5 % AA in corn oil/kg diet for periods from 132 up to 300 days, however the incidence of the lesion was rare. Hyalinosis of the mesenteric, pancreatic and testicular arteries was also observed in this study, but again these changes were rare. Lalich (1969) showed also that pulmonary oedema in association with the thickening of the pulmonary arteries was observed in 5 of 8 Sprague Dawley rats following AA feeding at a concentration of 30 ml of 5 % AA in corn oil/kg diet for 28 days.

7.1.7 Allylamine toxicity in other species

The studies mentioned above have reported AA-induced toxicity in the dog, the rat and the mouse, and Boor and Hysmith (1987) pointed out that AA demonstrated cardiovascular effects when administered by several different routes in the rat, the mouse and the rabbit. Studies investigating the myocardial and vascular effects of AA have also been performed in the turkey (Simpson, 1982), the calf (Boor and Hysmith, 1987) and the monkey (Guzman et al., 1961).
7.1.8 Allylamine toxicity in man

A case report by the Shell Oil Company in which workers were exposed to 0.2 ppm AA for 3 to 4 h a day showed that AA exposure at this concentration was not associated with worker detection or symptom reporting, but exposure to higher (undefined) concentrations by workers resulted in mucous membrane irritation (United States Environmental Protection Agency, 2000).

7.1.9 Aims of the present studies

The aims of the present studies were to assess the toxicity of AA on the rat myocardium following a single dose of the compound administered by gavage. We also wished to define the time window of cardiac injury using histopathological examination and serum clinical chemistry analysis; there are no reports in the literature on serum clinical chemistry changes in response to AA administration, apart from the response of serum total CK investigated by Boor et al. (1979) in the rat. In this way, it would be possible to study the time course of cardiac troponin I (cTnI) release, and also investigate the usefulness of heart fatty acid-binding protein (H-FABP) as a new serum marker in the diagnosis of cardiac injury.

A preliminary (abstract) report on these investigations has been published (Brady et al., 2006).
7.2 MATERIALS AND METHODS

7.2.1 Experimental protocols

7.2.1.1 Experiment 1; dose ranging study, 25 – 200 mg/kg

OBJECTIVE: To investigate whether myocardial lesions can be induced by a single gavage administration of allylamine at dose levels from 25 to 200 mg/kg.

7.2.1.1.1 Animal handling

Aqueous allylamine (AA) solutions were prepared (as described in Chapter 2.4.3) and a single dose administered by gavage to male Hanover Wistar rats (mean weight 181.6 g) at dose levels of 0 (vehicle control; deionised water), 25, 50, 75, 100, 125, 150, 175 and 200 mg/kg (n=5/group). Diet and water was provided ad libitum to all groups. Each group of n=5 rats occupied 1 cage. Body weights, clinical signs and water consumptions were monitored each day (Chapter 2.1, Chapter 2.3). Animals were autopsied at 48 h post-dosing for serum clinical chemistry and histopathological investigations.

7.2.1.1.2 Sample handling

At autopsy, animals were killed by exsanguination under deep isoflurane anaesthesia (as described in Chapter 2.6) and hearts removed, weighed and placed in formalin fixative (Chapter 2.10). Serum was prepared and clinical chemistry analysis was performed on all animals in all groups (Chapter 2.8.2). Parameters measured were: urea, creatinine (CREAT), albumin, total protein (TP), sodium, potassium, calcium, chloride, alanine aminotransferase (ALT), aspartate aminotransferase (AST), total alkaline phosphatase (ALP), total creatine kinase (CK), total lactate dehydrogenase (LD), glutamate dehydrogenase (GLD), aldolase and cTnI. Levels of cTnI were measured on the Bayer ACS: 180S system. Sections of hearts were examined histologically from all animals in all groups. Heart lesions were scored for severity on a scale of 0 to 5: 0 = no abnormalities detected (NAD), 1 = minimal, 2 = mild, 3 = moderate, 4 = marked and 5 = very marked.
7.2.1.2 Experiment 2; dose ranging study, 200 – 350 mg/kg

OBJECTIVE: To investigate whether myocardial lesions can be induced by a single gavage administration of allylamine at dose levels up to 350 mg/kg.

7.2.1.2.1 Animal handling

Aqueous AA solutions were prepared (as described in Chapter 2.4.3) and administered to male Hanover Wistar rats (mean weight 171.8 g) as in Experiment 1 (dose ranging study, 25 – 200 mg/kg) at dose levels of 0 (vehicle control), 200, 250, 300 and 350 mg/kg (n=5/group). Each treatment group occupied 1 cage. Diet and water was provided ad libitum to all groups. Body weights, clinical signs and water consumptions were monitored each day (Chapter 2.1, Chapter 2.3). Animals were autopsied at 48 h post-dosing for serum clinical chemistry and histopathological investigations. An in-life blood sample (see Chapter 2.5) was taken at 24 h post-dosing to investigate the timing of cardiac injury as assessed by serum cTnI levels.

7.2.1.2.2 Sample handling

Sample handling (serum clinical chemistry and histological assessment) was as for Experiment 1 (dose ranging study, 25 – 200 mg/kg) except for the in-life 24 h blood sample. Here, serum was prepared and cTnI levels determined (Bayer ACS: 180S).

7.2.1.3 Experiment 3; time course study

OBJECTIVE: To investigate the time course of myocardial injury induced by allylamine.

7.2.1.3.1 Animal handling

An aqueous AA solution was prepared (as described in Chapter 2.4.3) and administered as a single dose by gavage to male Hanover Wistar rats (mean weight 184.8 g) at a dose level of 300 mg/kg; animals (n=5) were autopsied at 4, 8, 12, 18, 24, 30, 36 and 48 h post-dosing. Control (vehicle-treated; deionised water) animals were autopsied at 0 h.
(n=5). Each group of n=5 animals occupied 1 cage. Samples were prepared for serum clinical chemistry and histopathological investigations.

7.2.1.3.2 Sample handling

At autopsy, animals were killed by exsanguination under deep isoflurane anaesthesia (as described in Chapter 2.6) and hearts removed, weighed and placed in formalin fixative (Chapter 2.10). Serum clinical chemistry analysis was performed on all animals in all groups; parameters measured were cTnI (on the Bayer ACS: 180S system) and heart fatty acid-binding protein (H-FABP) (Chapter 2.8.2).

7.2.2 Statistical analysis

Data from all studies were analysed using one-way analysis of variance (ANOVA) followed by Dunnett’s test for post hoc multiple comparison. In cases of violation of the assumptions for parametric testing, the Kruskal-Wallis test was used in combination with Dunn’s post-test. Statistical analysis was performed using GraphPad Prism version 3.00 for windows (GraphPad software, San Diego, California, USA).
7.3 RESULTS

7.3.1 Experiment 1; dose ranging study, 25 – 200 mg/kg

OBJECTIVE: To investigate whether myocardial lesions can be induced by a single gavage administration of allylamine at dose levels from 25 to 200 mg/kg.

7.3.1.1 Clinical signs and body weight changes

Male Hanover Wistar rats were given a single gavage dose of AA at dose levels of 0 (vehicle control; deionised water), 25, 50, 75, 100, 125, 150, 175 and 200 mg/kg. Immediately after dosing, animals were examined and clinical observations recorded at 1, 3 and 6 h post-dosing in addition to the routine daily checks. At 1 and 3 h post-dosing, all animals in all AA treatment groups appeared to be very warm to the touch, with the majority of animals in each treatment group displaying nose, ears and paws that were pinker than in the water-treated controls. However, by 6 h post-dosing, all AA-treated animals had recovered and were normal in appearance.

During the 48 h post-dosing period, all animals in all groups remained healthy and no clinical signs of AA toxicity were observed. Body weights were determined daily over the 48 h period following AA administration adversely and there was evidence that AA administration affected body weight gain in a general dose-dependent fashion. For example, over the 48 h period, control animals increased in mean body weight from 174.0 to 192.2 g (18.2 g gain; a 10.5 % increase), and in the 25, 100 and 200 mg/kg AA groups the comparable data was 155.4 to 166.8 g (11.4 g; 7.3 % increase); 170.0 to 177.2 g (7.2 g; 4.2 % increase); and 201.0 to 202.6 g (1.6 g; 0.8 % increase), respectively.

7.3.1.2 Water consumption

There were no notable differences in water consumption between the AA treatment groups. The mean water consumption over the 48 h post-dosing period for the control (vehicle-treated animals) was 22.8 ml/rat/day, and for the 25, 100 and 200 mg/kg AA-treated groups the consumptions were 21.2, 22.1 and 23.2 ml/rat/day, respectively.
7.3.1.3 Serum clinical chemistry

Results are summarised in Table 7.1. Administration of AA at dose levels from 25 to 200 mg/kg did not, in general, significantly affect mean serum levels at 48 h post-dosing of urea, CREAT, albumin, TP, sodium, potassium, calcium, chloride, AST, total ALP, total CK, total LD, GLD or aldolase. However, at 48 h post-dosing, the mean serum activity of ALT was decreased (generally) in a dose-related fashion in all AA-treated groups (Figure 7.1). The decrease was significant in the 125, 175 and 200 mg/kg AA groups (P<0.001, P<0.05 and P<0.001, respectively); the reductions in the mean activities were to 66.8 %, 72.2 % and 62.3 % of the mean control level, respectively.

Mean serum levels of cTnl at 48 h post AA dosing tended to be increased above baseline control values (0.034 µg/L) at AA dose levels of 125 mg/kg and above (Table 7.2). The increases were dose-related. However, the only statistically significant increase was in the 200 mg/kg AA group (P<0.001). Here, the group mean level of 1.146 µg/L was a 32.7-fold increase over the mean control level of 0.034 µg/L.

7.3.1.4 Gross pathology and histopathology

At autopsy, no macroscopic abnormalities were observed in any of the AA treatment groups.

Administration of AA at dose levels of 25 to 200 mg/kg had no effect on relative heart weight at 48 h post-dosing. For example, the group mean relative heart weights in the control vehicle-treated group, and in the 25, 100 and 200 mg/kg AA groups were 3.984, 3.869, 3.822 and 3.717 g/kg, respectively.

The incidence of microscopic findings in the heart is summarised in Table 7.3. There were 2 treatment-related changes present in the hearts of animals treated with AA: myocardial degeneration/necrosis (Figure 7.2) and vasculopathy. Both lesions increased in incidence and in severity grading with increasing AA dose level. Table 7.3 shows that a proportion of animals dosed with AA at levels of 100 mg/kg and above showed acute degeneration and necrosis in the myocardium, and at these dose levels, lesions were graded according to severity as minimal to marked. Myocardial degeneration/necrosis was characterised by increased myofibre eosinophilia, and vacuolation and fragmentation of the myofibres was evident in association with macrophage and...
neutrophil infiltrates (Figure 7.2). These lesions occurred predominantly in the apex, the interventricular septum and the left ventricular free wall of the heart. Lesions were less frequent in the papillary muscle and the right ventricular free wall. The lesions mainly involved the inner portion of the myocardium surrounding the ventricle; however in a small number of cases, the lesions were transmural. James’ reticulin staining demonstrated the collapse of the reticulin framework of the myofibres in areas of necrosis. Interstitial oedema and the activation of spindle cells (probably fibroblasts) were also evident in association with myocardial necrosis.

Vasculopathy, graded in severity as minimal to mild (Table 7.3), and characterised by mural disorganisation, perivascular oedema and the nuclear enlargement of medial cells, was present in 1 of the 5 animals dosed at 100 mg/kg AA, 1 of 5 animals at 125 mg/kg AA, and in a proportion of animals dosed at 150 mg/kg or higher. The affected vessels were located in parts of the myocardium separate from the areas of necrosis.

7.3.2 Experiment 2; dose ranging study, 200 – 350 mg/kg

OBJECTIVE: To investigate whether myocardial lesions can be induced by a single gavage administration of allylamine at dose levels up to 350 mg/kg.

7.3.2.1 Clinical signs and body weight changes

Following AA dosing at 0 (vehicle control), 200, 250, 300 and 350 mg/kg, clinical observations were recorded at 1, 3 and 6 h post-dosing and daily thereafter. At 1 to 3 h post-dosing, animals in all AA treatment groups appeared to be particularly warm, as previously observed (Experiment 1; dose ranging study, 25 – 200 mg/kg); the ears, nose and paws of the animals were markedly pink. However, at 6 h post-dosing, all animals appeared normal.

During the 48 h post-dosing period, animals treated with levels up to 300 mg/kg AA remained healthy and no clinical signs of significant toxicity were observed. However, at 24 h post-dosing, 2 rats from the 350 mg/kg treatment group were found dead; samples were not taken from these animals. The remaining 3 rats in the group demonstrated some evidence of toxicity. These animals showed a reduced activity, loss
of condition and piloerection. However, the condition of these animals was generally acceptable and allowed survival to the 48 h autopsy time point.

Body weights were determined daily over the 48 h period following AA administration (Figure 7.3). Over this period, control animals increased in mean body weight by 4.3%; at 200 mg/kg AA, the mean increase was 2.3%. However, at the 250, 300 and 350 mg/kg AA dose levels, mean body weights were decreased, and at the 48 h time point the mean body weights of the animals in these groups were reduced to 94.1%, 97.3% and 94.0% of the group mean body weight at the time of dosing, respectively.

At the autopsy at 48 h post-dosing, the 3 animals in the 350 mg/kg AA dose group, on close external examination, showed that the animals were subdued and had lost condition. All 3 animals had piloerection, and were walking with an abnormal gait: they were walking on "tip-toe" with their hind legs, as if walking on a hot surface.

7.3.2.2 Water consumption

Treatment with AA affected (reduced) water consumption in a general dose-related fashion. The mean water consumption over the 48 h post-dosing period for the control (vehicle-treated animals) was 23.2 ml/rat/day, and for the 200, 250 and 300 mg/kg AA-treated groups the mean consumptions were 22.6, 15.7 and 16.5 ml/rat/day, respectively.

7.3.2.3 Serum clinical chemistry

At the 48 h post-dosing autopsy time point, the administration of AA by gavage at 200, 250, 300 or 350 mg/kg did not significantly affect mean serum levels of urea, CREAT, potassium, chloride, AST, GLD or aldolase (Table 7.4). Mean levels of albumin and TP were generally decreased in a dose-related fashion and the reductions became significant at 300 and 350 mg/kg AA for albumin (P<0.001 at both dose levels), and at 350 mg/kg for TP (P<0.001). Likewise, there were dose-related decreases in mean serum sodium and calcium concentrations, which achieved significance at the 350 mg/kg dose level for sodium (P<0.05) and at the 300 and 350 mg/kg dose levels for calcium (P<0.05 and P<0.001, respectively).
Mean serum ALT, total ALP, total CK and total LD all showed general dose-related trends of decreasing activities with increasing doses of AA, however the changes were not always consistent (Table 7.4). The group mean ALT activities were decreased at the 200, 250 and 300 mg/kg dose levels (NS at any dose level), however the group mean ALT activity in the 350 mg/kg AA group was very close to the mean control level. Decreases were statistically significant for ALP at 350 mg/kg (P<0.05) and for LD at dose levels of 300 mg/kg (P<0.001) and 350 mg/kg (P<0.05). The decreases for mean total CK did not achieve statistical significance at any dose level of AA.

In-life tail vein blood samples were taken at 24 h post-dosing and serum assayed for cTnI. Group mean cTnI levels were raised above mean control levels (<0.030 µg/L) in all AA dose groups (Table 7.5). However, none of the increases in mean serum cTnI levels were statistically significant. Also, there appeared to be no clear dose response relationship in the mean cTnI levels. Furthermore, there was considerable variability in the individual cTnI values at 24 h, for example, at 350 mg/kg AA, cTnI levels of <0.03 µg/L were recorded in 2 animals, whereas values of 2.50 and 3.81 µg/L were seen in individual animals at 200 and 250 mg/kg AA, respectively.

Mean serum levels of cTnI at the 48 h post-dosing autopsy time point tended to be increased at all AA dose levels, however the changes were variable and not consistent (Table 7.6). Within all AA groups, there were a number of animals with cTnI levels equal to the mean control level of <0.030 µg/L. For example, levels of cTnI were at the base line control value in 2 of 5 animals in the 200 mg/kg AA group, 3 of 4 animals at 250 mg/kg, 1 of 5 animals at 300 mg/kg, and 2 of 3 animals at 350 mg/kg. Compared with the 24 h in-life blood samples (Table 7.5), both mean and individual values of cTnI were generally lower at the 48 h autopsy time point, suggesting that levels were returning towards control base line figures. In addition, the highest individual cTnI value observed at 24 h was 3.81 µg/L in an animal dosed with 250 mg/kg AA (Table 7.5), whereas the highest individual cTnI at 48 h was 1.75 µg/L in the same animal (Table 7.6). The mean cTnI value at 24 h for all AA-treated animals (n=14) was 0.816 µg/L; at 48 h, the mean level for all AA-treated rats (n=17) was 0.274 µg/L. There appeared to be no relationship between cTnI concentrations and AA dose level at the 48 h autopsy sampling point.
There were no macroscopic abnormalities observed in the AA-treated rats at the 48 h autopsy time point.

Administration of AA increased the mean relative heart weight in all treated groups in a statistically significant fashion (Figure 7.4). However, there was no evidence of a dose-related effect. The mean increase in relative heart weight at 200 mg/kg AA was 20.1 % above the mean control weight (3.24 g/kg), whereas at 300 mg/kg the increase was 17.2 % above control.

The incidence of microscopic findings in the heart is summarised in Table 7.7. There were 2 treatment-related changes present in the heart of animals treated with AA: first, myocardial degeneration with necrosis, and second, vasculopathy, as was seen in Experiment 1 (dose ranging study, 25 – 200 mg/kg). The first lesion, myocardial degeneration and necrosis, was graded in severity as minimal to moderate across the 4 AA dose groups. The lesions were characterised as myofibre vacuolation and fragmentation, in conjunction with the homogenous appearance of muscle fibres; interstitial oedema was also present, as was the activation of fibroblasts, and infiltration with small numbers of macrophages and neutrophils. The second lesion, vasculopathy, was graded in severity as minimal or mild across the 4 AA dose level groups. This lesion was present in the small intramural arteries throughout the myocardium. In addition, 1 animal dosed at 200 mg/kg AA showed minimal disorganisation of the media of the pulmonary artery, and 1 animal at 300 mg/kg displayed arteritis with fibrinoid necrosis in an isolated vessel close to the aortic root. In contrast to the findings in Experiment 1 (dose ranging study, 25 – 200 mg/kg), there were no clear dose-related increases in the incidence and severity of the 2 histopathological lesions with increasing dose levels of AA.
7.3.3 Experiment 3; time course study

OBJECTIVE: To investigate the time course of myocardial injury induced by allylamine.

7.3.3.1 Clinical signs and body weight changes

Following AA dosing at 300 mg/kg, animals were checked and clinical observations recorded 2 to 3 times daily. At 10 min to 1 h after AA administration, animals appeared to be excessively warm as reported previously (Experiment 1; dose ranging study, 25 – 200 mg/kg; Experiment 2; dose ranging study, 200 – 350 mg/kg). The ears, nose and paws of AA-treated animals were pinker than usual. In addition during this time period (to 1 h post-dosing), AA-treated rats were subdued and non-responsive to stimuli; however all animals had recovered and appeared normal by 1 h post-dosing.

During the 48 h post-dosing period, animals treated with 300 mg/kg AA remained healthy and there were no clinical signs of toxicity. However, AA administration did cause body weight losses (Table 7.8). By the 18 h autopsy time point, all 5 AA-treated animals had lost weight in the 18 h autopsy group following AA dosing, and were on average 95.5 % of their starting (pre-dosing) weights. The 48 h AA treatment group were on average 90.4 % of their pre-dosing weight at the time of autopsy.

7.3.3.2 Serum clinical chemistry

The normal analysis of serum clinical biochemistry parameters (e.g. as set out in Table 7.1 and 7.5) was not carried out in this experiment; H-FABP and cTnl assays were conducted. AA administration resulted in a clear increase in the group mean serum H-FABP level at 12 h post-dosing (Figure 7.5). At this time point, the group mean H-FABP value was 3.975 ng/mL compared to a group mean value of 2.860 ng/mL for control (vehicle-treated; time “0”) rats. The increase at 12 h was therefore 39.0 % above the mean control level. H-FABP levels remained elevated above the controls at 18 h post-dosing (a group mean of 6.258 ng/mL), an increase of 118.8 %, and the level appeared to peak at this time point. However, after 18 h (i.e. at 24, 30, 36 and 48 h post-dosing), mean serum H-FABP levels were lower than the control values (mean H-FABP levels of 1.610, 1.606, 1.548 and 1.546 ng/mL, decreases to 56.3 %, 56.1 %, 54.1 % and
54.1% of the control level, respectively). The increases in serum H-FABP seen at 12 and 18 h post-dosing were not statistically significant, reflecting the variability of the data. Indeed, when individual H-FABP values were examined at these time points, only 3 individual animals have levels clearly above the control base line values; these are levels of 9.91 (12 h), 18.09 (18 h) and 8.29 (18 h) ng/mL.

At time "0" (i.e. the autopsy of the vehicle-dosed control animals at the beginning of the study) and at 4 h post-dosing, serum cTnI individual values, and group mean values were at normal (control) base line levels of <0.030 μg/L (Table 7.9). Group mean serum cTnI levels were first increased at 8 h post-dosing, to 0.118 μg/L. This increase in the group mean was due to 2 individual animals of the 5 assayed, which had increases in cTnI values to 0.38 and 0.12 μg/L. Group mean cTnI levels continued to increase at 12 h and 18 h post-dosing, to 2.618 and 3.218 μg/L, respectively. At these time points, 2 particularly high cTnI levels were recorded, a value of 8.89 μg/L (at 12 h) and a value of 13.70 μg/L (at 18 h). After 18 h, there was some evidence of a fall in serum cTnI values to a mean of 0.892 μg/L (at 24 h) and to 1.778 μg/L (at 30 h). The highest cTnI values at these time points were 3.57 μg/L (24 h) and 3.35 μg/L (30 h). However at 36 h post-dosing, there seemed to be some evidence a second cTnI peak, with a mean level of 5.208 μg/L. There were 2 particularly high individual cTnI values at the 36 h autopsy time point, 8.28 and 14.70 μg/L (Table 7.9); the latter value was the highest cTnI level recorded in the experiment (a fold increase of 489.0-fold over the control base line level). At 48 h post-dosing, the group mean serum cTnI level (0.294 μg/L) was still increased above the mean control level (<0.030 μg/L). Although the group mean cTnI levels were increased above the mean control level at all time points from 8 h post-dosing, no increases were statistically significant, reflecting the variability of the individual cTnI values and therefore the relatively high SDs about the means (Table 7.9).

7.3.3.3 Gross pathology and histopathology

At autopsy, there were no macroscopic external or internal abnormalities in any animals at any time point.

In the 48 h time period following the administration of AA, the group mean relative heart weights increased over time (Figure 7.6). These increases were statistically
significant at 30, 36 and 48 h post-dosing (P<0.001 at each time point). The mean relative weight of the hearts from the control (vehicle-treated; time “0”) animals was 3.282 g/kg and at 30, 36 and 48 h post-dosing, the mean relative heart weights were 3.948, 3.836 and 3.867 g/kg, increases of 20.3, 16.9 and 17.8 % above the mean control value, respectively.

The incidence of microscopic findings in the hearts is summarised in Table 7.10. There were 3 treatment-related changes in the hearts of animals treated with AA: first, myocardial degeneration with necrosis, second, vasculopathy, and third, interstitial oedema. Treatment-related changes were present in the myocardium and in the small intramural arteries. At 4 and 8 h post-dosing, the hearts of AA-treated animals appeared histologically similar to the vehicle-treated (time “0”) controls (Table 7.10). The first lesions became apparent at the 12 h post-dosing autopsy time point, where 2 of the 5 animals (number 8 and 24) showed evidence of minimal myocardial degeneration/necrosis, 1 (number 28) showed minimal vasculopathy, and 2 (number 7 and 24) showed mild interstitial oedema. The 3 lesion types then increased in incidence and severity grading at 18, 24, 30 and 36 h post-dosing. At 30, 36 and 48 h post-dosing, all animals showed the 3 types of histological alterations in response to AA administration. The initial necrosis first evident at 12 h post-dosing was characterised as coagulative or lytic change that was variably accompanied by a minimal neutrophil infiltrate. By 24 h post-dosing, macrophages could be observed in some lesions. Interstitial fibroblast activation was first seen at 30 h post-dosing, and increased in incidence up to 48 h. At 48 h post-dosing, the necrotic lesions were also accompanied by interstitial (fibroblast) mitotic figures and a mainly mononuclear inflammatory infiltrate. The accompanying interstitial oedema, graded as minimal to marked, was first seen at 12 h, and affected all animals by 30 h post-dosing (Table 7.10). The evidence of vasculopathy in the small mural arteries appeared as increased vessel permeability with degeneration, and perivascular inflammation and fibroplasia. From 12 h post-dosing, perivascular oedema and a mononuclear cell infiltrate was present. Degenerative changes to the media and adventitia were also evident from 18 h post-dosing. These histological alterations were characterised by swollen nuclei in the media cells, disorganisation of the media structure, and (rarely) fibrinoid necrosis (Figure 7.7). From 24 h onwards, there was increasing perivascular fibroblast activation characterised by increased numbers of plump fibroblasts with mitotic figures. By 48 h, these changes had resulted in adventitial thickening.
AA is a reactive amine used in the manufacture of polymers and pharmaceutical agents (Boor et al., 1980). The compound is a severe respiratory, eye and skin irritant in man and experimental animals (Hine et al., 1960; United States Environmental Protection Agency, 2000), but most importantly in the present context, the compound is a potent cardiovascular toxicant, causing both myocardial and vascular changes (Guzman et al., 1961). The main histopathological alterations that occur in the rat following AA administration are myocardial necrosis/fibrosis, and cardiac vascular lesions (Hine et al., 1960). Myocardial necrosis has been reported to occur within 24 h of compound administration by gavage, or by 4 to 6 days following administration in the drinking water (Boor et al., 1979; Boor and Ferrans, 1982). However the vascular lesions have been reported to take a longer period of time to develop and the induction of such lesions in the rat has been reported to occur after 21 days of compound administration in the drinking water (Boor et al., 1979; Boor et al., 1980). For this reason, in the present work, in studies with agents other than AA, where the time course of cardiac lesion development was unknown, our experimental designs have tended to involve an initial autopsy at 24 h post-dosing. However, in planning experiments with AA, it was decided to take autopsy samples at 48 h post-dosing (Experiments 1 and 2), as it was considered that this would allow the induction of degeneration/necrosis changes, and also vascular lesion development, during the period 24 to 48 h post-dosing.

In the present studies, a principal aim was to attempt to induce cardiotoxicity in the rat with a single gavage dose of AA. In addition, we wished to define the time course of cardiac injury using serum clinical chemistry analysis and histopathological examination. In Experiment 1 (dose ranging study, 25 – 200 mg/kg), several findings of interest were identified in the AA-treated animals. However, the dose levels of AA employed in the study were not considered to be optimal. The dose levels used in Experiment 1 were chosen as it was believed that 200 mg/kg AA was probably very close to the maximum tolerated dose in the rat, as Boor et al. (1979) had shown that in this species a dose level of 150 mg/kg was, in some cases, a lethal dose. However, the results of Experiment 1 suggested that dose levels above 200 mg/kg AA would be well tolerated and thus higher levels of the compound were tested in Experiment 2.
AA administration as a single gavage dose had no effect on the relative heart weights of rats in Experiment 1 (dose ranging study, 25 – 200 mg/kg). Nevertheless, relative heart weights have been shown to be increased in rats treated with 0.1 % AA in the drinking water for 21 and 36 days (Boor et al., 1979).

In Experiment 1, following the administration of a single dose of AA at levels from 25 to 200 mg/kg, dose-related decreases were seen in the levels of serum ALT (Figure 7.1), and at 125, 175 and 200 mg/kg the reductions were statistically significant. ALT levels are known to decrease when animals are nutritionally imbalanced, e.g. in times of fasting (Jenkins and Robinson, 1975). Treatment with AA in Experiment 1 adversely affected body weight gain; for example, the control (vehicle-treated, 0 mg/kg) group gained a mean weight of 18.2 g (a 10.5 % increase), whereas the 200 mg/kg AA-treated animals gained a mean of 1.6 g (a 0.8 % increase) in the 48 h period following AA administration.

Increases in serum total CK levels have often been used in studies by other workers as a marker of cardiotoxicity using agents other than AA (Wexler, 1970; Barrett et al., 1988; Ray et al., 2005), however no changes were seen in serum total CK levels in Experiment 1 (Table 7.1). Boor et al. (1979) demonstrated that serum CK activities were raised to 260 % and 180 % above control levels following the administration of single gavage doses of 50 and 100 mg/kg AA, respectively; furthermore the increase, to 260 % at 50 mg/kg, was statistically significant.

In Experiment 1, Table 7.2 shows evidence of a general trend for increasing individual cTnI values, and group mean cTnI values, with increasing dose levels of AA at 125 mg/kg and above. Similarly, Table 7.3 demonstrates a general trend that with increasing AA dose levels of 100 mg/kg and above, both the myocardial severity grade and the incidence of degeneration/necrosis increase. These features also apply to the vasculopathy findings. The relationship between cTnI values and myocardial degeneration/necrosis severity grades are further examined in individual animals in Table 7.11. Although the data is not robust, both cTnI values and degeneration/necrosis severity grades are available for 43 individual animals (including controls). Of these 43 rats, 26 have severity grades of 0 (no abnormalities detected) and these 26 animals have a mean cTnI value of 0.050 µg/L; 3 animals have a severity grade of 1 (minimal) and a mean cTnI value of 0.183 µg/L; 6 animals have a severity grade of 2 (mild) and a mean
cTnl value of 0.147 µg/L; 5 animals have a severity grade of 3 (moderate) and a mean cTnl value of 0.830 µg/L; 3 animals have a severity grade of 4 (marked) and a mean cTnl value of 0.973 µg/L. Furthermore, if the data is grouped, for example if the 9 animals with a severity grade of 1 or 2 are grouped they are seen to have a mean cTnl value of 0.159 µg/L, and if the 8 individual animals with a severity grade of 3 or 4 are grouped they are seen to have a mean cTnl value of 0.884 µg/L. In this way it is considered reasonable to suggest that there is some evidence of a general trend for increasing cTnl values in individual animals with higher degeneration/necrosis severity grades. However, it is appreciated that discrepancies between cTnl elevations and histopathological evidence of myocardial injury may be related to the 48 h point of autopsy sampling. Levels of cTnl are known to increase in the serum soon after microscopic evidence of damage to the cardiomyocyte becomes evident (Chapter 4, Time course study on the cardiotoxicity of isoproterenol; Chapter 5, Time course study on the cardiotoxicity of isoproterenol and the utility of heart fatty acid-binding protein as an early serum marker of cardiac injury). Therefore, it is possible that the serum cTnl increases induced in the present study (Table 7.2, 7.11) may have occurred earlier and peaked earlier than the 48 h autopsy time point. Also, at the lower doses (i.e. 100 and 125 mg/kg), the increases in serum cTnl may have returned to baseline levels by 48 h post-dosing. Therefore, Experiment 2 (dose ranging study, 200 – 500 mg/kg) was designed to give an indication of the time course of myocardial injury following AA administration, by taking a 24 h in-life blood sample to assess cTnl levels. In addition, higher dose levels of AA were used in Experiment 2 in an attempt to gain a clearer picture of the effects of compound administration.

In Experiment 2 (dose ranging study, 200 – 350 mg/kg), AA administration caused a decrease in group mean body weight at dose levels of 250 mg/kg and above at 24 h and 48 h post-dosing (Figure 7.3). In addition, differences were seen between the vehicle-treated control group and the AA-treated groups in the amount of water consumed (consumption was reduced in the AA-treated rats). This agrees with the findings of Boor et al. (1979). These workers showed that rats on a regime of 0.1 % AA in the drinking water for 21 days had reductions in body weight, in conjunction with a decrease in water consumption.

AA administration, in general, caused a dose-related decrease in serum albumin (P<0.001), TP (P<0.001), ALT (NS), total ALP (P<0.05), total CK (NS) and total LD
(P<0.05) values (Table 7.4; P values refer to data at 350 mg/kg). These decreases in blood protein and enzyme levels at 48 h post-dosing may be associated with a decreased nutritional status of the animals, as there was body weight loss following compound administration (Figure 7.3). There were also dose-related decreases in serum sodium and calcium concentrations (Table 7.4). This last result is in contrast to the work of Conklin et al. (1999) who demonstrated that when 100 mg/kg AA was administered as a single gavage dose in the rat, no changes were seen in serum levels of sodium at 24 h post-dosing. However, the report of Conklin et al. (1999) does describe an increase in urinary sodium excretion at 24 h post-dosing.

Relative heart weights were increased in all AA treated groups in Experiment 2 (Figure 7.4). This result is in agreement with the work of Boor et al. (1979), who showed increases in relative heart weights of rats administered AA at a concentration of 0.1 % in the drinking water for 21 days. However, no studies have been identified in the literature where the relative heart weights of rats have been investigated following a single gavage dose of AA.

In serum samples obtained “in-life” at 24 h post-dosing in Experiment 2, increased mean cTnI values were seen at all dose levels of AA (200, 250, 300 and 350 mg/kg) (Table 7.5). Raised mean cTnI values were also evident at 48 h post-dosing (Table 7.6). However, the mean cTnI values, at each AA dose level, were lower at 48 h post-dosing than at 24 h. Also, for 13 individual animals where data was available at both 24 and 48 h post-dosing, 9 showed a decrease in cTnI levels at 48 h, 3 showed the same level, and 1 animal showed an increased level; the mean cTnI value for the 13 animals was 0.874 μg/L at 24 h and 0.301 μg/L at 48 h post-dosing.

In Experiment 2 (dose ranging study, 200 – 350 mg/kg), at 48 h post-dosing, histopathological evidence of myocardial necrosis and vasculopathy was seen in all AA treatment groups (Table 7.7). As 2 animals died at the 350 mg/kg dose level, a level of 300 mg/kg AA was chosen for use in Experiment 3 (time course study).

In Experiment 3 (time course study), the time course of the development of cardiac injury was investigated from 4 to 48 h post-dosing. There appear to be no other reports in the literature where the histopathological changes in the heart have been investigated relatively soon after the administration of a single gavage dose of AA. In the current investigation, rats were given a single gavage dose of AA at 300 mg/kg and killed at 0,
4, 8, 12, 18, 24, 30, 36 and 48 h post-dosing. At approximately 18 h following AA administration, animals showed evidence of a loss in body weight (Table 7.8). The decrease in group mean autopsy body weights was significant at 48 h post-dosing. In addition, the mean relative heart weights began to increase from the 4 h sampling point and this effect continued throughout the post-dosing period (Figure 7.6). These increases in relative heart weight were significant at 30, 36 and 48 h post-dosing.

In Experiment 3, myocardial degeneration/necrosis in the AA-treated rats was initially observed in 2 animals at 12 h post-dosing, and the lesions were given a severity grading of "minimal" (Table 7.10). If individual animal degeneration/necrosis severity grading scores at 12 h post-dosing are compared with individual cTnI values (Table 7.12), it can be seen that these 2 animals had increases in serum cTnI levels, to 8.89 and 1.52 µg/L; these were the only 2 cTnI increases observed at this time point. Histopathological lesions of the 3 types identified (myocardial degeneration/necrosis, vasculopathy and interstitial oedema) increased in incidence and severity after the 12 h autopsy time point, until 30 h post-dosing (Table 7.10). From the 30 h time point, all 5 AA-treated animals at each time point showed a histopathological response to AA administration. An examination of the data in Table 7.12 shows that there is, in general, some correlation between serum cTnI levels and the development of myocardial degeneration/necrosis, on an individual animal basis. For example, the highest cTnI value observed in the study, 14.70 µg/L, was in an animal sampled at 36 h post-dosing. This animal had a myocardial degeneration/necrosis severity grade of 3, signifying a moderate lesion; this was the highest severity grade recorded in the present study.

At 8 h post-dosing in Experiment 3, 2 individual animals had raised cTnI concentrations of 0.38 and 0.12 µg/L (Table 7.9). However, there was no histological evidence of cardiac injury in these individual animals at this time point (Table 7.10) and therefore, the increases in cTnI may be due to leakage from the small cytoplasmic pool of cTnI, which is known to be present in the myofibre; this concept of a cytoplasmic pool was put forward by Bleier et al. (1998). It is generally considered that normally, the contractile apparatus-bound cardiac troponins are only released into the bloodstream when there is breakdown of both the intracellular myofibrils and the structure of the cardiomyocyte itself (Wallace et al., 2004). Therefore the positive increases in serum cTnI levels in the 2 rats at 8 h post-dosing, without evidence of a histological cardiac lesion, may lend support to the idea of an early cTnI release from a cytoplasmic pool.
Measurement of serum H-FABP (Figure 7.5, Table 7.12) gave additional information in this experimental model of AA-induced cardiac injury. Group mean H-FABP levels were increased above control (0 h) values (a mean level of 2.860 ng/mL) at 12 h post-dosing (a mean level of 3.975 ng/mL; NS) and at 18 h post-dosing (a mean level of 6.258 ng/mL; NS). However these increases (39.0 % at 12 h; 118.8 % at 18 h) were not statistically significant. Figure 7.5 appears to show the peak level of serum H-FABP was at 18 h post-dosing. The first increases in serum H-FABP in individual animals occurred at 8 h post-dosing, when 1 animal had a serum H-FABP concentration of 5.92 ng/mL (Figure 7.5; Table 7.12). This animal also had a raised serum cTnl level (0.38 µg/L; Table 7.9, 7.12); however the cardiac histology of this rat was normal (Table 7.10, 7.12). The first time point where the group mean serum H-FABP value appeared to be raised above control levels was at 12 h post-dosing. At this sampling time point, 1 animal showed an H-FABP level (9.91 ng/mL) above the base line control value. This individual animal also showed an increase in serum cTnl at this time point (8.89 µg/L; Table 7.9, Table 7.12). Furthermore, this rat demonstrated minimal histological evidence of myocardial degeneration/necrosis at this sampling point (Table 7.12), the earliest time of histopathological evidence of myocardial injury. Therefore, H-FABP, as with cTnl, did assist in confirming the time of onset of cardiac injury.

The histopathological changes in the myocardium observed in the present 3 experiments agree with previous reports in the literature. Boor et al. (1979) demonstrated the appearance of myocardial necrosis in the rat at 24 h post-dosing following a single gavage dose of AA at 100 and 150 mg/kg. No lesions were observed at 50 mg/kg AA. In Experiment 1 (dose ranging study, 25 – 200 mg/kg), 100 mg/kg was the lowest AA dose where myocardial necrosis was observed at the 48 h time point (Table 7.3). Experiment 3 (time course study) allowed the onset and development of pathological lesions to be identified and followed. There are no studies in the literature which report on the development of AA-induced lesions in time and where the very early changes occurring in the myocardium are discussed. All of the single dose studies which are reported use the 24 h time point as the first sampling occasion (Boor et al., 1979; Boor and Ferrans, 1982; Conklin et al., 1999). Nevertheless, these studies all show that histological changes are present in the myocardium at 24 h following compound administration. In the present study (Experiment 3), we have clearly shown that evidence of myocardial degeneration/necrosis may be identified as early as 12 h post-dosing (Table 7.10).
The type of myocardial injury that is observed following the administration of AA is very similar to that seen following the treatment with tachycardia-inducing drugs, such as isoproterenol (ISO). That is, increased myofibre eosinophilia, vacuolation and fragmentation of the myofibres in association with inflammatory cell infiltration. However, the temporal pattern of pathogenesis differs between the 2 compounds. Following ISO administration, histopathological evidence of myocardial injury may be observed as early as 10 min post-dosing (Chapter 5; Early time course study on the cardiotoxicity of isoproterenol investigating the utility of heart fatty acid-binding protein as a serum marker of cardiac injury). Following AA administration, the first histopathological changes were evident at 12 h post-dosing in 2 out of 5 AA-treated rats (Table 7.10, 7.12). ISO is a synthetic catecholamine and potent non-selective β-adrenoceptor agonist. Through the β effects of ISO (i.e. increase in heart rate, decrease in blood pressure), the drug induces ischaemia in the myocardium, leading to myocardial necrosis. Sklar et al. (1991) showed in vitro that AA has positive inotropic effects. Conklin and Boor (1998) went on to demonstrate that AA has positive inotropic effects in vivo in the rat. In addition, acrolein has been identified as having some sympathomimetic actions (Egle and Hudgins, 1974). Therefore, the main mechanism of AA-induced cardiotoxicity may be related to the induction of myocardial necrosis resulting from hypoxia and ischaemia. ISO is a potent β agonist, and causes immediate effects on the cardiovascular system following administration (Conway et al., 1968; Dollery, 1998). The mean plasma half-life for an increase in heart rate of ISO by IV injection in man was shown by Conolly et al. (1972) to be 29.1 seconds, demonstrating the rapid effects of the drug. The temporal delay in AA-induced cardiotoxicity in the present studies may be related to the metabolism of the compound following administration.

In the current studies, AA-induced vasculopathy was identified at dose levels of 100 mg/kg and above (Experiment 1; Table 7.3) and the lesion was first present at 12 h post-dosing (Experiment 3; Table 7.10). No previous studies on AA in the literature have reported vascular changes so soon after compound administration. According to the published reports, AA-induced acute myocardial necrosis appears much earlier than histological vascular alterations (Boor et al., 1979; Boor and Ferrans, 1982). Boor et al. (1979) administered a single gavage dose of AA to rats at dose levels of 50, 100 and 150 mg/kg. At 24 h post-dosing, myocardial necrosis was present in some animals at the 100 and 150 mg/kg dose levels; however there was no evidence of microscopic vascular
abnormalities. When AA was administered to rats at a dose of 0.1 % AA in the drinking water, acute myocardial necrosis was observed after 4 days of AA administration, however vascular lesions were observed only when AA was administered for 21 days or longer (Boor et al., 1979). In the study by Boor and Ferrans (1982), rats were administered AA for 1 to 7 days at a concentration of 10.7 mM in the drinking water. Myocardial necrosis was evident after 6 to 7 days of compound administration; however there was no histological evidence of vasculopathy. Nevertheless, in all of the 3 present experiments, vasculopathy, characterised by mural disorganisation, perivascular oedema, and medial and adventitial degenerative changes, were seen accompanying the myocardial necrosis induced by AA (Figure 7.7). However, AA is known to cause coronary artery vasospasm (Conklin and Boor, 1998), and such an effect would suggest a direct action of AA on the vasculature. The induction of vasospasm in vivo may lead to ischaemia, again resulting in myocardial necrosis.

The results in Table 7.11 show that myocardial degeneration/necrosis and vasculopathy was evident in animals treated with dose levels of AA of 100 mg/kg and above. Therefore, it appeared that these 2 histological alterations occurred together. However, there appears to be no relationship between the severity gradings for degeneration/necrosis and those for vasculopathy. For example, although vasculopathy was present only in those animals that displayed evidence of myocardial degeneration/necrosis, in the 3 animals with myocardial/degeneration severity grading scores of 4 (moderate), the vasculopathy severity grading scores were 1 (minimal), 2 (mild) and 0 (no abnormalities detected). In addition, the affected vessels were located in parts of the myocardium separate from the areas of necrosis. It is suggested that the 2 histopathological lesions described here induced by AA are independent: it is not necessary for 1 of the lesions to appear before the other. Furthermore, the vascular changes occurring did not result in occlusion of the lumen, which would lead to a reduced blood supply to the tissue. Therefore, the degeneration/necrosis observed in the present studies was not a result of vessel occlusion with resulting tissue hypoxia.

The increase in relative heart weights observed in Experiment 2 (dose ranging study, 200 – 350 mg/kg) and in Experiment 3 (time course study) were significant at 48 h at AA dose levels of 200 (P<0.001), 250 (P<0.001), 300 (P<0.05) and 350 (P<0.001) mg/kg (Experiment 2) and at 30, 36, and 48 h post-dosing ((P<0.001 at each time point) at 300 mg/kg ISO. Cardiac hypertrophy may result in an increase in relative heart
weight (Kizaki et al., 2005). However, the myocardial injury observed in the present studies was characterised by myocardial degeneration/necrosis with neutrophil infiltration, and interstitial oedema. Therefore, it is suggested that the increases in group mean relative heart weight as induced by AA was due to the histopathological changes observed here, rather than cardiac hypertrophy.

AA administration appeared to cause clinical signs of an increase in the body temperature of the animals immediately after dosing in all of the present studies. This was identified only by the observation of the animals. No other studies appear to have mentioned such a phenomenon occurring following the administration of AA and the reasons for the response are unclear. Nevertheless, acetylcholine causes generalised vasodilation through the release of nitric oxide (Rang et al., 1999), and vasodilation of the face, ears and extremities may occur in the rat as a cholinomimetic effect (Ghanayem et al., 1991). Therefore, the perceived increase in body temperature in AA-treated rats in the present study may be related to acetylcholine effects.

In summary, the present studies with AA describe the development and investigation of a new model of compound-induced myocardial injury. When AA was administered as a single gavage dose to male Hanover Wistar rats at doses up to 350 mg/kg (Experiments 1 and 2), myocardial degeneration/necrosis was present at 48 h post-dosing in 72.1 % (31 out of a total of 43) of the animals dosed at 100 mg/kg and above. All rats (n=5) dosed at 300 mg/kg showed degeneration/necrosis at 48 h post-dosing (Experiment 2; Table 7.7). Vasculopathy affecting mainly the small intramural arteries of the heart was also present concurrently with the myocardial necrosis. The time course of AA-induced cardiotoxicity was followed over a 48 h period after Hanover Wistar rats were administered a single gavage dose of AA at 300 mg/kg. Here, myocardial necrosis was first seen at 12 h post-dosing, affecting a proportion of animals, and the lesions increased in incidence and severity over time (Table 7.10). cTnl was shown to be an excellent marker of cardiomyocyte necrosis, appearing at an earlier time point than the histological evidence of cardiac injury. Serum H-FABP was also identified as a marker of cardiac injury, however more work on this protein is required.
Figure 7.1. Experiment 1, dose ranging study, 25 – 200 mg/kg; group mean serum alanine aminotransferase (ALT) levels in rats treated with vehicle (control, 0 mg/kg) and increasing dose levels of allylamine (25–200 mg/kg). Values are means, SD as error bars; n=5 for control and allylamine-treated rats, except for 25 and 150 mg/kg, where n=4; animals were sampled at 48 h post-dosing. *Significantly different from control, P<0.05; ***P<0.001.
Figure 7.2. Experiment 1, dose ranging study, 25 – 200 mg/kg; (A) myocardium from a control rat dosed with vehicle and (B) myocardium from a rat treated with a single dose of 200 mg/kg allylamine and autopsied at 48 h post-dosing. The normal appearance and structure of the myofibres of the myocardium is shown in (A); in (B) the myofibres are fragmented with granular cytoplasm; inflammatory cells are evident between muscle fibres. H&E, x 400 original magnification.
Figure 7.3. Experiment 2, dose ranging study, 200 – 350 mg/kg; percentage mean body weight change in rats treated with allylamine (AA) at dose levels of 0 (vehicle-treated control), and 200, 250, 300 and 350 mg/kg. Percentage mean body weight gain or loss refers to the amount of weight gained or lost compared to the mean body weight at the time of AA dosing (time “0”). There were 5 animals in each dose level group, except in the 350 mg/kg AA group, where n=3 at 1 and 2 days post-dosing.
Figure 7.4. Experiment 2, dose ranging study, 200 – 350 mg/kg; group mean relative heart weights in control (vehicle-treated, 0 mg/kg) male Hanover Wistar rats and rats treated with allylamine (AA; 200 to 350 mg/kg). Values are means, SD as error bars; n=5 for all groups, except for 350 mg/kg AA, where n=3; animals were sampled at 48 h post-dosing. *Significantly different from control, P<0.05; ***P<0.001. Please note the origin of the Y axis is 3.0 g/kg.
Figure 7.5. Experiment 3, time course study; group mean serum heart fatty acid-binding protein (H-FABP) levels (ng/mL) in control (vehicle-treated, time “0”) male Hanover Wistar rats and rats treated with 300 mg/kg allylamine (AA) and sampled over a 48 h post-dosing period. Mean values with SD (as error bars) are presented; n=5 for control and AA-treated rats, except at 12 h post-dosing, where n=4.
Figure 7.6. Experiment 3, time course study; group mean relative heart weights in control (vehicle-treated, time "0") male Hanover Wistar rats and rats treated with 300 mg/kg allylamine and sampled over a 48 h post-dosing period. Values are means, SD as error bars; n=5 for all groups. ***Significantly different from control, P<0.001. Note the origin of the Y axis is 3.0 g/kg.
Figure 7.7. Experiment 3, time course study; (A) small myocardial arteriole from a rat treated with 0 mg/kg allylamine (control) and (B) from a rat treated with a single dose of 300 mg/kg allylamine and autopsied at 36 h post-dosing. The normal appearance and structure of the vessel is shown in (A); in (B) there is minimal fibrinoid necrosis of the vessel wall and swollen nuclei; the perivascular space is distended by oedema and contains inflammatory cells and activated fibroblasts. H&E, x 400 original magnification.
Table 7.1. Experiment 1, dose ranging study, 25 - 200 mg/kg; serum clinical chemistry results at 48 h post-dosing in control (vehicle-treated) male Hanover Wistar rats and rats treated with allylamine (AA)*, b.

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*Values are means, SD in parentheses; n=5 for control and AA-treated rats, except at 25 and 150 mg/kg, where n=4. Animals were given a single gavage administration of allylamine and autopsied at 48 h post-dosing; data for alanine aminotransferase (ALT) is presented separately (Figure 7.1). *Significantly different from control, P<0.05.

bAbbreviations and units: urea, mmol/L; CREAT, creatinine, μmol/L; albumin, g/L; TP, total protein, g/L; sodium, mmol/L; potassium, mmol/L; calcium, mmol/L; chloride, mmol/L; AST, aspartate aminotransferase, U/L; ALP, total alkaline phosphatase, U/L; CK, total creatine kinase, U/L; LD, total lactate dehydrogenase, U/L; GLD, glutamate dehydrogenase, U/L; aldolase, U/L.
Table 7.2. Experiment 1, dose ranging study, 25 – 200 mg/kg; serum cardiac troponin I (cTnI) results at 48 h post-dosing in individual control (vehicle-treated) male Hanover Wistar rats and rats treated with allylamine\(^a\).

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<td>0.03</td>
<td>0.03</td>
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<td>0.13</td>
<td>1.61</td>
<td>0.98</td>
</tr>
<tr>
<td></td>
<td>0.03</td>
<td>NS</td>
<td>0.03</td>
<td>0.03</td>
<td>0.03</td>
<td>0.03</td>
<td>0.45</td>
<td>0.03</td>
<td>0.98</td>
</tr>
</tbody>
</table>

| Mean                   | 0.034| 0.030| 0.030| 0.032| 0.038| 0.066| 0.285| 0.364| 1.146 *** |
| SD                     | 0.009| 0.000| 0.000| 0.004| 0.018| 0.065| 0.297| 0.151| 0.681 |

| Group mean fold increase in relation to mean control value | - | - | - | - | 0.1 | 0.9 | 7.4 | 9.7 | 32.7 |

\(^a\)Values are measured in \(\mu\)g/L. NS indicates no sample. ***Significantly different from control, \(P<0.001\).
<table>
<thead>
<tr>
<th>Microscopic finding</th>
<th>Severity grade</th>
<th>0</th>
<th>25</th>
<th>50</th>
<th>75</th>
<th>100</th>
<th>125</th>
<th>150</th>
<th>175</th>
<th>200</th>
</tr>
</thead>
<tbody>
<tr>
<td>Myocardial degeneration/necrosis</td>
<td>NAD</td>
<td>5</td>
<td>5</td>
<td>5</td>
<td>5</td>
<td>3</td>
<td>3</td>
<td>-</td>
<td>1</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Minimal</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
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<td>-</td>
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<td>-</td>
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<td>2</td>
<td>1</td>
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<td>1</td>
<td>2</td>
<td>2</td>
</tr>
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<td>-</td>
<td>-</td>
<td>-</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>Very marked</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
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</tr>
<tr>
<td><strong>Vasculopathy</strong></td>
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<td>4</td>
<td>2</td>
<td>1</td>
<td>2</td>
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<td>Minimal</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Mild</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
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<tr>
<td></td>
<td>Moderate</td>
<td>-</td>
<td>-</td>
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<td>-</td>
<td>-</td>
<td>-</td>
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</tr>
<tr>
<td></td>
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<td>-</td>
</tr>
<tr>
<td></td>
<td>Very marked</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

n=5 for animals in all groups. Rats were given a single gavage dose of AA at 25, 50, 75, 100, 125, 150, 175 or 200 mg/kg and autopsied at 48 h post-dosing.

NAD= no abnormalities detected.
Table 7.4. Experiment 2, dose ranging study, 200 – 350 mg/kg; serum clinical chemistry results at 48 h post-dosing in control (vehicle-treated) male Hanover Wistar rats and rats treated with allylamine (AA).^{a}

<table>
<thead>
<tr>
<th>Allylamine dose (mg/kg)</th>
<th>0</th>
<th>200</th>
<th>250</th>
<th>300</th>
<th>350</th>
</tr>
</thead>
<tbody>
<tr>
<td>Urea</td>
<td>7.28 (0.56)</td>
<td>5.78 (0.57)</td>
<td>6.45 (0.13)</td>
<td>6.60 (0.73)</td>
<td>7.37 (1.84)</td>
</tr>
<tr>
<td>CREAT</td>
<td>38.6 (1.5)</td>
<td>39.3 (1.0)</td>
<td>39.3 (2.5)</td>
<td>39.8 (2.8)</td>
<td>36.7 (4.5)</td>
</tr>
<tr>
<td>Albumin</td>
<td>32.2 (1.3)</td>
<td>30.3 (0.5)</td>
<td>29.5 (1.3)</td>
<td>27.8 (3.2)***</td>
<td>24.0 (2.0)***</td>
</tr>
<tr>
<td>TP</td>
<td>53.0 (2.0)</td>
<td>51.0 (1.2)</td>
<td>51.8 (2.8)</td>
<td>49.6 (3.6)</td>
<td>45.7 (3.2)***</td>
</tr>
<tr>
<td>Sodium</td>
<td>143.6 (0.5)</td>
<td>143.8 (0.5)</td>
<td>143.5 (1.0)</td>
<td>142.2 (2.0)</td>
<td>139.0 (4.6) *</td>
</tr>
<tr>
<td>Potassium</td>
<td>5.00 (0.12)</td>
<td>4.73 (0.29)</td>
<td>4.68 (0.22)</td>
<td>4.64 (0.88)</td>
<td>4.93 (0.45)</td>
</tr>
<tr>
<td>Calcium</td>
<td>2.74 (0.09)</td>
<td>2.70 (0.00)</td>
<td>2.60 (0.08)</td>
<td>2.56 (0.17) *</td>
<td>2.43 (0.06)***</td>
</tr>
<tr>
<td>Chloride</td>
<td>101.0 (0.7)</td>
<td>102.3 (1.7)</td>
<td>102.8 (1.3)</td>
<td>101.8 (1.1)</td>
<td>99.0 (3.5)</td>
</tr>
<tr>
<td>ALT</td>
<td>43.2 (4.2)</td>
<td>38.5 (1.9)</td>
<td>35.8 (8.8)</td>
<td>31.2 (19.1)</td>
<td>43.0 (33.6)</td>
</tr>
<tr>
<td>AST</td>
<td>64.0 (6.2)</td>
<td>57.0 (2.9)</td>
<td>64.3 (10.0)</td>
<td>55.0 (13.0)</td>
<td>60.3 (21.0)</td>
</tr>
<tr>
<td>ALP</td>
<td>471.0 (67.4)</td>
<td>549.0 (151.9)</td>
<td>430.3 (17.8)</td>
<td>347.4 (93.4)</td>
<td>282.3 (67.2) *</td>
</tr>
<tr>
<td>CK</td>
<td>465.4 (138.7)</td>
<td>379.8 (50.1)</td>
<td>303.0 (104.1)</td>
<td>226.6 (138.1)</td>
<td>307.7 (210.9)</td>
</tr>
<tr>
<td>LD</td>
<td>1225.4 (459.1)</td>
<td>915.8 (119.5)</td>
<td>824.3 (353.3)</td>
<td>431.2 (394.9)***</td>
<td>537.7 (129.2) *</td>
</tr>
<tr>
<td>GLD</td>
<td>3.66 (1.59)</td>
<td>2.50 (1.02)</td>
<td>3.88 (2.32)</td>
<td>3.94 (2.89)</td>
<td>2.17 (1.52)</td>
</tr>
<tr>
<td>Aldolase</td>
<td>37.4 (4.5)</td>
<td>35.8 (4.6)</td>
<td>38.3 (6.8)</td>
<td>39.0 (10.0)</td>
<td>37.3 (8.6)</td>
</tr>
</tbody>
</table>

Values are means, SD in parentheses; n=5/group, except for 200 and 250 mg/kg AA, where n=4 and 350 mg/kg, where n=3. *Significantly different from control, P<0.05, ***P<0.001.

Abbreviations and units: as for Table 7.1, plus ALT, alanine aminotransferase, U/L.
Table 7.5. Experiment 2, dose ranging study, 200 – 350 mg/kg; serum cardiac troponin I (cTnl) levels in individual control (vehicle-treated) male Hanover Wistar rats and rats treated with allylamine and sampled at 24 h post-dosing.

<table>
<thead>
<tr>
<th>Allylamine dose (mg/kg)</th>
<th>0</th>
<th>200</th>
<th>250</th>
<th>300</th>
<th>350</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt;0.03</td>
<td>0.36</td>
<td>0.15</td>
<td>1.18</td>
<td>&lt;0.03</td>
<td></td>
</tr>
<tr>
<td>&lt;0.03</td>
<td>2.50</td>
<td>NS</td>
<td>NS</td>
<td>ICD</td>
<td></td>
</tr>
<tr>
<td>&lt;0.03</td>
<td>0.17</td>
<td>3.81</td>
<td>0.62</td>
<td>0.68</td>
<td></td>
</tr>
<tr>
<td>&lt;0.03</td>
<td>1.13</td>
<td>0.67</td>
<td>NS</td>
<td>ICD</td>
<td></td>
</tr>
<tr>
<td>&lt;0.03</td>
<td>0.03</td>
<td>0.06</td>
<td>NS</td>
<td>&lt;0.03</td>
<td></td>
</tr>
<tr>
<td>Mean</td>
<td>&lt;0.03</td>
<td>0.840</td>
<td>1.173</td>
<td>0.900</td>
<td>0.250</td>
</tr>
<tr>
<td>SD</td>
<td>0.000</td>
<td>1.020</td>
<td>1.779</td>
<td>0.396</td>
<td>0.380</td>
</tr>
</tbody>
</table>

cTnl values are measured in µg/L. NS indicates no sample for analysis. ICD indicates intercurrent death animal, and therefore no sample was available for analysis. Blood samples were obtained “in-life”, from the tail vein and serum prepared for cTnl analysis.

Table 7.6. Experiment 2, dose ranging study, 200 – 350 mg/kg; serum cardiac troponin I (cTnl) levels in individual control (vehicle-treated) male Hanover Wistar rats and rats treated with allylamine and sampled at 48 h post-dosing.

<table>
<thead>
<tr>
<th>Allylamine dose (mg/kg)</th>
<th>0</th>
<th>200</th>
<th>250</th>
<th>300</th>
<th>350</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt;0.03</td>
<td>&lt;0.03</td>
<td>&lt;0.03</td>
<td>&lt;0.03</td>
<td>&lt;0.03</td>
<td>&lt;0.03</td>
</tr>
<tr>
<td>&lt;0.03</td>
<td>0.08</td>
<td>&lt;0.03</td>
<td>0.43</td>
<td>ICD</td>
<td></td>
</tr>
<tr>
<td>&lt;0.03</td>
<td>1.49</td>
<td>1.75</td>
<td>0.17</td>
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<td></td>
</tr>
<tr>
<td>&lt;0.03</td>
<td>0.06</td>
<td>&lt;0.03</td>
<td>0.13</td>
<td>ICD</td>
<td></td>
</tr>
<tr>
<td>&lt;0.03</td>
<td>&lt;0.03</td>
<td>NS</td>
<td>0.16</td>
<td>&lt;0.03</td>
<td></td>
</tr>
<tr>
<td>Mean</td>
<td>&lt;0.03</td>
<td>0.338</td>
<td>0.460</td>
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<td>0.070</td>
</tr>
<tr>
<td>SD</td>
<td>0.000</td>
<td>0.644</td>
<td>0.860</td>
<td>0.148</td>
<td>0.099</td>
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</table>

cTnl values are measured in µg/L. NS indicates no sample for analysis. ICD indicates intercurrent death animal, and therefore no sample was available for analysis. Blood samples were obtained at autopsy, from the inferior vena cava and serum prepared for cTnl analysis.
Table 7.7. Experiment 2, dose ranging study, 200 – 350 mg/kg; incidence of microscopic findings in the heart of control (vehicle-treated) male Hanover Wistar rats and rats treated with allylamine (AA)\(^a\).

<table>
<thead>
<tr>
<th>Microscopic finding</th>
<th>Severity grade</th>
<th>0</th>
<th>200</th>
<th>250</th>
<th>300</th>
<th>350</th>
</tr>
</thead>
<tbody>
<tr>
<td>Myocardial degeneration/necrosis</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>NAD</td>
<td>5</td>
<td>1</td>
<td>3</td>
<td>-</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Minimal</td>
<td>-</td>
<td>1</td>
<td>-</td>
<td>2</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Mild</td>
<td>-</td>
<td>1</td>
<td>2</td>
<td>2</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Moderate</td>
<td>-</td>
<td>2</td>
<td>-</td>
<td>1</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Marked</td>
<td>-</td>
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<td>-</td>
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</tr>
<tr>
<td>Very marked</td>
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</tr>
<tr>
<td>Vasculopathy</td>
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<tr>
<td>NAD</td>
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<td>4</td>
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<tr>
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<td>Moderate</td>
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<td>-</td>
<td>-</td>
<td>-</td>
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</tr>
</tbody>
</table>

\(^a\) n=5 for animals in all groups, except for 350 mg/kg AA, where n=3. Rats were given a single gavage dose of allylamine at 200, 250, 300 or 350 mg/kg and autopsied at 48 h post-dosing.

\(^b\) NAD = no abnormalities detected.
Table 7.8. Experiment 3, time course study; body weight of individual control (vehicle-treated; time “0”) male Hanover Wistar rats and rats treated with allylamine at 300 mg/kg before dosing and at autopsy 4 to 48 h post-dosing.

<table>
<thead>
<tr>
<th>Time point (h)</th>
<th>Weight pre-dosing (g)</th>
<th>Weight at autopsy (g)</th>
<th>Weight change (g)</th>
<th>% group mean body weight change in relation to pre-dosing body weight</th>
</tr>
</thead>
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<td>183</td>
<td>183</td>
<td>0</td>
<td>0.0</td>
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<td>193</td>
<td>193</td>
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<td>178</td>
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<tr>
<td>12</td>
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<td>+5</td>
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</tr>
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<td>191</td>
<td>181</td>
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<td>185</td>
<td>176</td>
<td>-9</td>
<td></td>
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<td>189</td>
<td>177</td>
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<td></td>
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<td>24</td>
<td>180</td>
<td>178</td>
<td>-2</td>
<td></td>
</tr>
<tr>
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<td>171</td>
<td>163</td>
<td>-8</td>
<td></td>
</tr>
<tr>
<td>24</td>
<td>194</td>
<td>182</td>
<td>-12</td>
<td>-5.5</td>
</tr>
<tr>
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<td>183</td>
<td>165</td>
<td>-18</td>
<td></td>
</tr>
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Table 7.9. Experiment 3, time course study; serum cardiac troponin I (cTnI) results in individual control (vehicle-treated, time “0”) male Hanover Wistar rats and rats treated with 300 mg/kg allylamine and sampled over a 48 h post-dosing period.

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Mean: <0.030 <0.030 0.118 2.618 3.218 0.892 1.778 5.208 0.294
SD: 0.000 0.000 0.152 4.240 5.913 1.512 1.174 6.200 0.306

*Values are measured in µg/L. NS indicates no sample for analysis. n=5/group, except at 12 h post-dosing, where n=4.
Table 7.10. Experiment 3, time course study; incidence of microscopic findings in the hearts of control (vehicle-treated; time “0”) male Hanover Wistar rats and rats treated with 300 mg/kg allylamine and sampled over a 48 h post-dosing period*.

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<th>36</th>
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<tbody>
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* n=5/group. NAD = no abnormalities detected.
Table 7.11. Experiment 1, dose ranging study, 25 – 200 mg/kg; cardiac troponin I (cTnl) responses and pathology scores in individual control (vehicle-treated; time 0 h) male Hanover Wistar rats and in rats treated with allylamine.

<table>
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<th>Allylamine dose (mg/kg)</th>
<th>cTnl level (µg/L)</th>
<th>Mean (SD) cTnl</th>
<th>Myocardial degeneration/necrosis score Mean (SD)</th>
<th>Vasculopathy score Mean (SD)</th>
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*NS indicates no sample available for analysis. Myocardial degeneration/necrosis and vasculopathy severity grade scores: 0= no abnormalities detected; 1= minimal; 2= mild; 3= moderate; 4= marked. *Significantly different from control, P<0.05, ***P<0.001.
Table 7.12. Experiment 3, time course study; cardiac troponin I (cTnl) and heart fatty acid-binding protein (H-FABP) responses and pathology scores in individual control (vehicle-treated; time 0 h) male Hanover Wistar rats and in rats treated with 300 mg/kg allylamine and sampled over a 48 h period**.

<table>
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<th>Time point (h)</th>
<th>cTnl level (μg/L)</th>
<th>Mean (SD)</th>
<th>H-FABP level (ng/mL)</th>
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<th>Myocardial degeneration/ necrosis score</th>
<th>Mean (SD)</th>
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**NS indicates no sample. Myocardial degeneration/necrosis scores: 0= no abnormalities detected; 1= minimal; 2= mild; 3= moderate. ***Significantly different from control, P<0.001.
CHAPTER 8: STUDIES ON THE CARDIOTOXICITY OF ERUCIC ACID

8.1 INTRODUCTION

8.1.1 Background

Erucic acid (EA; cis-13-docosenoic acid; 22:1 \( n-9 \)) is a monounsaturated long chain fatty acid naturally occurring at high concentrations in the seed oils of rape, nasturtium, lupin, wallflower and mustard (McCutcheon \textit{et al.}, 1976; Ackman, 1983). EA has also been found in some marine animal oils (Food Standards Australia New Zealand, 2003). The first reports of the poor nutritional effects of EA appeared in the 1940’s and 1950’s, where it was shown by a number of groups that the feeding of high EA rapeseed (HEAR) oil retarded the rate of growth in young rats (Boer \textit{et al.}, 1947; Thomasson and Boldingh, 1955). However, the first group to observe that the feeding of both low EA rapeseed (LEAR) oil and HEAR oil to experimental animals (the rat and the pig) caused histopathological changes in the heart was Roine \textit{et al.} (1960). The main histopathological lesions observed in the heart following the administration of EA are myocardial lipidosis and myocardial necrosis (Roine \textit{et al.}, 1960; Abdellatif and Vles, 1970; Charlton \textit{et al.}, 1975; Chien \textit{et al.}, 1983).

8.1.2 Myocardial lipidosis

The short-term feeding of HEAR oil or glyceryl trierucate (pure EA) to rats results in a transient, rapid and severe fat infiltration of the myocardium (Abdellatif and Vles, 1970; Charlton \textit{et al.}, 1975; Chien \textit{et al.}, 1983), with the severity of the lesion being proportional to the amount of EA administered (Abdellatif and Vles, 1973). In the initial stages of lipidosis, the myocardium may be pale macroscopically (Johnston and Grice, 1991). Microscopically, the lipid droplets are round, with no discernible membrane, and vary in diameter up to approximately 8 \( \mu \text{m} \) (Sauer and Kramer, 1983). The fat droplets are arranged in a linear fashion along the myofibrils, and are sometimes associated with mitochondria (Umemura \textit{et al.}, 1978; Bhatnagar and Yamashiro, 1979; Yamashiro and Clandinin, 1980). Charlton \textit{et al.} (1975) showed that the foci of lipidosis were most prevalent in the ventricular walls and the interventricular septum. Some authors report
an increase in the number of mitochondria following the feeding of HEAR oil (Bhatnagar and Yamashiro, 1979; Svaar and Langmark, 1980), but this is disputed by others (Caselli et al., 1990). In addition, the appearance of "megamitochondria" has been reported by Bhatnagar and Yamashiro (1979). Chien et al. (1983) showed that the lipid droplets were not associated with necrosis or inflammation in the very early stages of lesion development.

Abdellatif and Vles (1970) fed male Wistar rats 50 % of their calories as HEAR oil for up to 7 days to follow the time course of lesion progression. Fatty infiltration was present after only 1 day of feeding and increased in severity after 3 to 6 days. It was also shown that the discontinuation of the feeding of HEAR oil resulted in the regression of the fatty lesions (i.e. the change was reversible). The time course of fatty lesion formation was also followed by Chien et al. (1983) who fed HEAR oil as 20 % of the calorie intake to male Sprague Dawley rats. Myocardial lipidosis was observed after 1 day of feeding, and peaked in severity at 3 to 5 days. By day 10 however, cardiomyocytes displayed a less severe lipid accumulation, and by day 16 onwards, only mild focal lipidosis was present. The earlier results of Charlton et al. (1975) were similar to the findings of Chien et al. (1983). Charlton et al. (1975) reported that male and female Sprague Dawley rats fed 20 % HEAR oil by weight of the diet developed a very marked lipid accumulation which peaked at day 7 of feeding, but regressed after this point. By day 28 of feeding, no lipid was observed in the myocardium of HEAR oil-treated animals. Nevertheless, the results of Abdellatif and Vles (1970) and Chien et al. (1983) do suggest that cardiac lipid levels may never completely return to normal (i.e. the absence of fat droplets in the myocardium) with the continued feeding of HEAR oil.

8.1.2.1 Metabolic aspects of myocardial lipidosis

In rats fed HEAR oil, there is an accumulation of EA within the cardiac lipid profile, which then declines as lipidosis regresses (Hulan et al., 1976). The early deposition of lipid within the myocardium is therefore considered to be due to the inefficient metabolism of the long chain fatty acid (i.e. the EA molecule; 22:1) within the mitochondria and this represents an imbalance between the uptake and oxidation of fatty acids. EA has been shown to be oxidised at a lower rate in the heart mitochondria in comparison to other long chain fatty acids (Galli Kienle et al., 1976). In addition,
Christophersen and Bremer (1972) showed that the oxidation of palmitoyl carnitine by cardiac mitochondria is inhibited in the presence of EA, thus the metabolism of EA itself may be the rate limiting step in the oxidation of fatty acids.

The early myocardial lipidosis seen with the administration of EA is only a transient change during a period of adaptation to the metabolism of the EA molecule. The presence of EA in the cardiomyocytes induces a system to shorten the long chain fatty acid (C22:1) to eicosenoic acid (C20:1) and to oleic acid (C18:1) so that these acids can be utilised by the mitochondria (Pinson and Padieu, 1974; Norseth et al., 1979). It is suggested that the chain shortening of EA is carried out by an extra-mitochondrial (peroxisomal) \(-\)oxidation system (Norseth and Christophersen, 1978). An increase in mitochondrial number has been observed in some HEAR oil feeding studies (Bhatnagar and Yamashiro, 1979; Svaar and Langmark, 1980), but not in others (Caselli et al., 1990). Heart mitochondrial oxidative activity however has been demonstrated not to be altered with the feeding of HEAR oil (Galli Kienle et al., 1976). In addition, Caselli et al. (1990) found no increase in the number or activity of myocyte peroxisomes following the feeding of HEAR oil. However, Norseth and Thomassen (1983) demonstrated the presence of inducible heart “microperoxisomes” which are stimulated by the feeding of HEAR oil, with the increase in \(-\)oxidation being directly correlated to the amount of HEAR oil in the diet. Christiansen and Thomassen (1979) concluded however that the reversal of myocardial lipidosis was mainly due to an increased capacity for chain shortening in the liver.

### 8.1.3 Myocardial necrosis

The longer term feeding of EA as LEAR oil and HEAR oil has been reported to result in the regression of myocardial lipidosis and the appearance of focal myocardial necrosis and fibrosis (Abdellatif and Vles, 1973; Charlton et al., 1975; Chien et al., 1983). Although lipidosis appears to affect both sexes and all strains of rat studied, the subsequent necrosis appears only to develop in male albino animals, of the Wistar and Sprague Dawley strains (Charlton et al., 1975; Umemura et al., 1978; Kramer et al., 1979). Microscopically, these lesions are characterised by cardiomyocyte degeneration, the loss of myocytes and the accumulation of macrophages, followed by “scars” composed of loose, fibrous tissue (Chien et al., 1983). As with lipidosis, these later
lesions are also mainly present in the ventricular walls and interventricular septum (Kramer et al., 1979).

Charlton et al. (1975) fed male and female Sprague Dawley rats corn oil (i.e. an oil containing no EA), LEAR oil or HEAR oil (20 % of diet by weight) and killed animals at day 112 (16 weeks). The male rats killed at this time point had distinct foci of myocardial necrosis throughout the myocardium, however these lesions were rarely observed in female rats. A few scattered, focal, interstitial accumulations of mononuclear cells were seen in both sexes however (incidence not reported). Lesions were present in both the LEAR oil and HEAR oil groups, but also in a small proportion of animals fed corn oil (mostly males).

The development of myocardial necrosis in 2 strains of rats was compared by Kramer et al. (1979). Male weanling Sprague Dawley and Chester Beatty rats were fed corn oil (control), LEAR oil or HEAR oil (20 % by weight of diet) for 16 weeks. There was a significantly lower incidence of focal myocardial necrosis in the Chester Beatty animals compared with the Sprague Dawley rats. The incidence of necrosis was similar between all of the diets in the Chester Beatty strain; however a higher incidence was observed in the Sprague Dawley rats fed LEAR oil and HEAR oil, compared with the corn oil-fed animals.

Umemura et al. (1978) fed male Wistar rats either corn oil (control), LEAR oil or HEAR oil (20 % by weight of diet) for 18 weeks. Foci of myocardial necrosis were present in all 3 treatment groups, with the incidence in the LEAR and HEAR oil groups being twice that of the corn oil group. In addition, the lesions were also of a higher severity grade in the 2 rapeseed oil groups. The myofibres showed increased eosinophilia, separation of the myofibres and vacuolation. Mast cells and macrophages were often also present. The older foci showed lymphocyte and neutrophil involvement and an increase in immature collagen fibre formation. As there was some incidence of lesions in the corn oil (control) group, the presence of a high amount of lipid in the diet must therefore also be a causative factor in the development of myocardial necrosis.

The above studies therefore appear to show that myocardial necrosis and fibrosis occurs in male albino rats fed for a long term on a high lipid diet, with an increased incidence occurring when rapeseed oil is administered. Svaar and Langmark (1980) showed in addition that lesions also occur in male rats fed high fat (lipid) diets, irrespective of the
specific dietary oil administered. Furthermore, myocardial necrosis was seen in male Sprague Dawley rats fed rapeseed oil, partially hydrogenated fish oil or peanut oil for 30 weeks (Svaar and Langmark, 1980). It was also demonstrated by Hulan et al. (1976) that necrotic lesions developed in rats fed lard or rapeseed oil, with the incidence and severity being highest in the rapeseed oil-fed animals.

Some long term feeding studies have also produced evidence that myocardial necrosis and fibrosis appears to occur independently of an initial stage myocardial lipidosis: for example, necrotic and fibrotic lesions have been observed in rats fed LEAR oil, although LEAR oil has been shown by some groups not to induce an initial cardiac fatty infiltration (Kramer et al., 1979), although this finding is disputed by others (Abdellatif and Vles, 1973). However, the presence of EA does appear to increase the incidence and severity of the lesions in a dose related fashion (Abdellatif and Vles, 1970; Charlton et al., 1975). Therefore, it is concluded that the pathophysiology of myocardial lesion formation in this area of research on dietary lipid administration is complex and controversial, with both EA and high amounts of fats appearing to contribute to lesion development. Finally, it is well known that spontaneous cardiomyopathy is a common age-related degenerative disease in laboratory rats, which is particularly prevalent in male animals (Ayers and Jones, 1978; Burek, 1978; Kemi et al., 2000).

8.1.4 Myocardial performance

A study in the rat by de Wildt and Speijers (1984) appears to be the only investigation looking at whether the long term feeding of pure EA and HEAR oil, or sunflower oil has any effect on myocardial performance. These authors reported that feeding HEAR oil for 24 to 26 weeks caused focal myocardial fibrotic lesions to develop, however this did not occur in the groups fed pure EA or animals fed sunflower oil. There were no changes to the intrinsic myocardial contractility of the heart when tested both in vitro and in vivo, in any of the treatment groups. Furthermore, no electrocardiogram changes were observed in any group. The HEAR oil-fed animals did show less contractile reserve capacity following inotropic stimulation, but no changes were observed in the sunflower oil or pure EA-treated groups. The vasoconstrictor responses to noradrenaline were reduced in both the pure EA and HEAR oil groups, suggesting that pure EA may interfere with the contractile elements of the peripheral vascular system. Isoproterenol (ISO) reduced myocardial contractility in both the sunflower oil and HEAR oil groups.
ISO is a non-selective \( \beta \)-adrenoceptor agonist, which will increase energy demand through \( \beta_1 \) stimulation, whilst causing a concurrent decrease in blood pressure (and therefore a decrease in perfusion pressure) through \( \beta_2 \) stimulation (Sweetman, 2006). Therefore, in this study (de Wildt and Speijers, 1984), it was concluded that HEAR oil, but not pure EA, causes a loss of contractile reserve capacity without changes in the myocardial conduction system. However, reduced myocardial function during periods of energy demand appeared to be due to feeding a diet high in fat, rather than the presence of EA in the diet (either as pure EA or as HEAR oil).

### 8.1.5 Other target organs

In addition to the fatty infiltration of the myocardium observed with short term lipid administration, other target organs are reported to be susceptible to the lipotoxic effects of EA and HEAR oil. The feeding of HEAR oil for 2 weeks to male Wistar rats resulted in fat deposition in the myocardium, but also in skeletal muscle and adrenal glands, with no changes observed in the liver, spleen or kidneys (Abdellatif and Vles, 1970). In contrast, increased fat accumulation was seen in the livers of male and female Sprague Dawley rats fed HEAR oil for 1 and 2 weeks (Kramer et al., 1973). Hepatic lipidosis was also observed in male Sprague Dawley and Chester Beatty rats fed HEAR oil for 16 weeks (Kramer et al., 1979). Charlton et al. (1975) observed the slight accumulation of lipid in skeletal muscles (diaphragm, masseter, and semimembranosus muscles) of male and female Sprague Dawley rats fed HEAR oil for up to 112 days. Finally, the feeding of HEAR oil or glyceryl trierucate for 24 weeks to male Wistar rats was reported to result in several histological changes in the kidney, namely tubular dilatation, proteinaceous cast formation and interstitial foci of fibrosis (Abdellatif and Vles, 1973).

### 8.1.6 Other species

In the studies mentioned above, the great majority of the work discussed has been conducted in the rat. However, the very first study linking EA with myocardial lipidosis was carried out in the rat and in the pig (Roine et al., 1960). Therefore, the feeding of EA does not only produce myocardial lesions in the rat. Myocardial lipidosis can also be induced by feeding EA to the duckling (Abdellatif and Vles, 1971), the chicken and the turkey (Charlton et al., 1975), the gerbil (Beare-Rogers et al., 1972), the monkey (Beare-Rogers and Nera, 1972), and the pig (Roine et al., 1960; Kramer et al., 1990).
However, the pig is much less sensitive to the lipotoxic effects of EA in comparison to the rat (Hulan et al., 1976). Myocardial necrosis has also been reported in the rabbit following 87 weeks of feeding HEAR oil (Vles and Abdellatif, 1970) and in the pig after 16 weeks of feeding HEAR oil (Hulan et al., 1976).

8.1.7 Relevance to man

Although there is much evidence that the feeding of HEAR oil induces myocardial lipidosis in experimental animals, there appears to be no evidence that EA is toxic to man. Rapeseed oil has been used as a cooking oil since the 13th century, and no records show any nutritional or health problems associated with its use (Sauer and Kramer, 1983). Holmes (1918) showed that HEAR oil had a digestibility of 98.8 % in man. In contrast, HEAR oil has a digestibility coefficient of only 83.0 % for the Wistar rat, and 64.8 % for the Sprague Dawley rat (Beare et al., 1960). HEAR oil is also poorly digested in the rabbit and guinea pig (Carroll, 1957), whereas in the pig, HEAR oil is almost completely digested (Sauer and Kramer, 1983). The lack of sensitivity of the pig to EA cardiotoxicity (Hulan et al., 1976) may be related to the high level of digestibility of HEAR oil in swine. Similarly, this high level of digestibility may also be the reason that man does not appear to be susceptible to EA-induced myocardial lipidosis.

8.1.8 Aims of the present studies

No studies have been identified in the literature that have used the gavage route of administration for EA; all have employed a dietary regimen of administration. Therefore, the aims of the present studies were to investigate the toxic effects on rat heart of LEAR oil, HEAR oil, and pure EA mixed with LEAR oil ("Spike oil", as described in Chapter 2.4.6) administered in the diet or by gavage over a period of 96 h. We also wished to follow the development of cardiac lipidosis over the 96 h period. Similarly, there appears to be no information in the literature reporting serum clinical chemistry changes following EA administration in the rat. Therefore, we wished to evaluate possible changes in serum levels of cardiac troponin I (cTnI) and other clinical chemistry parameters in myocardial lipidosis.

A preliminary report of these findings has been published in abstract form (Brady et al., 2007).
8.2 MATERIALS AND METHODS

8.2.1 Experimental protocols

8.2.1.1 Experiment 1, preliminary 96 h feeding study

OBJECTIVE: To investigate whether myocardial lipidosis can be induced via the dietary administration of LEAR oil, Spike oil or HEAR oil over a 96 h period.

8.2.1.1.1 Animal handling

Control ground diet, LEAR oil diet, Spike oil diet and HEAR oil diet were prepared as described in Chapter 2.4.6. Male Hanover Wistar rats (n=20; mean weight 100.0 g) were randomly divided into 4 groups (n=5/group). Group 1 received Control ground diet, Group 2 received LEAR oil diet, Group 3 received Spike oil diet and Group 4 received HEAR oil diet. All groups received 100 g of fresh diet per cage every 24 h (Chapter 2.2). Each group of n=5 rats occupied 1 cage. Water was provided ad libitum to all groups. Body weights, diet and water consumptions were monitored over each 24 h period (Chapter 2.1, 2.2, 2.3). Animals were maintained on each dietary treatment for 96 h and then autopsied for clinical chemistry and histopathological investigations.

8.2.1.1.2 Sample handling

Serum clinical chemistry analysis was performed on all animals in all groups (Chapter 2.8.2). Parameters measured were: urea, creatinine (CREAT), albumin, total protein (TP), sodium, potassium, chloride, total cholesterol (CHOL), triglycerides (TRIG), non-esterified fatty acids (NEFA), alanine aminotransferase (ALT), aspartate aminotransferase (AST), total creatine kinase (CK), total lactate dehydrogenase (LD), glutamate dehydrogenase (GLD), aldolase and cTnl. cTnl levels were measured on the Bayer ACS: 180S system (Chapter 2.8.2). At autopsy, animals were killed by intraperitoneal (IP) injection of Euthatal (as described in Chapter 2.6) and hearts removed, weighed and placed in fixative (Chapter 2.10). Sections of hearts were examined from all animals in all groups. Heart lesions were scored on a severity scale of
0 to 5: 0 = no abnormalities detected (NAD), 1 = minimal; 2 = mild; 3 = moderate; 4 = marked and 5 = very marked.

8.2.1.2  Experiment 2, preliminary 96 h gavage study

OBJECTIVE: To investigate whether myocardial lipidosis can be induced via the gavage administration of HEAR oil over a 96 h period.

8.2.1.2.1  Animal handling

Table 8.1 sets out the experimental design of the study. HEAR oil diet was prepared as described in Chapter 2.4.6. Male Hanover Wistar rats (n=35; mean weight 145.9 g) were randomly divided into 7 groups (n=5/group). Each group of n=5 rats occupied 1 cage. Groups 1 – 6 were treated with oil by gavage at a dose level of 31 ml oil/kg body weight/24 h. This dose level was calculated as below:

In Experiment 1 (preliminary 96 h feeding study), it was found that a 116 g rat consumed 16.3 g HEAR oil diet per 24 h period.

\[
\text{116 g rat consumed} = 3.26 \text{ g of HEAR oil (20 \% [w/w] of HEAR oil diet).}
\]

\[
\text{100 g rat would receive} = 2.81 \text{ g of HEAR oil.}
\]

\[
\text{2.81 g HEAR oil} = 3.1 \text{ ml of HEAR oil (as 1 g HEAR oil = 1.1 ml).}
\]

Therefore, rats consumed 3.1 ml of oil per 100 g body weight (31 ml/kg) per 24 h period.

A timetable of gavage dosing is presented in Table 8.1. Group 1 received 1 dose of LEAR oil per 24 h (31 ml/kg), Group 2 received 1 dose of HEAR oil per 24 h (31 ml/kg), Group 3 received 2 doses of HEAR oil per 24 h (31 ml/kg divided into 2 equal doses, i.e. of 15.5 ml/kg), Group 4 received 3 doses of HEAR oil per 24 h (31 ml/kg divided into 3 equal doses), Group 5 received 4 doses of HEAR oil per 24 h and Group 6 received 5 doses of HEAR oil per 24 h. Groups 1 to 6 received 100 g of fresh Control ground diet per cage every 24 h during the period of treatment (0 h to 96 h; Chapter 2.2). Group 7 received HEAR oil diet. All treatment groups had free access to drinking water, and water consumption was monitored every 24 h (Chapter 2.3). Treatment was
for 96 h and animals were then autopsied for clinical chemistry and histopathological investigations.

8.2.1.2.2 Sample handling

Sample handling (i.e. clinical chemistry analysis and histopathological examination) was the same as for Experiment 1 (preliminary 96 h feeding study). Levels of cTnI were measured on the Bayer ACS: 180S system (Chapter 2.8.2).

8.2.1.3 Experiment 3, 96 h gavage dosing evaluation study

OBJECTIVE: To evaluate the comparative effectiveness in the induction of cardiac lipidosis of gavage dosing Spike oil and HEAR oil over a 96 h study period, using administration regimens over an 8 h period (09.00 h to 17.00 h) and over a 12 h period (09.00 h to 21.00 h).

8.2.1.3.1 Animal handling

Table 8.2 sets out the experimental design of the study. Sunflower oil diet, LEAR oil diet, Spike oil diet, HEAR oil diet and Spike oil were prepared as described in Chapter 2.4.6. Male Hanover Wistar rats (n=40; mean weight 98.0 g) were randomly divided into 8 groups (n=5/group). Each group of n=5 rats occupied 1 cage. The timetable of gavage dosing is presented in Table 8.2. Group 1 received Sunflower oil diet (control); Group 2 received LEAR oil diet; Group 3 received Spike oil diet; Group 4 received HEAR oil diet; Groups 5 and 6 received Spike oil by gavage, 3 times per 24 h period and Groups 7 and 8 received HEAR oil by gavage, 3 times per 24 h period. Groups 5 and 7 were dosed at 09.00 h, 13.00 h and 17.00 h; these times are referred to as “social hours”. Groups 6 and 8 were dosed at 09.00 h, 15.00 h and 21.00 h; these times are referred to as “unsocial hours”. Groups 1 to 4 received 150 g of fresh diet every 24 h period (Chapter 2.2). Groups 5 to 8 received extruded Global Rodent Diet ad libitum. All groups had unrestricted access to water. Diet (Groups 1 to 4) and water (all groups) consumptions were monitored every 24 h (Chapter 2.2, 2.3). Animals were maintained on dietary or gavage treatment for 96 h and were then autopsied for clinical chemistry and histopathological investigations.
8.2.1.3.2 Sample handling

Serum clinical chemistry analysis was performed on all animals in all groups (Chapter 2.8.2). Parameters measured were: urea, CREAT, albumin, TP, sodium, potassium, chloride, CHOL, high density lipoprotein CHOL (HDL-CHOL), TRIG, NEFA, ALT, AST, CK, LD, GLD, aldolase and cTnI. Levels of cTnI were measured on the Bayer Centaur CP (Chapter 2.8.2). At autopsy, animals were killed by IP injection of Euthatal (as described in Chapter 2.6) and hearts removed, weighed and placed in fixative (Chapter 2.10). Sections of hearts were examined histologically from all animals in all groups and were scored for lesion severity on a scale of 0 to 5, as in Experiment 1 (preliminary 96 h feeding study).

8.2.1.4 Experiment 4, time course study

OBJECTIVE: To assess the time course of cardiac lesion development and to identify histopathological changes occurring in skeletal muscle and liver over a 96 h period.

8.2.1.4.1 Animal handling

Table 8.3 presents the experimental design of the study with autopsy time points. Spike oil and HEAR oil diet were prepared as described in Chapter 2.4.6. Male Hanover Wistar rats (n=65; mean weight 92.8 g) were randomly divided into 13 groups (n=5/group; Table 8.3). Each group of n=5 rats occupied 1 cage. Groups 1, 2, 5, 8 and 11 received extruded Global Rodent Diet; Groups 3, 6, 9 and 12 received Spike oil by gavage, 3 times per 24 h period, at 09.00 h, 13.00 h and 17.00 h. Groups 4, 7, 10 and 13 received HEAR oil diet and were given 150 g of fresh diet per cage every 24 h (Chapter 2.2). The Spike oil gavage groups had unrestricted access to extruded Global Rodent Diet. Water was provided *ad libitum* to all groups, and water consumptions were monitored daily (Chapter 2.3). Animals were maintained on experimental treatments for 0 h (Group 1), 24 h (Group 2, 3 and 4), 48 h (Group 5, 6 and 7), 72 h (Group 8, 9 and 10) and 96 h (Group 11, 12 and 13) and were then autopsied for clinical chemistry and histopathological investigations.
8.2.1.4.2 Sample handling

Sample handling (i.e. clinical chemistry analysis and histopathological examination) was the same as for Experiment 3 (96 h gavage dosing evaluation study). Levels of cTnI were measured on the Bayer Centaur CP (Chapter 2.8.2). Hearts were taken at each autopsy time point (0, 24, 48, 72 and 96 h). At the 96 h autopsy, the liver, kidneys, adrenal glands, spleen, thymus and testes were also removed and weighed from all 3 treatment groups (Group 11, 12 and 13); the left hind limb was also removed and placed in fixative for the examination of skeletal muscle; livers were placed in formalin after weighing. Sections of heart from all groups were examined at all autopsy time points. Sections of liver and skeletal muscle were examined at 96 h. Lesions were scored on a scale of severity from 0 to 5: 0 = no abnormalities detected (NAD), 1 = minimal; 2 = mild; 3 = moderate; 4 = marked and 5 = very marked.

8.2.2 Statistical analysis

Data from all studies were analysed using one-way analysis of variance (ANOVA) followed by Dunnett’s test for post hoc multiple comparison. In cases of violation of the assumptions for parametric testing, the Kruskal-Wallis test was used in combination with Dunn’s post-test. Statistical analysis was performed using GraphPad Prism version 3.00 for windows (GraphPad software, San Diego, California, USA).
8.3 RESULTS

8.3.1 Experiment 1, preliminary 96 h feeding study

OBJECTIVE: To investigate whether myocardial lipidosis can be induced via the dietary administration of LEAR oil, Spike oil or HEAR oil over a 96 h period.

8.3.1.1 Clinical signs and body weight changes

The diets administered to Groups 1 to 4 in Experiment 1 were: Control ground diet, LEAR oil diet, Spike oil diet and HEAR oil diet, respectively. Animals in all groups were fed Control ground diet from -120 h to 0 h, and then oil-enriched diets (Groups 2, 3 and 4) from 0 h to 96 h. All animals (n=5 per dietary group) remained healthy over the 96 h period of EA treatment and no major clinical signs of toxicity were observed. However, during the period 72 h to 96 h, some animals fed the oil-enriched diets (Groups 2, 3 and 4) had oily fur (approximately 3 of the 5 animals in each group). Animals were weighed on 4 occasions from -120 h to 0 h (at -120, -96, -72 and 0 h), and on 4 occasions from 24 h to 96 h (24, 48, 72 and 96 h; Table 8.4A). A comparative examination of the mean body weights of animals in each dietary treatment group produced some evidence that feeding the high EA-containing experimental diets (Groups 3 and 4) possibly caused a slight reduction in body weight increases (Table 8.4B). For example, the mean percentage body weight increases during the period from -120 h to 0 h for Groups 1 to 4 (fed Control ground diet) were, respectively, 37.4, 39.3, 37.8 and 40.5 %; from 0 h to 96 h the relevant figures were 24.3, 20.2, 16.6 and 16.8 %, respectively. Also, it is seen (Table 8.4B) that in the period -120 h to 0 h, the mean body weight increases for Groups 1 to 4 over a 24 h period were 5.24, 5.60, 5.48 and 6.04 g, respectively. In the period 0 h to 96 h, the comparable figures were 5.85, 5.00, 4.15 and 4.40 g, respectively.

8.3.1.2 Diet and water consumption

Diet consumptions were carried out over 24 h and 72 h periods on 3 occasions before the animals were maintained on the experimental diets (i.e. in the period from -120 h to 0 h); during this period all groups were fed the Control ground diet (Table 8.5A). In the
period 0 h to 96 h, during the feeding of the experimental diets, 24 h diet consumptions were conducted on 4 occasions. The results presented in Table 8.5A show that, in general, the mean quantities of oil-enriched diets consumed by Groups 2, 3 and 4 during the period 0 h to 96 h compared well with the mean consumption of the Group 1 control animals, and also with the mean consumption for each group in the period -120 h to 0 h. Therefore, this suggests that the oil-enriched experimental diets were acceptably palatable. The mean diet consumptions from -120 h to 0 h for Groups 1 to 4 were, respectively, 15.0, 15.3, 15.0 and 14.7 g diet consumed/rat/24 h; from 0 h to 96 h the figures were 18.4, 16.4, 14.2 and 16.3 g diet consumed/rat/24 h, respectively (Table 8.5A).

Water consumptions are set out in Table 8.5B. Although the data shows variability, and there is an anomalous result (33.7 ml water consumed at 24 h to 48 h; Group 2), the consumptions for Groups 3 and 4 in the period 0 h to 96 h generally compare with the findings at -120 h to 0 h for those groups; also the 0 h to 96 h data (Groups 3 and 4) are similar to the Group 1 results for that time period. It is therefore concluded that there is no obvious indication that the maintenance of animals on the experimental oil-enriched diets greatly affected water consumption.

8.3.1.3 Serum clinical chemistry

Results are summarised in Table 8.6. The feeding of oil-enriched diets (Groups 2, 3 and 4; LEAR oil diet, Spike oil diet and HEAR oil diet, respectively) did not affect mean serum levels of albumin, TP, sodium, potassium, chloride, CHOL, CK or GLD at 96 h. However, mean serum urea levels were decreased significantly in Group 2 compared with the control Group 1 result (P<0.001); however no changes were seen in the urea levels in the other treatment groups (Groups 3 and 4). In Group 3, serum CREAT levels were significantly increased above the mean control value (P<0.05), however no changes were observed in Groups 2 or 4.

Mean serum TRIG levels in Groups 2 and 4 were decreased significantly (P<0.001) compared to the controls (Group 1). Mean serum TRIG levels were also decreased below control levels in Group 3, but this was not statistically significant. The decreases were to 47.6 % (Group 2), 73.8 % (Group 3) and 38.1 % (Group 4) of the mean control
values. There was also some evidence that serum NEFA levels were decreased in Groups 2 and 4, but these reductions were not statistically significant.

The mean serum activity of ALT was increased significantly in both high EA-treated groups (Groups 3 and 4, P<0.05 and P<0.001, respectively) compared with the controls. These increases were 49.6 % (Group 3) and 63.2 % (Group 4) above the mean control (Group 1) value. In addition, levels of ALT were also increased in Group 2, but this elevation was not significant. Mean serum AST levels were increased above control (Group 1) levels in both high EA-treated groups (Groups 3 and 4), but this was only significant in Group 4 (a 38.0 % increase; P<0.001). There was some evidence that mean serum LD activity was increased in Group 3 (Spike oil diet) and 4 (HEAR oil diet), but these increases were not statistically significant. There was a trend for serum aldolase activity to be increased in the high EA-treated groups, but this was statistically significant only in Group 3 (a 41.4 % increase; P<0.05).

Serum cTnI results, at the end of the 96 h feeding period, showed a trend of increased levels in all the oil-enriched diet groups (Groups 2, 3 and 4; Table 8.7). However the individual data was inconsistent and the mean levels were not statistically significantly increased for any dietary group. Nevertheless, the mean serum cTnI level for Group 4 (0.222 μg/L) was increased 6.4-fold above the mean control value (<0.030 μg/L); however this elevation was due mainly to an individual animal in Group 4 with a cTnI result of 0.77 μg/L, giving a 24.7-fold increase above the mean control value.

**8.3.1.4 Gross pathology and histopathology**

At autopsy, animals in Groups 1 and 2 (Control ground diet and LEAR oil diet, respectively), showed no macroscopic abnormalities. However, in Groups 3 and 4 (Spike oil diet and HEAR oil diet, respectively), the hearts of all animals appeared noticeably pale, with the ventricular myocardium almost cream in colour and the coronary vessels were very prominent. The atria were not affected in this way and were normal in colour and appearance. In some animals in Group 3 (1 of 5) and Group 4 (5 of 5), the liver also was pale in colour.

The feeding of high EA-containing diets (Group 3, Spike oil diet; Group 4, HEAR oil diet) had an effect on the relative heart weights (Figure 8.1). The mean relative heart weights were significantly increased to 5.32 g/kg (a 15.9 % increase; P<0.05) and 5.62
g/kg (a 22.4 % increase; P<0.001) for Groups 3 and 4, respectively, compared with the (Group 1) controls (4.59 g/kg). The mean relative heart weight of animals in Group 2 was also increased (to 5.14 g/kg, a 12.0 % increase) but this elevation was not statistically significant.

The incidence of microscopic findings in the heart is summarised in Table 8.8. All animals treated with high levels of EA (Groups 3 and 4) showed a diffuse microvesicular myocardial vacuolation (Figure 8.2), graded as minimal to moderate. Cardiac lesions were predominantly present in the subepicardial area of the apical portion of the heart. The vacuoles were arranged in linear rows parallel to the cellular long axis and Oil Red O (ORO) staining demonstrated that the vacuoles contained neutral lipid (Figure 8.3). Of the animals with lipidosis, the majority also demonstrated focal and multifocal myocardial degeneration/necrosis, with a mononuclear cell infiltrate (4 out of 5 in Group 3; 4 out of 5 in Group 4). Myocardial degeneration/necrosis was characterised by increased eosinophilia, fragmentation of the myofibres and nuclear pyknosis (Figure 8.4). The inflammatory cells present consisted mainly of macrophages, with some lymphocytes. It is considered that there were no clear differences in the severity of the cardiac lesions (both myocardial vacuolation, and also degeneration/necrosis) in the Group 3 (Spike oil diet) compared with the Group 4 (HEAR oil diet) rats (Table 8.8). No cardiac lesions were identified in the hearts of Group 1 (Control ground diet) and Group 2 (LEAR oil diet) animals; although some rats in Group 2 had shown evidence of increased cTnI values (Table 8.7).

8.3.2  Experiment 2, preliminary 96 h gavage study

OBJECTIVE: To investigate whether myocardial lipidosis can be induced via the gavage administration of HEAR oil over a 96 h period.

8.3.2.1  Clinical signs and body weight changes

All animals in all groups (n=5 rats in each of 7 experimental groups; Table 8.1) remained healthy over the period of treatment. By 96 h however, some animals treated with HEAR oil by gavage (Groups 2 to 6) or HEAR oil diet (Group 7) had oily fur. A proportion of animals in Groups 2 to 6 also displayed piloerection. In addition, there was some evidence of urogenital soiling and soft faecal pellets in Group 6 (5 gavage
administrations of HEAR oil in a 24 h period) throughout the treatment period. Body weights were determined every 24 h over the 96 h treatment period, but there was no clear evidence that treatment had any adverse effect on body weight gain, although the data was quite variable (Table 8.9).

8.3.2.2 Diet and water consumption

Diet consumptions were carried out over 24 h and 72 h periods on 3 occasions before the animals were treated with LEAR oil and HEAR oil by gavage, or HEAR oil by dietary administration, and on 4 occasions during the treatment period (Table 8.10A). During the pre-treatment period (-120 h to 0 h), all groups were fed Control ground diet. During the 0 h to 96 h treatment period, Groups 1 to 6 were fed Control ground diet, and Group 7 HEAR oil diet. The results in Table 8.10A show that, in general, the amounts of diet consumed by all groups was similar; there appears to be no clear reduction in diet consumption in rats treated with HEAR oil by gavage or in the diet.

Water consumptions were carried out during the period of gavage and dietary administration of LEAR oil and HEAR oil (0 h to 96 h; Table 8.10B). The quantities of water consumed by each group was generally similar, suggesting that LEAR oil and HEAR oil treatment had no significant effect on water consumption.

8.3.2.3 Serum clinical chemistry

Results are summarised in Table 8.11. In comparison with Group 1 (LEAR oil by gavage), the administration of HEAR oil by gavage and in the diet for a period of 96 h did not affect serum levels of urea, CREAT, albumin, sodium, potassium, chloride, TRIG, NEFA, ALT, AST, CK, LD, GLD or aldolase in a statistically significant manner.

However, mean serum TP was increased above the mean level of 48.3 g/L (Group 1) in all HEAR oil-treated groups (gavage and dietary administration) and this elevation was significant (P<0.001) in Groups 4 and 7. In addition, mean levels of serum CHOL were significantly increased (P<0.05 or P<0.001) in all HEAR oil-treated groups (Groups 2 to 7). However, the increases in serum TP and CHOL in the gavage dosed HEAR oil groups (Groups 2 to 6) did not appear to be related to the number of gavage doses administered in a 24 h period.
In Group 1 (LEAR oil by gavage), the levels of serum cTnI were <0.03 µg/L for all animals (Table 8.12). In Groups 2 to 6 (HEAR oil by gavage), a total of 8 individual animals had serum cTnI values above 0.03 µg/L, and 4 of these rats had levels above 0.10 µg/L, out of a total of 23 individual serum samples assayed. In Group 7 (HEAR oil diet), 3 of 5 animals demonstrated cTnI values above 0.03 µg/L. The four highest individual values of cTnI recorded in Groups 2 to 7 were 1.19 (Group 3), 0.48 (Group 5), and 0.32 and 0.49 (Group 7) µg/L, fold increases of 38.7, 15.0, 9.7 and 15.3, respectively. However, statistical analysis demonstrated that the mean values for cTnI in the HEAR oil groups (Groups 2 to 7) were not significantly increased above the Group 1 values. Therefore, although the findings are inconsistent, the administration of HEAR oil by gavage induced increases in cTnI levels in some individual animals, but these elevations showed no clear relationship with the number of gavage doses of HEAR oil administered during a 24 h period. In addition, the administration of HEAR oil in the diet (Group 7) also induced some individual increases in serum levels of cTnI, giving a group mean cTnI concentration of 0.196 µg/L.

8.3.2.4 Gross pathology and histopathology

At autopsy, animals in Group 1 (LEAR oil by gavage, 1 dose per 24 h) showed no macroscopic abnormalities. However, in the HEAR oil gavage groups (Groups 2 to 6) and the HEAR oil diet group (Group 7), the hearts from all rats were pale in appearance. The paleness of the myocardia was most pronounced in Group 7 (HEAR oil diet), and the degree of paleness in Groups 2 to 6 was similar. The atria of the hearts of HEAR-oil treated rats were normal in appearance. The coronary vessels were also prominent in all HEAR oil groups. In Group 6, the intestines were hyperaemic.

Gavage and dietary administration of HEAR oil appeared to induce some increase in relative heart weights, but this was only statistically significant in Group 3, where the relative weight was increased from 3.77 g/kg (Group 1) to 4.67 g/kg (a 23.9 % increase; P<0.001; Figure 8.5).

The incidence of microscopic findings in the hearts is summarised in Table 8.13. All animals treated with HEAR oil by gavage or HEAR oil diet (Groups 2 to 7) had a diffuse microvesicular myocardial vacuolation, graded as minimal to moderate. ORO staining was performed in 1 out of 5 animals in Groups 1, 2 and 3, confirming the
vacuoles contained neutral lipid. The vacuoles were arranged in linear rows, parallel to the myofibre long axis. The vacuolation observed in the animals dosed with HEAR oil by gavage (Groups 2 to 6) was generally milder in severity than that observed in the animals fed the HEAR oil diet (Group 7). This finding therefore correlates with the macroscopic observations. There appeared to be no clear connection between the number of gavage doses of HEAR oil administered in a 24 h period in Groups 2 to 6 and the severity of the myocardial vacuolation. A proportion of the animals with lipidosis also showed focal and multifocal myocardial degeneration/necrosis with a mononuclear cell infiltrate (Table 8.13). The myocardial vacuolation and degeneration/necrosis were predominantly located in the subepicardial and central mural area in the apex of the heart.

Transmission electron microscopy (TEM; Chapter 2.10) was used to examine the heart of an animal from Group 1 (LEAR oil by gavage) and an animal from Group 7 (HEAR oil diet). In the sample from the HEAR oil diet animal, the myocardial fibres contained numerous lipid droplets of variable size, mostly between 500 nm and 2 μm in diameter (Figure 8.6). The lipid droplets were situated mainly within the extracellular sarcoplasm, in close association with the mitochondria and in places, the myofibres appeared to be displaced by the larger lipid droplets, giving a rather disorganised appearance. However, the mitochondria did not appear to be structurally disrupted by the presence of the lipid droplets (Figure 8.7).

8.3.3 Experiment 3, 96 h gavage dosing evaluation study

OBJECTIVE: To evaluate the comparative effectiveness in the induction of cardiac lipidosis of gavage dosing Spike oil and HEAR oil over a 96 h study period, using administration regimens over an 8 h period (09.00 h to 17.00 h) and over a 12 h period (09.00 h to 21.00 h).

8.3.3.1 Clinical signs and body weight changes

All animals in the 8 experimental groups (n=5/group; Table 8.2) remained generally healthy throughout the 96 h treatment period. However, animals treated with EA-containing oils (Spike oil and HEAR oil) by gavage (Groups 5 to 8) showed loose stools
by 24 h, which developed into mild diarrhoea by 48 h. Also, from 72 h, animals in Groups 1 to 4 (oil-enriched diets) had oily fur and soft stools. The stools were more loose/less formed in Groups 5 to 8 compared with Groups 1 to 4.

Body weights were recorded on 4 occasions from -120 h to 0 h (at -120, -96, -72 and 0 h), and on 4 occasions from 24 h to 96 h (24, 48, 72 and 96 h; Table 8.14A, 8.14B). A comparative examination of the mean body weights of animals in each treatment group shows that the dosing of animals with Spike oil by gavage (Group 5 and 6) appeared to have an effect on body weight gain. The mean percentage body weight increase for Group 1 (Sunflower oil diet) over a 24 h period from 0 h to 96 h was 4.83 %; whereas the increases for Group 5 (Spike oil by gavage, “social hours”) and Group 6 (Spike oil by gavage, “unsocial hours”) were 1.70 % and 1.60 %, respectively (Table 8.14B). The values of 1.70 % and 1.60 % also contrast with the group mean 24 h percentage increases for Groups 5 and 6 before the treatment period; these percentages were 7.48 and 7.14, respectively. The mean percentage body weight increases over a 24 h period from 0 h to 96 h for Groups 2, 3 and 4, and 7 and 8 were generally similar to the Group 1 value of 4.83 %.

8.3.3.2 Diet and water consumption

Diet consumptions were carried out in Groups 1 to 4 (i.e. treatments with dietary oils) on 4 occasions over 24 h periods during the 0 h to 96 h treatment period (Table 8.15A). During the 96 h treatment period, Group 1 were fed Sunflower oil diet, Group 2 were fed LEAR oil diet, Group 3 were fed Spike oil diet and Group 4 were fed HEAR oil diet. The results in Table 8.15A suggest that the feeding of high EA-containing diets (Groups 3 and 4) may have had a very small effect in reducing diet consumption (mean 24 h consumptions per rat were 13.1, 13.4, 11.4 and 12.1 g for Groups 1 to 4, respectively). Furthermore, when the relative diet consumptions were calculated (Chapter 2.2) in g diet consumed/kg body weight/24 h period (Table 8.15B), it can be seen that there is some evidence that the quantities of diet consumed by Group 3 (Spike oil diet) and Group 4 (HEAR oil diet) were slightly reduced compared to Group 1 (Sunflower oil diet).

Water consumptions were carried out over 24 h and 72 h periods on 3 occasions (-120 h to 0 h) before animals were placed on dietary oil treatments (Groups 1 to 4), or
administered high EA-containing oils by gavage (Groups 5 to 8). Consumptions were also conducted on 4 occasions during the 96 h treatment period (0 h to 96 h; Table 8.15C). The results suggest that dietary and gavage oil treatment had no significant effect on water consumption.

8.3.3.3 Serum clinical chemistry

Results are summarised in Table 8.16. In comparison with Group 1 (Sunflower oil diet), the administration of LEAR oil, Spike oil or HEAR oil in the diet (Group 2, 3 and 4, respectively) or by gavage (Groups 5, 6, 7 and 8, respectively) did not significantly affect mean serum levels of CREAT, albumin, TP, potassium, CHOL, HDL-CHOL, TRIG, NEFA, LD or aldolase. Mean serum urea levels were increased in all groups (Groups 2 to 8) in comparison with Group 1, however this was only significant (P<0.05) in Group 7 (HEAR oil by gavage, “social hours”). This increase was 46.1 % above the mean Group 1 value.

Group mean serum levels of sodium were raised significantly (P<0.05) in Group 6 (Spike oil by gavage, “unsocial hours”) and Group 7 (HEAR oil by gavage, “social hours”). However, the increases in both groups were only 2.3 % above Group 1 levels. There were no significant changes in serum sodium in the other treatment groups, although the mean levels were increased in all other groups in comparison with Group 1. Serum chloride levels in Groups 5 and 6 were decreased significantly (P<0.001 and P<0.05, respectively) in comparison to the Group 1 animals. These values represented decreases of 5.6 % (Group 5) and 4.4 % (Group 6) compared with Group 1. No significant changes in serum chloride were observed in Groups 2, 3, 4, 7 or 8, although there appeared to be an overall trend for values to be reduced compared with Group 1.

Group mean ALT activities were elevated above the Group 1 mean value in Groups 3 to 8, however these increases were only significant in Group 5 (P<0.05; Spike oil by gavage, “social hours”) and Group 6 (P<0.001; Spike oil by gavage, “unsocial hours”). The serum ALT activities in these groups were increased 1.7-fold (Group 5) and 3.3-fold (Group 6) above the mean Group 1 level. There was a trend for serum AST activity to be increased above the mean Group 1 level in the gavage-treated animals (Groups 5 to 8); however this was significant only in Group 6 (a 50.8 % increase; P<0.05). Serum CK activity was significantly reduced (P<0.05 or P<0.001) below the Group 1 mean.
value in the HEAR oil diet and HEAR oil gavage-treated groups (Groups 4, 7 and 8, respectively). These values represented decreases of 47.9 % (Group 4), 48.2 % (Group 7) and 77.8 % (Group 8) in comparison with Group 1. Levels of serum GLD were increased in all groups compared with the mean Group 1 level, however increases were only significant in Groups 4, 5 and 6 (P<0.001 for all 3 groups).

Levels of serum cTnI were assayed with the Bayer Centaur CP (Table 8.16). Group mean serum cTnI concentrations remained at Group 1 levels (0.010 µg/L) in all animals in all groups, except for Groups 3 and 4, where there were very small increases in mean cTnI levels. The Group 3 mean cTnI level was 0.018 µg/L and the Group 4 mean cTnI level was 0.026 µg/L. The increase in the Group 3 mean was due to 2 animals in the Group (n=4 samples) with cTnI levels of 0.02 µg/L and 0.03 µg/L. The remaining 2 animals in Group 3 had base line cTnI values of <0.01 µg/L and 0.01 µg/L. In Group 4, 2 of 5 animals had cTnI concentrations of 0.02 µg/L and 0.08 µg/L; the other 3 animals in the Group all had base line cTnI concentrations of 0.01 µg/L.

8.3.3.4 Gross pathology and histopathology

At autopsy, animals in Group 1 (Sunflower oil diet) and Group 2 (LEAR oil diet) showed no macroscopic abnormalities. However, the hearts of all animals treated with high EA diets (Groups 3 and 4) and EA-containing oils by gavage (Groups 5 to 8) were pale in appearance and the coronary blood vessels were prominent. The degree of paleness was greatest in the HEAR oil diet animals (Group 4), with the other EA-treated groups (Group 3, 5, 6, 7 and 8) displaying a similar grade of pallor. No other macroscopic abnormalities were observed in any animal.

Treatment of rats with oil-enriched diets, or EA-containing oils by gavage, had no significant effect on relative heart weights in any group (Figure 8.8).

The incidence of microscopic findings is summarised in Table 8.17. All animals treated with Spike oil or HEAR oil in the diet (Group 3 and 4, respectively) or by gavage (Groups 5 and 6, and 7 and 8) had evidence of myocardial vacuolation consistent with lipid accumulation, except for 1 of 5 rats in Group 5 and 1 of 5 rats in Group 6. The severity of the lipidosis was graded as minimal to moderate, and the most severe vacuolation was seen in the animals given Spike oil or HEAR oil in the diet (Groups 3 and 4). This corresponds with the macroscopic observations and the presence of some
individual elevated cTnl levels in Groups 3 and 4 (Table 8.16). There was no clear difference in the severity of vacuolation between Groups 5, 6, 7 and 8 (treated with Spike oil or HEAR oil by gavage). Furthermore, there was no clear difference between Spike oil and HEAR oil gavaged at 09.00 h, 13.00 h and 17.00 h (“social hours”) in Group 5 and 7, respectively, in comparison with these 2 oils gavaged at 09.00 h, 15.00 h and 21.00 h (“unsocial hours”) in Groups 6 and 8, respectively. Enrichment of the diet with Sunflower oil (Group 1) or LEAR oil (Group 2) did not result in myocardial lipid accumulation.

No evidence of myocardial degeneration/necrosis was observed in the hearts of animals from any group in the present study.

8.3.4 Experiment 4, time course study

OBJECTIVE: To assess the time course of cardiac lesion development and to identify histopathological changes occurring in skeletal muscle and liver over a 96 h period.

8.3.4.1 Clinical signs and body weight changes

Experimental design is set out in Table 8.3. All animals (n=5 per group) in the 13 groups generally remained healthy over the period of treatment. However, from 24 h onwards, animals treated with Spike oil by gavage had oily fur. This change was also noted in HEAR oil diet-treated animals from 48 h into the treatment period. Some evidence of gastrointestinal (GI) disturbance (e.g. urogenital soiling, soft faecal pellets and mild diarrhoea) was present in Spike oil-treated animals (gavage) at 24 h and continued until 96 h. These clinical signs also appeared in animals fed the HEAR oil diet at 48 h. The degree of GI disturbance was more significant in the HEAR oil diet-treated animals.

Animals were weighed on 4 occasions in the pre-treatment period from -120 h to 0 h, and on 4 occasions (at 24, 48, 72 and 96 h) during the treatment period (Table 8.18). The body weight data suggest that treating rats by gavage with Spike oil on 3 occasions every 24 h may have adversely affected body weight gain. The percentage increases in group mean body weight for each group at 24, 48, 72 and 96 h, in relation to the group mean body weight at 0 h (the beginning of the treatment period), were: 5.0 %, 13.2 %,
18.4 % and 26.1 % (control); 1.0 %, 6.1 %, 6.8 % and 0.3 % (Spike oil by gavage); 2.0 %, 8.7 %, 14.1 % and 26.4 % (HEAR oil diet), respectively.

8.3.4.2 Water consumption

Water consumptions were carried out over 24 h and 72 h periods on 3 occasions (-120 h to 0 h) before EA treatment commenced and on 4 occasions during the 96 h treatment period (at 24, 48, 72 and 96 h). The results are presented in Table 8.19. The data suggests that the administration of EA by gavage or in the diet had no major effect on water consumption.

8.3.4.3 Serum clinical chemistry

Results are summarised in Table 8.20A to 8.20E. In animals autopsied at 24, 48, 72 and 96 h, in comparison with the animals fed control diet (extruded Global Rodent Diet), the administration of EA by gavage (Spike oil) or in the diet (HEAR oil diet) did not appear to affect serum values of CREAT, potassium, chloride, TRIG, NEFA, AST, CK or LD at any time point. However, at the 24 h autopsy (Table 8.20B), mean serum urea levels were increased slightly above the control level in both EA-treated groups. This was significant only in the Spike oil gavage group (P<0.05). However, the increases were only 24.1 % and 19.8 % above the mean control value for the Spike oil by gavage and HEAR oil diet groups, respectively. No effect was observed on the mean serum urea levels at any other autopsy time point.

Mean serum albumin levels were decreased significantly (P<0.05 at both time points) in the Spike oil by gavage group at 48 h (Table 8.20C) and 96 h (Table 8.20E). However the mean decreases were only 6.0 % (48 h) and 7.0 % (96 h) below the controls. The feeding of the HEAR oil diet did not affect serum albumin levels at any time point. Mean levels of serum TP were significantly decreased in the Spike oil gavage group from the 48 h autopsy onwards (P<0.001, 48 h; P<0.001, 72 h; P<0.05, 96 h). Levels of TP were not significantly affected in rats fed the HEAR oil diet.

Mean serum sodium concentrations were increased significantly in both EA-treated groups at 24 h (P<0.05 for each group; Table 8.20B). The group mean values were 147.2 and 146.8 mmol/L for the Spike oil by gavage and the HEAR oil diet groups, respectively, in comparison to the control level of 145.4 mmol/L. Group mean serum
sodium levels were not altered by EA treatment at the 48 h or 72 h autopsy; however, at 96 h, the group mean concentrations were decreased by 0.7 and 1.4 % for the Spike oil by gavage and the HEAR oil diet groups, respectively. The decrease at 96 h in the HEAR oil diet group mean was statistically significant (P<0.05) (Table 8.20 E).

The group mean level of CHOL in the HEAR oil diet group was decreased, compared with the mean control value, at the 24 h autopsy (P<0.05; Table 8.20B). Mean CHOL levels were not affected at the other autopsy time points.

Serum ALT activities were increased significantly in both EA-treated groups at 24 h (Spike oil by gavage, P<0.001; HEAR oil diet, P<0.05; Table 8.20B). These increases were 48.9 % (Spike oil by gavage) and 26.6 % (HEAR oil diet) above the mean control value. At 48 h, the group mean ALT level for the Spike oil by gavage group was increased 26.8 % above the control mean; however this increase was not significant (Table 8.20C). No changes in serum ALT levels were observed at the 72 h autopsy; however, at 96 h, the group mean ALT for the Spike oil gavage group was significantly raised (a 66.9 % increase; P<0.001) above the controls (Table 8.20E). The HEAR oil diet ALT group mean for 96 h was increased 31.0 % above the controls; however this elevation was not statistically significant.

The group mean activity of GLD at 24 h in the HEAR oil diet group was increased significantly (P<0.05) in comparison with the control value (a 33.2 % increase; Table 8.20B). No changes were seen in the Spike oil by gavage group at this time point. At the 72 h autopsy (Table 8.20D), the Spike oil by gavage GLD group mean was increased 24.0 % above the mean control activity; however, this increase was not significant. By the 96 h autopsy point, only the Spike oil by gavage GLD group mean was increased significantly (P<0.05) above the mean control value; this represented a 26.8 % increase (Table 8.20E).

Mean serum aldolase activity was significantly increased in both EA-treated groups at the 24 h autopsy time point (Spike oil by gavage, P<0.05; HEAR oil diet, P<0.001; Table 8.20B). Levels were significantly increased in both groups also at 72 h (Spike oil gavage, P<0.001; HEAR oil diet, P<0.05). Group mean aldolase levels were not significantly affected by EA administration at 48 h or 96 h.
The mean serum cTnI concentrations assayed with the Bayer Centaur CP remained at control baseline levels (0.010 μg/L) in both EA treatment groups, at all autopsy time points, except for the 24 h autopsy, where the HEAR oil diet group mean was increased to 0.030 μg/L (Table 8.20B). The increase in the group mean was due to 1 animal, of the 5 animals in the group, having a cTnI concentration of 0.11 μg/L, that is, a 10.0-fold increase above the base line control value.

8.3.4.4 Gross pathology and histopathology

Animals in the control groups (Groups 1, 2, 5, 8 and 11) autopsied at 0, 24, 48, 72 and 96 h of treatment showed no macroscopic abnormalities at any time point. However, in 2 of 5 animals in the HEAR oil diet group at 24 h, the coronary vessels of the heart appeared more prominent than in the control animals; the external appearance of the hearts of the 5 rats in the Spike oil gavage group were normal at this time point. At 48 h, the heart in 1 of 5 animals in each group of the EA-treated animals appeared pale in appearance with prominent blood vessels. By 72 h, this macroscopic change was present in 1 of 5 animals in the Spike oil by gavage group, and in all 5 animals in the HEAR oil diet-fed group. At the final 96 h autopsy point, the adrenal glands were pale in 2 of 5 animals treated with Spike oil by gavage, however this change was not observed in the HEAR oil diet group animals. The hearts of the Spike oil by gavage animals were normal in appearance at 96 h; however, the hearts of the HEAR oil diet animals were all pale with prominent coronary vessels.

The administration of EA by gavage, or in the diet, had no significant effect on relative heart weights at any of the autopsy time points (Figure 8.9). At the 96 h time point, the liver, kidneys, adrenal glands, spleen, thymus and testes were also removed from the 5 animals in each treatment group and weighed. The mean relative weights of these organs are presented in Table 8.21. EA treatment did not affect the mean relative weights of the liver, kidneys, adrenal glands, thymus or testes; however in the Spike oil by gavage group, the mean relative spleen weight was decreased significantly (P<0.05). This was a 25.5 % decrease in comparison with the mean control weight.

The incidence of microscopic findings in the hearts of the EA-treated animals at 0, 24, 48, 72 and 96 h is presented in Table 8.22. A number of animals treated with Spike oil by gavage or the HEAR oil diet had a diffuse microvesicular vacuolation of the
myofibre cytoplasm consistent with lipid accumulation, graded as minimal to moderate. Lipidosis was present in 4 of 5 animals in both the Spike oil by gavage and the HEAR oil diet groups (Groups 3 and 4, respectively) after 24 h of treatment. In the HEAR oil diet-treated animals (Groups 4, 7, 10 and 13), the incidence and severity of the myocardial changes increased with the duration of dosing. However, findings in the Spike oil by gavage animals (Groups 3, 6, 9 and 12) were more variable, and there was no clear increase in the incidence or severity of lipidosis with the duration of dosing after the 24 h time point. The administration of Spike oil by gavage or HEAR oil in the diet for periods up to 96 h did not induce myocardial degeneration/necrosis at any time point (Table 8.22). The individual animal in the HEAR oil diet group autopsied at 24 h with a cTnI value of 0.11 µg/L showed no microscopic changes consistent with myocardial degeneration/necrosis; 4 step sections from this animal were examined.

Samples of skeletal muscle (quadriceps femoris) and liver were examined at the 86 h autopsy from animals given the Control diet, Spike oil by gavage, or HEAR oil diet. Results for the skeletal muscle sections are presented in Table 8.23. Skeletal myofibre vacuolation, graded as minimal, was observed in the 5 animals administered the Control diet (Group 11). Increased skeletal myofibre vacuolation, graded as mild, was evident in 1 of 5 animals in the Spike oil by gavage group (Group 12); the remaining 4 animals were graded as minimal. All 5 animals in the HEAR oil diet group (Group 13) showed myofibre vacuolation graded as mild. Vacuoles were present mainly in the small calibre fibres, and were larger and more numerous in the HEAR oil diet group (Group 13) compared with the Control diet animals (Group 11) or in the 4 Spike oil by gavage group animals (Group 12), graded as minimal (Figure 8.10). Vacuoles were evenly dispersed in cross sections of the small calibre fibres and were arranged linearly in longitudinal sections along the fibres. The vacuoles in animals of all groups contained neutral lipid, as confirmed by ORO staining (Figure 8.11).

Sections of liver from all animals at 96 h showed no microscopic abnormalities when stained with H&E. However, ORO staining demonstrated minimal staining in 1 of 5 animals in the Spike oil by gavage group (Group 12), and in 2 of 5 animals in the HEAR oil diet group (Group 13). However, this amount of lipid in liver sections is within the normal limits of historic control animals from other studies and is considered to be a background change. Animals fed the Control diet (Group 11) did not show minimal staining in the liver.
8.4 DISCUSSION

EA is a long chain fatty acid found at high concentrations in rapeseed oil. The dietary administration of HEAR oils has been shown to cause histopathological alterations in the heart in a number of species, including the rat, the pig, the monkey, and various species of bird (Roine et al., 1960; Beare-Rogers and Nera, 1972; Charlton et al., 1975). LEAR oils may also cause such cardiac changes: the long term feeding of LEAR oil has been associated with the development of myocardial lipidosis (Abdellatif and Vles, 1973; Charlton et al., 1975; Chien et al., 1983).

The main aim of the present studies was to establish a model of cardiac lipidosis in the male Hanover Wistar rat. A survey of the literature showed that all previous studies on EA cardiotoxicity in the rat used dietary administration regimens of HEAR oil, and other routes of administration have not been investigated. However, dietary administration studies have some disadvantages (e.g. they are very labour-intensive) and consequently we wished to investigate, firstly, whether the gavage administration of HEAR oil would induce cardiac lipidosis in the rat. Secondly, it was not possible to identify any reports in the literature giving information on serum clinical biochemistry changes as a result of EA-induced cardiotoxicity; therefore we also wished to characterise the changes in a number of clinical chemistry parameters following EA administration.

When attempting to source HEAR oil, it became apparent that although LEAR oil could be obtained easily from commercial suppliers in the UK, in the (relatively) small quantities required (e.g. 500 ml to 5 L), the purchase of such small volumes of HEAR oil commercially was not straightforward. Therefore, as pure EA could be bought from a commercial supplier (Fluka/Sigma-Aldrich), we wished to investigate whether the addition of pure EA to LEAR oil, to give a 45 % (w/w) EA content (i.e. the “spiking” of the LEAR oil with pure EA to produce “Spike oil”), when administered to rats would cause lipidosis in the heart.

4 experiments were carried out. The first assessed whether the dietary administration of HEAR oil and Spike oil would induce cardiac lipidosis in the rat in a 96 h period. The second experiment investigated whether the gavage administration of HEAR oil would induce cardiac lipidosis in a 96 h period. Experiment 3 was designed to determine whether the timetable of gavage administration of Spike oil affected the development of
cardiac lipidosis; in Experiment 4, the time course of the development of lipidosis over a 96 h period was studied.

In the present investigations, EA has been demonstrated to cause cardiac lipidosis and myocardial degeneration/necrosis. The cardiac lesions reported here are well characterised in the literature (Abdellatif and Vles, 1973; Charlton et al., 1975; Yamashiro and Clandinin, 1980; Chien et al., 1983), however the pathogenesis of lesion development observed in the present studies is different to that reported previously. Early reports in the literature demonstrated that the short-term dietary administration of HEAR oil or pure EA in the rat induces a rapid but transient severe fatty infiltration within the myocardium; with longer term feeding, the cardiac lipidosis regresses and may be replaced by myocyte degeneration and ultimately the loss of myocytes, which is followed by fibrous scarring (Abdellatif and Vles, 1970). Lipidosis may be present after 1 day of HEAR oil administration, but this early histopathological change has been reported not to be associated with necrosis (Chien et al., 1983). However, in the present studies (Experiment 1 and 2), myocardial necrosis was present in conjunction with myocardial lipidosis at 96 h.

In the first experiment with EA (Experiment 1, preliminary 96 h feeding study), there was an indication that the feeding of high EA-containing diets (Spike oil diet and HEAR oil diet) had some adverse effect on body weight gain (Table 8.4A, 8.4B), however there appeared to be no major differences in the diet and water consumptions in the various treatment groups (Table 8.5A, 8.5B). Therefore, the experimental diets appeared to be palatable. The apparent digestibility of HEAR oil is reported to be only 83.0 % in the Wistar rat (Beare et al., 1960), compared with 98.8 % in man (Holmes, 1918). In addition, the feeding of HEAR oil has been shown to have growth-retarding effects in the rat, and it is the presence of EA itself that is considered to be the cause (Thomasson and Boldingh, 1955). Abdellatif and Vles (1973) also reported that the feeding of pure EA and HEAR oil for 24 weeks caused a decrease in body weight gain. In addition, rats given a LEAR oil diet also gained less weight than sunflower oil-fed controls (Abdellatif and Vles, 1973). Similar results to those of Abdellatif and Vles (1973) were seen in the present study (Experiment 1) where the presence of EA in the diet caused a reduction in body weight gain, and this was possibly related to aspects of digestibility.
The feeding of LEAR, Spike and HEAR oils for 96 h (Experiment 1, preliminary 96 h feeding study) caused decreases in serum TRIG levels, with significance (P<0.001) in Group 2 (LEAR oil diet) and in Group 4 (HEAR oil diet) (Table 8.6). In malnutrition in man, serum TRIG levels may be reduced as much as 50 % compared with healthy individuals (Young and Bermes, 2006). In addition, in a study by Kmiec et al. (2005) the fasting of rats for 48 h resulted in a significant decrease in serum TRIG levels. It was seen in the present study that the feeding of high EA-containing diets had an adverse effect on body weight gain; therefore it is possible that the decreases in serum TRIG in these animals (Table 8.6) was due to malnutrition. However, serum TP, albumin and CHOL are also decreased in malnutrition (Young and Bermes, 2006), and no changes were seen in these parameters in the present study (Table 8.6). Moreover, the feeding of a high fat diet may cause an increase in serum TRIG levels (Young and Bermes, 2006). However, Watkins et al. (1995) fed male Wistar rats diets supplemented with corn oil, LEAR oil, HEAR oil or mustard seed oil (mustard seed oil is high in EA) for 8 weeks and it was reported that the feeding of LEAR oil caused a decrease in serum HDL-CHOL levels, whereas the feeding of LEAR and mustard seed oils caused a decrease in serum TRIG values compared with the corn oil-fed (control) animals. There was some evidence in the present study (Experiment 1) that serum levels of NEFA were decreased in response to the feeding of oil-enriched diets (Table 8.6), however the reductions were not statistically significant. Kmiec et al. (2005) showed that serum NEFA levels were increased following the fasting of Wistar rats. It is also possible that the accumulation of neutral lipid within the cardiomyocytes may also account for the drop in circulating TRIG levels (Table 8.6); however there was a significant decrease in serum TRIG values in the Group 2 (LEAR oil diet) animals and these animals did not exhibit cardiac lipidosis (Table 8.6).

In Experiment 1, there were slight increases in serum ALT and AST activities in Group 3 (Spike oil diet) and in Group 4 (HEAR oil diet), with no concurrent increases in serum GLD (Table 8.6). With liver injury, an increase in serum activities of ALT, AST and GLD is observed, and it was possible that the slight increases in ALT and AST observed here (Table 8.6) were due to hepatic damage (Woodman, 1996). Abdellatif and Vles (1970) showed that the feeding of HEAR oil for 2 weeks caused no hepatic changes, however fat accumulation in the liver was observed by Kramer et al. (1973) following the feeding of HEAR oil for 1 and 2 weeks, and also for 16 weeks (Kramer et al., 1979).
At autopsy, the hearts of the high EA-treated rats were often pale in appearance, with the myocardium almost cream in colour. Similar observations were reported by Abdellatif and Vles (1970) in the hearts of rats fed 50% of their dietary calories as HEAR oil for 3 days, or 60% of calories for 2 weeks. In the present studies (Experiments 1 to 4), lipidosis was observed macroscopically in the ventricles rather than in the atria, and microscopically (seen as myocardial vacuolation) mainly in the subepicardial area of the apex of the heart (Figure 8.2, 8.3). Microscopically, Charlton et al. (1975) demonstrated that in the rat, fatty infiltration mainly occurred in the ventricular walls and in the interventricular septum, rather than in the atria. In the present studies, lipid droplets within the myocardium were evident in linear rows parallel to the cellular long axis, and this arrangement has also been reported by others (Bhatnagar and Yamashiro, 1979; Chien et al., 1983). Therefore, the results in the present studies (Experiments 1 to 4) agree with previous findings. Furthermore, in Experiment 1, the severity of the cardiac lesions observed in Group 3 (Spike oil diet) compared closely with the severity grade of cardiac changes in the Group 4 (HEAR oil diet) animals. Therefore, the feeding of Spike oil diet did induce cardiac lipidosis in Experiment 1 (preliminary 96 h feeding study). This appears to be a novel method of inducing lipidosis in the rat.

The relative heart weights of rats treated with Spike oil diet (Group 3) and HEAR oil diet (Group 4) were increased significantly compared with the Group 1 control animals in Experiment 1 (Figure 8.1). The Group 3 and Group 4 animals showed histopathological evidence of cardiac injury, with all animals displaying myocardial vacuolation, and 8 of 10 animals showing myocardial degeneration/necrosis with mononuclear cell infiltration (Table 8.8). It is possible that the fatty infiltration of the myocardium caused an increase in relative heart weight. In addition, the infiltration of inflammatory cells observed as a result of the myocardial degeneration/necrosis may have contributed (with some fluid accumulation) to the increase in relative heart weight in the high EA-treated animals. It was reported that the relative heart weights of rats fed HEAR oil and pure EA for a period of 24 weeks were increased significantly in comparison with animals fed LEAR oil and sunflower oil (Abdellatif and Vles, 1973). Furthermore, when Kramer et al. (1979) fed Sprague Dawley and Chester Beatty rats a HEAR oil diet (20% oil by weight of diet) for 1 and 2 weeks, the relative heart weights of rats fed HEAR oil were higher than animals given corn oil or LEAR oil.
In Experiment 1, although animals fed the LEAR oil diet (Group 2) for 96 h had no cardiac lesions, there was some evidence that serum cTnI levels were raised in some individual animals, with 2 rats having cTnI concentrations of 0.10 and 0.14 µg/L (Table 8.7) (mean control value: <0.030 µg/L). In the LEAR oil diet group (Group 2), it is possible that there was a degree of ultrastructural damage to the cardiomyocytes, producing some clinical biochemical evidence of myocardial injury (i.e. an increase in serum cTnI), but no light microscopic evidence of cardiac injury. Nevertheless, the reasons for the increase in serum cTnI in the animals fed LEAR oil diet in Experiment 1 remain unclear.

If the individual animal cTnI levels are compared with the individual histopathological findings in the hearts of treated rats (Table 8.24), it can be seen that there is no clear correlation between individual cTnI values and the histopathological score of myocardial injury for either myocardial vacuolation or degeneration/necrosis. The 4 highest cTnI values in Group 3 and 4 were 0.09 (rat 12), 0.77 (rat 17), 0.08 (rat 18) and 0.13 (rat 19) µg/L; the severity grades for myocardial vacuolation in these animals were mild, minimal, moderate and mild, respectively; the severity grades for myocardial degeneration/necrosis in these animals were minimal, mild, moderate and minimal, respectively. Furthermore, 2 rats in Group 2 demonstrated cTnI values of 0.10 (rat 9) and 0.14 (rat 10) µg/L, respectively, however these animals showed no cardiac lesions.

Experiment 2 (preliminary 96 h gavage study) was designed to determine whether the gavage administration of HEAR oil over a 96 h period would induce myocardial lipidosis. During the experiment, animals generally remained healthy, however in Group 6 (5 gavage administrations of HEAR oil/24 h; Table 8.1), there was some evidence of GI disturbance, with animals showing soft stools and urogenital soiling. Repeated oral administrations of vegetable oils in the rat have been associated with diarrhoea in other studies (Turton, 2007).

In Experiment 2 at the 96 h autopsy, serum clinical chemistry revealed increases in serum TP and CHOL in all groups treated with HEAR oil (Groups 2 to 7; Table 8.11). Unlike Experiment 1 (preliminary 96 h feeding study) however, no changes in serum TRIG were observed in any group. Moreover, the increases in serum TP and CHOL in Experiment 2 were not seen in Experiment 1.
Table 8.25 sets out a comparison between individual animal cTnl values and the individual myocardial vacuolation scores and myocardial degeneration/necrosis scores. An examination of these findings shows, firstly, that of a total of 35 rats, complete data is available for 32 animals. Of these 32 rats, those animals with a base line cTnl value of <0.03 or 0.03 μg/L (n=21), have a mean myocardial vacuolation score of 1.0, and a mean myocardial degeneration/necrosis score of 0.2. There are 4 rats with cTnl values greater than 0.03 and less than 0.10 μg/L; the mean myocardial vacuolation score of these animals is 1.5, and a mean myocardial degeneration/necrosis score of 0.0. Finally, there are 7 rats with cTnl values greater than 0.10 μg/L; the mean myocardial vacuolation score of these 7 animals is 2.7, and the mean myocardial degeneration/necrosis score is 1.2. Looking at the results on this way, it is considered that there is some evidence to suggest that animals with higher cTnl values tend to have higher vacuolation scores and these animals also have higher degeneration/necrosis scores.

Secondly, of the 32 rats with complete data in Experiment 2 (preliminary 96 h gavage study), 4 animals have a vacuolation score of 0 (i.e. NAD), and 28 rats have vacuolation scores of 1, 2 and 3. Of these 28 rats with positive vacuolation scores, 9 animals also have degeneration/necrosis scores of 1 or 2 (i.e. are positive for degeneration/necrosis). This finding may indicate a sequence in the pathogenesis of the lesions, i.e. that vacuolation may lead to degeneration/necrosis. (Note: there is only 1 animal, rat number 3, of the 32, which is positive for degeneration/necrosis but is negative for vacuolation.).

In Experiment 2, the gavage administration of HEAR oil and the feeding of HEAR oil diet for 96 h resulted in myocardial lipidosis in all animals (Table 8.13). Myocardial vacuolation was generally milder in severity in the gavage-treated groups compared with the HEAR oil diet-fed animals. All animals in Groups 2 to 7 with degeneration/necrosis also showed vacuolation (Table 8.13), and moderate vacuolation (grade 3) was in all cases accompanied by degeneration/necrosis. The majority of animals (12 of 15) with minimal (grade 1) or mild (grade 2; 7 of 10) vacuolation showed no evidence of degeneration/necrosis. Myocardial lesions were located predominantly in the subepicardial and central mural area in the apical portion of the heart. The lipid droplets were arranged in linear rows, parallel to the myofibre long axis, as was seen in the previous study (Experiment 1, preliminary 96 h feeding study); this pattern of lipid droplet arrangement has also been reported by other investigators (Bhatnagar and Yamashiro, 1979; Chien et al., 1983).
In Experiment 2, TEM was used to examine the heart of a single control animal from Group 1 (LEAR oil by gavage) and the heart of one animal from Group 7 (HEAR oil diet). Ultrastructural examination revealed that the myocardial fibres in the heart of the HEAR oil diet-treated rat contained lipid droplets varying in size between 500 nm and 2 µm in diameter (Figure 8.6). Sauer and Kramer (1983) report that the lipid droplets observed in myocardial lipidosis following the feeding of HEAR oil have no discernible membrane, and vary in diameter up to approximately 8 µm. A close examination of the lipid droplets observed using TEM in the present study also showed that the droplets are not membrane-bound (Figure 8.6, 8.7). Previous ultrastructural studies on EA-induced cardiac lipidosis in the rat have shown that lipid droplets were found in close association with mitochondria (Chien et al., 1983) and this localisation of droplets was also noted in the present study (Figure 8.7). However, other workers report that with the long-term feeding of high EA-containing diets, the mitochondria may become structurally distorted or increase in number (Bhatnagar and Yamashiro, 1979; Yamashiro and Clandinin, 1980). However in Experiment 2 (preliminary 96 h gavage study), there was no evidence of structural disruption or distortion of the mitochondria. Nevertheless, no quantitative study was performed on the TEM samples from Experiment 2 to identify whether EA treatment caused an alteration in the number of mitochondria in the cardiac myofibres.

From the information gained from Experiment 2, Experiment 3 (96 h gavage dosing evaluation study) was performed to evaluate the comparative effectiveness of the induction of cardiac lipidosis via the gavage dosing of Spike oil and HEAR oil using 2 administration regimens (over an 8 h or a 12 h period), in comparison with the feeding of Spike oil diet and HEAR oil diet (Table 8.2). The administration of EA-containing oils by gavage (Groups 5 to 8) caused some GI disturbance at 24 h, as was seen in the previous study (Experiment 2, preliminary 96 h gavage study); GI tract changes were also seen in the experimental diet-fed animals (Groups 1 to 4) at 72 h. The GI disturbance was most significant in the gavage-treated animals (Groups 5 to 8). The gavage administration of Spike oil (Group 5 and 6) also appeared to adversely affect body weight increases, but no body weights changes were seen in the other treatment groups compared with the sunflower oil diet-fed (control) animals (Table 8.14B).

In Experiment 3, at the 96 h autopsy, increases in serum urea above the mean levels of the sunflower oil diet-fed control animals were observed in all groups. Elevations in
serum urea levels may be due to kidney damage and decreased renal clearance (Lyman, 1986), but no confirmatory changes were observed in serum CREAT values. Serum urea concentration is known to be affected by non-renal factors, such as a high-protein diet, increased protein catabolism, GI bleeding and myocardial infarction (MI); however in these cases, serum CREAT may be normal (Stonard, 1996; Lamb et al., 2006). Therefore, in the present study (Experiment 3), there may have been non-renal factors contributing to the increase in serum urea. However, Abdellatif and Vles (1973) fed Wistar rats pure EA or HEAR oil for 24 weeks, and renal histopathological changes (tubular dilatation, proteinaceous cast formation, interstitial foci of necrosis) were observed. Although no histology was performed in Experiment 3 other than on the heart, it is possible that the feeding and gavage dosing of EA-containing oils caused damage to the kidney.

In Experiment 3, there were some increases in serum sodium values at 96 h, with statistical significance in Group 6 and Group 7 (Spike oil by gavage, HEAR oil by gavage, respectively; Table 8.16). Furthermore, the serum chloride levels were decreased significantly in Groups 5 and 6 (Spike oil by gavage). Hypernatraemia and hypochloraemia may be observed when there is GI fluid loss (York and Evans, 1996). Animals treated with EA-containing oils by gavage (Groups 5 to 8) did have some clinical evidence of GI disturbance, and this effect may have contributed to the changes seen in the serum electrolytes.

In Experiment 3, serum ALT values were increased in all groups at 96 h above the mean level in the Group 1 sunflower oil diet animals (Table 8.16); serum AST activities were also increased significantly in Groups 5 and 6 (Spike oil by gavage, “social hours” and “unsocial hours”, respectively) (Table 8.16). Furthermore, serum GLD levels were increased in all groups above the Group 1 (control) value. Increases in ALT, AST and GLD are indicative of hepatocellular injury (Woodman, 1996). Increases in ALT and AST were observed in Experiment 1 (preliminary 96 h feeding study); therefore, EA administration by gavage or in the diet may have caused some damage to the livers of treated animals. The liver has been reported to be a target organ for the toxicity of EA (Kramer et al., 1973; 1979). However, no hepatic lesions were identified in Experiment 4 in EA-treated animals, apart from minimal lipid deposition which was considered to be within normal background limits.
There were some small increases in Experiment 3 in serum cTnl concentrations in Group 3 (Spike oil diet) and in Group 4 (HEAR oil diet); however the highest individual cTnl level was 0.08 μg/L, in a rat from Group 4. Microscopic examination of the hearts of treated animals revealed the presence of myocardial vacuolation consistent with lipid accumulation in all animals given Spike oil diet or HEAR oil diet (Groups 3 and 4), and this change was seen in the majority of rats given Spike oil or HEAR oil by gavage (Groups 5 to 8) (Table 8.17). The vacuolation was most severe in the Spike oil diet and in the HEAR oil diet-fed animals, and there was no clear difference between the degree of vacuolation in the various gavage-treated groups. In addition, there was no evidence of myocardial degeneration/necrosis in any animal in Experiment 3.

The time course of the development of myocardial lipidosis was investigated in Experiment 4. In this study, animals were given extruded Global Rodent Diet (control), Spike oil by gavage (3 times per 24 h period) or HEAR oil diet, for 0 h, 24 h, 48 h, 72 h or 96 h. Myocardial lipidosis was first observed at the 24 h autopsy point in 4 of 5 animals in both the Spike oil by gavage and the HEAR oil diet groups (Table 8.22), and there was no difference in the severity of the lesion between the 2 groups. Abdellatif and Vles (1970) observed myocardial lipidosis after 1 day of feeding HEAR oil as 50 % of the calories in male Wistar rats. Furthermore, Chien et al. (1983) also saw lipidosis after 1 day of feeding HEAR oil as 20 % by weight of diet. The incidence and severity of myocardial lipidosis increased with time in the HEAR oil diet animals in the present study (Experiment 4; Table 8.22), but there was no correlation with the passage of time for the incidence and severity of lesions in the Spike oil by gavage animals. It is possible that the variability in the cardiac histological findings seen in the Spike oil by gavage rats in this experiment and in the previous studies is related to the method of administration. There would possibly be an increase (shortening) in the gut transit time with the gavage administration of oil, and therefore it is possible that there would be less time for the absorption of EA using the gavage method of administration.

At the 96 h autopsy in Experiment 4, other possible target organs of EA toxicity were weighed, and livers and samples of skeletal muscle were taken into fixative for histology examination. Other workers have reported histopathological changes following EA treatment in skeletal muscle, adrenal glands, liver and kidney (Abdellatif and Vles, 1970; Abdellatif and Vles, 1973; Kramer et al., 1973; Charlton et al., 1975). In the present study (Experiment 4, time course study), the group mean relative spleen
weight was decreased at 96 h in the Spike oil by gavage-treated animals. Atrophy of the spleen occurs in old age, in anaemia, in some wasting diseases, and also in coeliac disease, a condition of intestinal malabsorption (Anderson, 1985). However, the feeding of HEAR oil for 2 weeks to male Wistar rats resulted in no observed microscopic changes in the spleen (Abdellatif and Vles, 1970). Histopathological examination of the spleen was not possible in the present study, and therefore the reasons for the decrease in the group mean relative spleen weight observed here are unclear. No other changes in organ relative weights were observed in the Experiment 4.

The histopathological examination of the liver of Spike oil by gavage and HEAR oil diet-treated animals at 96 h showed minimal ORO staining in 1 of 5 Spike oil by gavage and in 2 of 5 HEAR oil diet animals, although these changes were considered to be a background, non treatment-related change. At 96 h, there were slight increases in serum levels of ALT, with statistical significance in the Spike oil by gavage group (P<0.001). In addition, the previous studies (Experiments 1 and 3) showed some changes in liver serum enzymes (ALT, AST, GLD). The lack of clear hepatic histopathological changes is in contrast to previously reported results. For example, Kramer et al. (1973) administered HEAR oil in the diet to male and female Sprague Dawley rats for 1 and 2 weeks, and reported the presence of hepatic lipidosis. In Experiment 4, microscopic examination of samples of the quadriceps femoris at 96 h revealed increased skeletal muscle vacuolation in 1 (of 5) Spike oil by gavage-treated animal, and in all 5 rats fed HEAR oil diet. The vacuoles were demonstrated to contain neutral lipid by ORO staining and were mainly present in the small calibre muscle fibres. It is postulated that these fibres are Type I fibres (slow twitch fibres which derive energy from fat via oxidative metabolism; Widmaier et al., 2005). Nevertheless, it has been reported that the feeding of HEAR oil for 32 weeks to Sprague Dawley rats resulted in no alterations in the skeletal muscle (Abdellatif and Vles, 1973). However, in Wistar rats fed HEAR oil for 2 weeks, slight fatty infiltration of skeletal muscle (diaphragm, masseter and the semimembranosus muscle) was observed after 1 day of feeding, with red muscle fibres principally showing fatty infiltration (Abdellatif and Vles, 1970); therefore, the results of the present study lend support to these findings.

In the Experiment 3 and Experiment 4, rats treated with EA-containing oils showed cardiac lipidosis, but myocardial degeneration/necrosis, initially observed in Experiments 1 and 2, was absent. However, the same batches of pure EA and HEAR oil
were used in all 4 of the present experiments. The reasons for the absence of degeneration/necrosis in Experiments 3 and 4 are not understood. It is possible that there was oxidation of the pure EA and the EA in the HEAR oil over time, and this may have decreased the cardiotoxic properties of the compound. However, Charlton et al. (1975) compared the properties of unoxidised and oxidised rapeseed oils containing various levels of EA with the feeding of HEAR oil diets (20 % rapeseed oil by weight) for up to 112 days. It was found that the incidence of myocardial necrosis was not affected by the oxidation state of the oils.

Therefore, it is concluded that in the present studies, a novel method of inducing myocardial lipidosis has been established, namely the gavage administration of EA-containing oils (Spike oil and HEAR oil). The biochemical changes associated with EA administration have been investigated, as have the serum levels of cTnl in rats with cardiac lipidosis. However, emphatic statements about a clear correlation between cTnl levels and histopathological lesion severity (both lipidosis and degeneration/necrosis) are not possible at this stage, and further investigations are required. The reasons for the non-identification of myocardial degeneration/necrosis in Experiments 3 and 4 are also not understood. The incidence and severity of myocardial lipidosis appears to be more predictable and more reproducible using a regimen of EA dietary administration (rather than gavage dosing), however there is no difference in the severity of lesions when rats are fed the Spike oil diet or the HEAR oil diet.

Further studies on EA-induced cardiac damage are required to firstly, fully define the time course of lesion development, secondly, to investigate the alterations in serum biochemistry parameters over a longer period of time than 96 h, and thirdly to study the reversibility of EA-induced cardiac lesions in the post-dosing period.
Figure 8.1. Experiment 1, preliminary 96 h feeding study; group mean relative heart weights in control male Hanover Wistar rats and rats maintained on erucic acid- (EA-) containing diets. Values are means, SD as error bars; n=5 for each group. Group 1, Control ground diet; Group 2, low EA rapeseed (LEAR) oil diet, (LEAR oil diet); Group 3, LEAR oil with added EA (Spike oil) diet, (Spike oil diet); Group 4, high EA rapeseed (HEAR) oil diet, (HEAR oil diet). *Significantly different from control, P<0.05; ***P<0.001. Note the origin of the Y axis is 3.0 g/kg.
Figure 8.2. Experiment 1, preliminary 96 h feeding study; (A) myocardium from a rat of Group 1 (Control ground diet) to show the normal appearance of cardiac myofibres; (B) myocardium from a rat of Group 4 maintained on a high erucic acid rapeseed (HEAR) oil diet for 96 h. There is a significant amount of clearly delineated cytoplasmic vacuoles in the myofibres. The vacuoles are in a linear arrangement, parallel to the longitudinal axis of the cell. H&E, x 400 original magnification.
Figure 8.3. Experiment 1, preliminary 96 h feeding study; (A) myocardium from a rat of Group 1 (Control ground diet) to show the normal appearance of cardiac myofibres; (B) myocardium from a rat of Group 4 maintained on a high erucic acid rapeseed (HEAR) oil diet for 96 h. The red colour indicates the presence of neutral lipid; no lipid is demonstrated in the control tissue. Oil Red O staining, x 200 original magnification.
Figure 8.4. Experiment 1, preliminary 96 h feeding study; (A) myocardium from a rat of Group 1 (Control ground diet) to show the normal appearance of cardiac myofibres; (B) myocardium from a rat of Group 4 maintained on a high erucic acid rapeseed (HEAR) oil diet for 96 h. Myocardial inflammation is present in association with myocardial lipidosis, and focally replaces myofibres. H&E, x 400 original magnification.
Figure 8.5. Experiment 2, preliminary 96 h gavage study; group mean relative heart weights in male Hanover Wistar rats treated with low erucic acid (EA) rapeseed (LEAR) oil and high EA rapeseed (HEAR) oil by gavage, and dietary HEAR oil (HEAR oil diet). Values are means, SD as error bars; n=5 for control and EA-treated rats. Group 1 = LEAR oil (1 dose per 24 h), Group 2 = HEAR oil (1 dose per 24 h), Group 3 = HEAR oil (2 doses per 24 h), Group 4 = HEAR oil (3 doses per 24 h), Group 5 = HEAR oil (4 doses per 24 h), Group 6 = HEAR oil (5 doses per 24 h), Group 7 = HEAR oil diet. ***Significantly different from Group 1, P<0.001. Note the origin of the Y axis is 3.0 g/kg.
Figure 8.6. Experiment 2, preliminary 96 h gavage study; (A) myocardium from a rat of Group 1 (LEAR oil by gavage) to show the normal appearance of cardiac myofibres; (B) myocardium from a rat of Group 7 maintained on a high erucic acid rapeseed (HEAR) oil diet for 96 h. In (B), there is a significant accumulation of fat droplets of varying size within the myofibres; droplets are arranged in a linear fashion parallel to the myofibrils. TEM longitudinal section, (A) x 5000 original magnification; (B) x 3500 original magnification.
Figure 8.7. Experiment 2, preliminary 96 h gavage study; (A) myocardium from a rat of Group 1 (LEAR oil by gavage) to show the normal appearance of cardiac myofibres; (B) myocardium from a rat of Group 7 maintained on a high erucic acid rapeseed (HEAR) oil diet for 96 h. In (B), the lipid droplets are present in close association with the mitochondria; however the structure of the mitochondria appears normal. TEM transverse section, (A) x 30000 original magnification; (B) x 25000 original magnification.
Figure 8.8. Experiment 3, 96 h gavage dosing evaluation study; relative heart weights in male Hanover Wistar rats treated with diets containing sunflower oil or erucic acid (EA), or with EA-containing oils by gavage. Values are means, SD as error bars; n=5 for each group. Group 1 = Sunflower oil diet, Group 2 = LEAR oil diet, Group 3 = Spike oil diet, Group 4 = HEAR oil diet, Group 5 = Spike oil (3 gavage doses per 24 h, “social hours”), Group 6 = Spike oil (3 gavage doses per 24 h; “unsocial hours”), Group 7 = HEAR oil (3 gavage doses per 24 h, “social hours”), Group 8 = HEAR oil (3 gavage doses per 24 h; “unsocial hours”). Note the origin of the Y axis is 3.0 g/kg.
Figure 8.9. Experiment 4, time course study; group mean relative heart weights in control male Hanover Wistar rats and animals treated with erucic acid. Values are means, SD as error bars; n=5 for each group. Animals were given extruded Global Rodent Diet (control), Spike oil by gavage 3 times per 24 h, or high erucic acid rapeseed (HEAR) oil diet. Treatment was for up to 96 h, and animals were autopsied at 0 h (controls), and at 24, 48, 72 and 96 h. Note the origin of the Y axis is 3.0 g/kg.
Figure 8.10. Experiment 4, time course study; (A) skeletal muscle (quadriceps femoris) from a rat of Group 11 (Control diet) to show the normal appearance of myofibres; (B) skeletal muscle from a rat of Group 13 maintained on a high erucic acid rapeseed (HEAR) oil diet for 96 h. In (B), there is an increased accumulation of fat droplets, mainly within the small calibre myofibres. H&E, x400 original magnification (A and B).
Figure 8.11. Experiment 4, time course study; (A) skeletal muscle (quadriceps femoris) from a rat of Group 11 (Control diet) to show the normal appearance of myofibres; (B) skeletal muscle from a rat of Group 13 maintained on a high erucic acid rapeseed (HEAR) oil diet for 96 h. In (B), positive staining with Oil Red O (ORO) confirms the increased accumulation of neutral lipid in the vacuoles. ORO, x200 original magnification.
Table 8.1. Experiment 2, preliminary 96 h gavage study; experimental design and timetable of gavage dosing \(^{a,b}\).

<table>
<thead>
<tr>
<th>Group</th>
<th>Treatment</th>
<th>Number of gavage doses over 24 h period</th>
<th>09.00 h</th>
<th>12.00 h</th>
<th>13.00 h</th>
<th>15.00 h</th>
<th>17.00 h</th>
<th>18.00 h</th>
<th>21.00 h</th>
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<tbody>
<tr>
<td>1</td>
<td>LEAR oil</td>
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<td>X</td>
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<td></td>
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</tr>
<tr>
<td>3</td>
<td>HEAR oil</td>
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<td></td>
<td>X</td>
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<td></td>
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</tr>
<tr>
<td>4</td>
<td>HEAR oil</td>
<td>3</td>
<td>X</td>
<td></td>
<td></td>
<td>X</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>HEAR oil</td>
<td>4</td>
<td>X</td>
<td></td>
<td></td>
<td></td>
<td>X</td>
<td></td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>HEAR oil</td>
<td>5</td>
<td>X</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>X</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>HEAR oil diet</td>
<td>-</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

\(^{a}\) \(n=5\) animals in all groups. Abbreviations: LEAR oil, low erucic acid rapeseed oil; HEAR oil, high erucic acid rapeseed oil. Groups 1 and 2 were given a single gavage dose of 31 ml oil/kg body weight at 09.00 h. For Group 3, the volume of the dose of 31 ml oil/kg body weight was divided into 2 equal aliquots, which were administered at 09.00 h and 21.00 h. For Group 4, the 31 ml volume was divided into 3 equal aliquots and administered at 09.00 h, 15.00 h and 21.00 h, etc. The regimens of gavage administration were carried out every 24 h for 96 h. Groups 1 to 6 were fed Control ground diet.

\(^{b}\) X represents carry out procedure.
Table 8.2. Experiment 3, 96 h gavage dosing evaluation study; experimental design and timetable of gavage dosing\textsuperscript{a,b}.

<table>
<thead>
<tr>
<th>Group</th>
<th>Treatment</th>
<th>Number of gavage doses over 24 h period</th>
<th>09.00 h</th>
<th>13.00 h</th>
<th>15.00 h</th>
<th>17.00 h</th>
<th>21.00 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Sunflower oil diet</td>
<td>-</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>LEAR oil diet</td>
<td>-</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>Spike oil diet</td>
<td>-</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>HEAR oil diet</td>
<td>-</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>Spike oil</td>
<td>3</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td></td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>Spike oil</td>
<td>3</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td></td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>HEAR oil</td>
<td>3</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td></td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>HEAR oil</td>
<td>3</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

\textsuperscript{a} n=5 animals in all groups. Abbreviations: Spike oil, low erucic acid (EA) rapeseed oil mixed with pure EA to give the oil an EA content of 45 \%; HEAR oil, high EA rapeseed oil; Sunflower oil diet, sunflower oil mixed with ground diet to give an oil content of 20 \% (w/w); LEAR oil diet, low EA rapeseed (LEAR) oil mixed with ground diet to give an oil content of 20 \% (w/w); Spike oil diet, Spike oil mixed with ground diet to give an oil content of 20 \% (w/w); HEAR oil diet, HEAR oil mixed with ground diet to give an oil content of 20 \% (w/w). Groups 5 and 7 were given a dose volume of oil at 31 ml oil/kg body weight, which was divided into 3 equal aliquots, and an aliquot administered at 09.00 h, 13.00 h and 17.00 h ("social hours"). For Groups 6 and 8, treatment was as for Groups 5 and 7, but the 3 equal aliquots were administered at 09.00 h, 15.00 h and 21.00 h ("unsocial hours"). The regimens of gavage administration were carried out every 24 h for 96 h.

\textsuperscript{b} X represents carry out procedure.
### Table 8.3. Experiment 4, time course study; experimental design\(^a\).

<table>
<thead>
<tr>
<th>Group</th>
<th>Treatment</th>
<th>Autopsy time point (h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Control</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>Control</td>
<td>24</td>
</tr>
<tr>
<td>3</td>
<td>Spike oil by gavage</td>
<td>24</td>
</tr>
<tr>
<td>4</td>
<td>HEAR oil diet</td>
<td>24</td>
</tr>
<tr>
<td>5</td>
<td>Control</td>
<td>48</td>
</tr>
<tr>
<td>6</td>
<td>Spike oil by gavage</td>
<td>48</td>
</tr>
<tr>
<td>7</td>
<td>HEAR oil diet</td>
<td>48</td>
</tr>
<tr>
<td>8</td>
<td>Control</td>
<td>72</td>
</tr>
<tr>
<td>9</td>
<td>Spike oil by gavage</td>
<td>72</td>
</tr>
<tr>
<td>10</td>
<td>HEAR oil diet</td>
<td>72</td>
</tr>
<tr>
<td>11</td>
<td>Control</td>
<td>96</td>
</tr>
<tr>
<td>12</td>
<td>Spike oil by gavage</td>
<td>96</td>
</tr>
<tr>
<td>13</td>
<td>HEAR oil diet</td>
<td>96</td>
</tr>
</tbody>
</table>

\(^a\)n=5 animals in all groups. Groups 1, 2, 5, 8 and 11 (control) received extruded Global Rodent Diet *ad libitum*. Groups 3, 6, 9 and 12 were treated with Spike oil by gavage at a dose of 31 ml oil/kg body weight, divided into 3 equal aliquots, at 09.00 h, 13.00 h and 17.00 h. Groups 4, 7, 10 and 13 were fed HEAR oil diet. The regimens of gavage and dietary administration were carried out every 24 h for 96 h. Abbreviations: Spike oil, low erucic acid (EA) rapeseed oil mixed with pure EA to give the oil an EA content of 45 %; HEAR oil, high EA rapeseed oil; HEAR oil diet, HEAR oil mixed with ground diet to give an oil content of 20 % (w/w).
Table 8.4A. Experiment 1, preliminary 96 h feeding study; mean (SD) body weights (g) of control male Hanover Wistar rats and animals maintained on diets containing erucic acid (EA)

<table>
<thead>
<tr>
<th>Group</th>
<th>Diet</th>
<th>Time (h)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>-120</td>
</tr>
<tr>
<td>1</td>
<td>Control ground diet</td>
<td>70.0 (8.6)</td>
</tr>
<tr>
<td>2</td>
<td>LEAR oil diet</td>
<td>71.2 (6.5)</td>
</tr>
<tr>
<td>3</td>
<td>Spike oil diet</td>
<td>72.4 (9.8)</td>
</tr>
<tr>
<td>4</td>
<td>HEAR oil diet</td>
<td>74.6 (3.5)</td>
</tr>
</tbody>
</table>

*All 4 dietary groups were maintained on Control ground diet for the 24 h periods from -120 h to 0 h. Animals were fed experimental diets for the 24 h periods from 0 h; Group 1, Control ground diet; Group 2, low EA rapeseed (LEAR) oil diet (LEAR oil diet); Group 3, LEAR oil with added EA (Spike oil) diet (Spike oil diet); Group 4, high EA rapeseed (HEAR) oil diet (HEAR oil diet). There were n=5 rats in each group. Data was not analysed statistically.
Table 8.4B. Experiment 1, preliminary 96 h feeding study; analysis of body weight data.

<table>
<thead>
<tr>
<th>Group</th>
<th>Diet</th>
<th>Mean absolute body weight increase from -120 h to 0 h (g)</th>
<th>Mean absolute body weight increase from -120 h to 0 h as % of body weight at -120 h</th>
<th>Mean absolute body weight increase from 0 h to 96 h (g)</th>
<th>Mean absolute body weight increase from 0 h to 96 h as % of body weight at 0 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Control</td>
<td>26.2 (5.24)</td>
<td>37.4 (7.48)</td>
<td>23.4 (5.85)</td>
<td>24.3 (6.08)</td>
</tr>
<tr>
<td>2</td>
<td>LEAR</td>
<td>28.0 (5.60)</td>
<td>39.3 (7.86)</td>
<td>20.0 (5.00)</td>
<td>20.2 (5.05)</td>
</tr>
<tr>
<td>3</td>
<td>Spike</td>
<td>27.4 (5.48)</td>
<td>37.8 (7.56)</td>
<td>16.6 (4.15)</td>
<td>16.6 (4.15)</td>
</tr>
<tr>
<td>4</td>
<td>HEAR</td>
<td>30.2 (6.04)</td>
<td>40.5 (8.10)</td>
<td>17.6 (4.40)</td>
<td>16.8 (4.20)</td>
</tr>
</tbody>
</table>

*All other information as Table 8.4A. Data in parentheses give the mean figures for a 24 h period from -120 h to 0 h, and from 0 h to 96 h.*
Table 8.5A. Experiment 1, preliminary 96 h feeding study; absolute diet consumptions (g diet consumed/rat/24 h) for control male Hanover Wistar rats and animals maintained on diets containing erucic acid (EA)*.

<table>
<thead>
<tr>
<th>Group</th>
<th>Diet</th>
<th>-120 to -96</th>
<th>-96 to -72</th>
<th>-72 to 0&lt;sup&gt;h&lt;/sup&gt;</th>
<th>Mean&lt;sup&gt;a&lt;/sup&gt;</th>
<th>0 to 24</th>
<th>24 to 48</th>
<th>48 to 72</th>
<th>72 to 96</th>
<th>Mean&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Control ground diet</td>
<td>14.3</td>
<td>15.2</td>
<td>15.6</td>
<td>15.0</td>
<td>17.1</td>
<td>17.9</td>
<td>18.9</td>
<td>19.8</td>
<td>18.4</td>
</tr>
<tr>
<td>2</td>
<td>LEAR oil diet</td>
<td>13.9</td>
<td>15.2</td>
<td>16.8</td>
<td>15.3</td>
<td>15.5</td>
<td>16.7</td>
<td>17.7</td>
<td>15.7</td>
<td>16.4</td>
</tr>
<tr>
<td>3</td>
<td>Spike oil diet</td>
<td>14.0</td>
<td>15.0</td>
<td>16.1</td>
<td>15.0</td>
<td>13.7</td>
<td>15.4</td>
<td>15.6</td>
<td>12.2</td>
<td>14.2</td>
</tr>
<tr>
<td>4</td>
<td>HEAR oil diet</td>
<td>13.5</td>
<td>14.4</td>
<td>16.1</td>
<td>14.7</td>
<td>14.8</td>
<td>17.5</td>
<td>17.7</td>
<td>15.1</td>
<td>16.3</td>
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</tbody>
</table>

*All other information as Table 8.4A.

<sup>a</sup>The diet consumption for -72 h to 0 h was a 72 h diet consumption which has been averaged to provide a 24 h consumption figure.

<sup>b</sup>The mean of 3 diet consumptions, -120 h to 0 h.

<sup>c</sup>The mean of 4 diet consumptions, 0 h to 96 h.
Table 8.5B. Experiment 1, preliminary 96 h feeding study; absolute water consumptions (ml water consumed/rat/24 h) for control male Hanover Wistar rats and animals maintained on diets containing erucic acid (EA)\(^a\).

<table>
<thead>
<tr>
<th>Group</th>
<th>Diet</th>
<th>Time (h)</th>
<th></th>
<th></th>
<th></th>
<th></th>
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<tbody>
<tr>
<td></td>
<td></td>
<td>-120 to -96</td>
<td>-96 to -72</td>
<td>-72 to 0(^b)</td>
<td>Mean(^c)</td>
<td>0 to 24</td>
<td>24 to 48</td>
<td>48 to 72</td>
<td>72 to 96</td>
<td>Mean(^d)</td>
</tr>
<tr>
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<td>Control ground diet</td>
<td>13.6</td>
<td>14.9</td>
<td>15.0</td>
<td>14.5</td>
<td>16.1</td>
<td>17.4</td>
<td>20.1</td>
<td>18.3</td>
<td>18.0</td>
</tr>
<tr>
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<td>LEAR oil diet</td>
<td>16.5</td>
<td>19.3</td>
<td>19.4</td>
<td>18.4</td>
<td>15.9</td>
<td>33.7</td>
<td>21.6</td>
<td>16.7</td>
<td>22.0</td>
</tr>
<tr>
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<td>Spike oil diet</td>
<td>13.3</td>
<td>14.7</td>
<td>16.1</td>
<td>14.7</td>
<td>12.8</td>
<td>16.4</td>
<td>17.4</td>
<td>15.6</td>
<td>15.6</td>
</tr>
<tr>
<td>4</td>
<td>HEAR oil diet</td>
<td>14.3</td>
<td>15.6</td>
<td>17.3</td>
<td>15.7</td>
<td>14.7</td>
<td>18.5</td>
<td>17.8</td>
<td>16.9</td>
<td>17.0</td>
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</table>

\(^a\)All other information as Table 8.4A.

\(^b\)The water consumption for -72 h to 0 h was a 72 h water consumption which has been averaged to provide a 24 h consumption figure.

\(^c\)The mean of 3 water consumptions, -120 h to 0 h.

\(^d\)The mean of 4 water consumptions, 0 h to 96 h.
Table 8.6. Experiment 1, preliminary 96 h feeding study; serum clinical chemistry results in control male Hanover Wistar rats and animals maintained on diets containing erucic acid (EA)$^{a,b}$.

<table>
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<th>Treatment group</th>
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<td></td>
<td>1</td>
</tr>
<tr>
<td>Urea</td>
<td>7.20 (0.47)</td>
</tr>
<tr>
<td>CREAT</td>
<td>37.4 (1.8)</td>
</tr>
<tr>
<td>Albumin</td>
<td>30.6 (1.1)</td>
</tr>
<tr>
<td>TP</td>
<td>48.2 (1.6)</td>
</tr>
<tr>
<td>Sodium</td>
<td>144.6 (1.5)</td>
</tr>
<tr>
<td>Potassium</td>
<td>4.58 (0.24)</td>
</tr>
<tr>
<td>Chloride</td>
<td>101.2 (0.8)</td>
</tr>
<tr>
<td>CHOL</td>
<td>2.08 (0.18)</td>
</tr>
<tr>
<td>TRIG</td>
<td>0.84 (0.21)</td>
</tr>
<tr>
<td>NEFA</td>
<td>0.512 (0.072)</td>
</tr>
<tr>
<td>ALT</td>
<td>51.6 (8.3)</td>
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<tr>
<td>AST</td>
<td>93.2 (9.9)</td>
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<tr>
<td>CK</td>
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<td>2704.4 (844.9)</td>
</tr>
<tr>
<td>GLD</td>
<td>5.66 (0.38)</td>
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<tr>
<td>Aldolase</td>
<td>47.8 (6.4)</td>
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</tbody>
</table>

$^{a}$Values are means, SD in parentheses; n=5 for all groups. Group 1, Control ground diet; Group 2, low EA rapeseed (LEAR) oil diet (LEAR oil diet); Group 3, LEAR oil with added EA (Spike) diet (Spike oil diet); Group 4, high EA rapeseed (HEAR) oil diet (HEAR oil diet). *Significantly different from control, P<0.05; ***P<0.001.

$^{b}$Abbreviations and units: urea, mmol/L; CREAT, creatinine, μmol/L; albumin, g/L; TP, total protein, g/L; sodium, mmol/L; potassium, mmol/L; chloride, mmol/L; CHOL, total cholesterol, mmol/L; TRIG, triglyceride, mmol/L; NEFA, non-esterified fatty acids, mmol/L; ALT, alanine aminotransferase, U/L; AST, aspartate aminotransferase, U/L; CK, total creatine kinase, U/L; LD, total lactate dehydrogenase, U/L; GLD, glutamate dehydrogenase, U/L; aldolase, U/L.
Table 8.7. Experiment 1, preliminary 96 h feeding study; serum cardiac troponin I (cTnl) results in individual control male Hanover Wistar rats and animals maintained on diets containing erucic acid (EA)\textsuperscript{a,b}.

<table>
<thead>
<tr>
<th></th>
<th>Group 1</th>
<th>Group 2</th>
<th>Group 3</th>
<th>Group 4</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>&lt;0.03</td>
<td>0.06 (1.0)\textsuperscript{c}</td>
<td>NS</td>
<td>0.07 (1.3)</td>
</tr>
<tr>
<td></td>
<td>&lt;0.03</td>
<td>0.03 (→)</td>
<td>0.09 (2.0)</td>
<td>0.77 (24.7)</td>
</tr>
<tr>
<td></td>
<td>&lt;0.03</td>
<td>0.06 (1.0)</td>
<td>0.07 (1.3)</td>
<td>0.08 (1.7)</td>
</tr>
<tr>
<td></td>
<td>&lt;0.03</td>
<td>0.10 (2.3)</td>
<td>0.03 (→)</td>
<td>0.13 (3.3)</td>
</tr>
<tr>
<td></td>
<td>&lt;0.03</td>
<td>0.14 (3.7)</td>
<td>0.03 (→)</td>
<td>0.06 (1.0)</td>
</tr>
<tr>
<td>Mean</td>
<td>&lt;0.030</td>
<td>0.078 [1.6]\textsuperscript{d}</td>
<td>0.055 [0.8]</td>
<td>0.222 [6.4]</td>
</tr>
<tr>
<td>SD</td>
<td>0.000</td>
<td>0.043</td>
<td>0.030</td>
<td>0.308</td>
</tr>
</tbody>
</table>

\textsuperscript{a}Group 1, Control ground diet; Group 2, low EA rapeseed (LEAR) oil diet (LEAR oil diet); Group 3, LEAR oil with added EA (Spike) diet (Spike oil diet); Group 4, high EA rapeseed (HEAR) oil diet (HEAR oil diet). n=5 for all groups.

\textsuperscript{b}Values for cTnl are measured in \(\mu g/L\). NS indicates no sample for analysis.

\textsuperscript{c}{} indicates the "fold increase" of an individual value over the mean control group value.

\textsuperscript{d}[ ] indicates the "fold increase" of a group mean value over the mean control value.
Table 8.8. Experiment 1, preliminary 96 h feeding study; incidence of microscopic findings in the hearts of control male Hanover Wistar rats and animals maintained on diets containing erucic acid (EA)\(^a\).

<table>
<thead>
<tr>
<th>Microscopic finding</th>
<th>Severity grade</th>
<th>Treatment group</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Number of rats in each group with histological changes</td>
<td></td>
</tr>
<tr>
<td>Myocardial vacuolation</td>
<td></td>
<td>5</td>
</tr>
<tr>
<td>NAD(^a)</td>
<td>Minimal</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Mild</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Moderate</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Marked</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Very marked</td>
<td>-</td>
</tr>
<tr>
<td>Myocardial degeneration/necrosis</td>
<td></td>
<td>5</td>
</tr>
<tr>
<td>NAD</td>
<td>Minimal</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Mild</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Moderate</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Marked</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Very marked</td>
<td>-</td>
</tr>
<tr>
<td>Oil Red O positive staining</td>
<td></td>
<td>5</td>
</tr>
<tr>
<td>NAD</td>
<td>Minimal</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Mild</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Moderate</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Marked</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Very marked</td>
<td>-</td>
</tr>
</tbody>
</table>

\(^a\)n=5 for animals in all groups. Group 1, Control ground diet; Group 2, low EA rapeseed (LEAR) oil diet (LEAR oil diet); Group 3, LEAR oil with added EA (Spike oil) diet (Spike oil diet); Group 4, high EA rapeseed (HEAR) oil diet (HEAR oil diet).

^bNAD= no abnormalities detected.
Table 8.9. Experiment 2, preliminary 96 h gavage study; mean body weight increases in male Hanover Wistar rats treated with low erucic acid (EA) rapeseed (LEAR) oil and high EA rapeseed (HEAR) oil by gavage, and dietary HEAR oil®.

<table>
<thead>
<tr>
<th>Group</th>
<th>Treatment</th>
<th>Method of administration</th>
<th>Number of gavage doses over 24 h period</th>
<th>Mean body weight at 0 h (g)</th>
<th>Mean body weight at 96 h (g)</th>
<th>Mean body weight increase from 0 h to 96 h as % of body weight at 0 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>LEAR oil&lt;sup&gt;c&lt;/sup&gt;</td>
<td>Gavage</td>
<td>1</td>
<td>141.6</td>
<td>151.4</td>
<td>6.9</td>
</tr>
<tr>
<td>2</td>
<td>HEAR oil&lt;sup&gt;d&lt;/sup&gt;</td>
<td>Gavage</td>
<td>1</td>
<td>148.8</td>
<td>167.4</td>
<td>12.5</td>
</tr>
<tr>
<td>3</td>
<td>HEAR oil</td>
<td>Gavage</td>
<td>2</td>
<td>140.0</td>
<td>156.6</td>
<td>11.9</td>
</tr>
<tr>
<td>4</td>
<td>HEAR oil</td>
<td>Gavage</td>
<td>3</td>
<td>147.4</td>
<td>164.6</td>
<td>11.7</td>
</tr>
<tr>
<td>5</td>
<td>HEAR oil</td>
<td>Gavage</td>
<td>4</td>
<td>148.4</td>
<td>160.6</td>
<td>8.2</td>
</tr>
<tr>
<td>6</td>
<td>HEAR oil</td>
<td>Gavage</td>
<td>5</td>
<td>150.0</td>
<td>162.6</td>
<td>8.4</td>
</tr>
<tr>
<td>7</td>
<td>HEAR oil diet&lt;sup&gt;e&lt;/sup&gt;</td>
<td>Diet</td>
<td>-</td>
<td>145.0</td>
<td>164.8</td>
<td>13.7</td>
</tr>
</tbody>
</table>

<sup>a</sup>Details of gavage dosing regimens are presented in Table 8.1; n=5 rats per group.

<sup>b</sup>0 h = beginning of treatment period; 96 h = end of treatment period.

<sup>c</sup>LEAR oil = low EA rapeseed oil (<2 % EA).

<sup>d</sup>HEAR oil = high EA rapeseed oil (45 % EA).

<sup>e</sup>HEAR oil diet = HEAR oil mixed with Control ground diet to give an oil content (w/w) of 20 %.
Table 8.10A. Experiment 2, preliminary 96 h gavage study; absolute diet consumptions (g diet consumed/rat/24 h) of male Hanover Wistar rats treated with low erucic acid (EA) rapeseed (LEAR) oil and high EA rapeseed (HEAR) oil by gavage, and dietary HEAR oil.

<table>
<thead>
<tr>
<th>Group</th>
<th>Treatment</th>
<th>Method of administration</th>
<th>Number of gavage doses over 24 h period</th>
<th>Time (h)</th>
<th>Mean&lt;sup&gt;c&lt;/sup&gt;</th>
<th>0 to 24</th>
<th>24 to 48</th>
<th>48 to 72</th>
<th>72 to 96</th>
<th>Mean&lt;sup&gt;d&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>LEAR oil&lt;sup&gt;e&lt;/sup&gt;</td>
<td>Gavage</td>
<td>1</td>
<td>-120 to -96</td>
<td>-96 to -72</td>
<td>-72 to 0&lt;sup&gt;b&lt;/sup&gt;</td>
<td>Mean&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0 to 24</td>
<td>24 to 48</td>
<td>48 to 72</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>14.3</td>
<td>15.2</td>
<td>15.6</td>
<td>15.0</td>
<td>17.1</td>
<td>17.9</td>
<td>18.9</td>
</tr>
<tr>
<td>2</td>
<td>HEAR oil&lt;sup&gt;f&lt;/sup&gt;</td>
<td>Gavage</td>
<td>1</td>
<td>-120 to -96</td>
<td>-96 to -72</td>
<td>-72 to 0&lt;sup&gt;b&lt;/sup&gt;</td>
<td>Mean&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0 to 24</td>
<td>24 to 48</td>
<td>48 to 72</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>13.9</td>
<td>15.2</td>
<td>16.8</td>
<td>15.3</td>
<td>15.5</td>
<td>16.7</td>
<td>17.7</td>
</tr>
<tr>
<td>3</td>
<td>HEAR oil</td>
<td>Gavage</td>
<td>2</td>
<td>-120 to -96</td>
<td>-96 to -72</td>
<td>-72 to 0&lt;sup&gt;b&lt;/sup&gt;</td>
<td>Mean&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0 to 24</td>
<td>24 to 48</td>
<td>48 to 72</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>14.0</td>
<td>15.0</td>
<td>16.1</td>
<td>15.0</td>
<td>13.7</td>
<td>15.4</td>
<td>15.6</td>
</tr>
<tr>
<td>4</td>
<td>HEAR oil</td>
<td>Gavage</td>
<td>3</td>
<td>-120 to -96</td>
<td>-96 to -72</td>
<td>-72 to 0&lt;sup&gt;b&lt;/sup&gt;</td>
<td>Mean&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0 to 24</td>
<td>24 to 48</td>
<td>48 to 72</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>13.5</td>
<td>14.4</td>
<td>16.1</td>
<td>14.7</td>
<td>14.8</td>
<td>17.5</td>
<td>17.7</td>
</tr>
<tr>
<td>5</td>
<td>HEAR oil</td>
<td>Gavage</td>
<td>4</td>
<td>-120 to -96</td>
<td>-96 to -72</td>
<td>-72 to 0&lt;sup&gt;b&lt;/sup&gt;</td>
<td>Mean&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0 to 24</td>
<td>24 to 48</td>
<td>48 to 72</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>14.3</td>
<td>15.2</td>
<td>15.6</td>
<td>15.0</td>
<td>17.1</td>
<td>17.9</td>
<td>18.9</td>
</tr>
<tr>
<td>6</td>
<td>HEAR oil</td>
<td>Gavage</td>
<td>5</td>
<td>-120 to -96</td>
<td>-96 to -72</td>
<td>-72 to 0&lt;sup&gt;b&lt;/sup&gt;</td>
<td>Mean&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0 to 24</td>
<td>24 to 48</td>
<td>48 to 72</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>13.9</td>
<td>15.2</td>
<td>16.8</td>
<td>15.3</td>
<td>15.5</td>
<td>16.7</td>
<td>17.7</td>
</tr>
<tr>
<td>7</td>
<td>HEAR oil diet&lt;sup&gt;g&lt;/sup&gt;</td>
<td>Diet</td>
<td>-</td>
<td>-120 to -96</td>
<td>-96 to -72</td>
<td>-72 to 0&lt;sup&gt;b&lt;/sup&gt;</td>
<td>Mean&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0 to 24</td>
<td>24 to 48</td>
<td>48 to 72</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>14.0</td>
<td>15.0</td>
<td>16.1</td>
<td>15.0</td>
<td>13.7</td>
<td>15.4</td>
<td>15.6</td>
</tr>
</tbody>
</table>

<sup>a</sup>Details of gavage dosing regimens are presented in Table 8.1; n=5 rats per group; Groups 1 to 6 were maintained on Control ground diet throughout the experiment.

<sup>b</sup>The diet consumption for -72 h to 0 h was a 72 h diet consumption which has been averaged to provide a 24 h consumption figure.

<sup>c</sup>The mean of 3 diet consumptions, -120 h to 0 h.

<sup>d</sup>The mean of 4 diet consumptions, 0 h to 96 h.

<sup>e</sup>LEAR oil = low EA rapeseed oil (<2 % EA).

<sup>f</sup>HEAR oil = high EA rapeseed oil (45 % EA).

<sup>g</sup>HEAR oil diet = HEAR oil mixed with Control ground diet to give an oil content (w/w) of 20 %.
Table 8.10B. Experiment 2, preliminary 96 h gavage study; absolute water consumptions (ml water consumed/rat/24 h) of male Hanover Wistar rats treated with low erucic acid (EA) rapeseed (LEAR) oil and high EA rapeseed (HEAR) oil by gavage, and dietary HEAR oil*.

<table>
<thead>
<tr>
<th>Group</th>
<th>Treatment</th>
<th>Method of administration</th>
<th>Number of gavage doses over 24 h period</th>
<th>Time (h)</th>
<th>Mean^b</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0 to 24</td>
<td>24 to 48</td>
</tr>
<tr>
<td>1</td>
<td>LEAR oil®</td>
<td>Gavage</td>
<td>1</td>
<td>20.7</td>
<td>20.4</td>
</tr>
<tr>
<td>2</td>
<td>HEAR oil®</td>
<td>Gavage</td>
<td>1</td>
<td>26.2</td>
<td>27.4</td>
</tr>
<tr>
<td>3</td>
<td>HEAR oil</td>
<td>Gavage</td>
<td>2</td>
<td>19.0</td>
<td>20.4</td>
</tr>
<tr>
<td>4</td>
<td>HEAR oil</td>
<td>Gavage</td>
<td>3</td>
<td>19.1</td>
<td>21.8</td>
</tr>
<tr>
<td>5</td>
<td>HEAR oil</td>
<td>Gavage</td>
<td>4</td>
<td>20.8</td>
<td>20.9</td>
</tr>
<tr>
<td>6</td>
<td>HEAR oil</td>
<td>Gavage</td>
<td>5</td>
<td>24.8</td>
<td>22.8</td>
</tr>
<tr>
<td>7</td>
<td>HEAR oil diet®</td>
<td>Diet</td>
<td>-</td>
<td>21.1</td>
<td>21.1</td>
</tr>
</tbody>
</table>

*Details of gavage dosing regimens are presented in Table 8.1; n=5 rats per group; Groups 1 to 6 were maintained on Control ground diet throughout the experiment.

^bThe mean of 4 water consumptions, 0 h to 96 h.

LEAR oil® = low EA rapeseed oil (<2 % EA).

HEAR oil® = high EA rapeseed oil (45 % EA).

HEAR oil diet® = HEAR oil mixed with ground diet to give an oil content (w/w) of 20 %.
Table 8.11. Experiment 2, preliminary 96 h gavage study; serum clinical chemistry results in male Hanover Wistar rats treated with low erucic acid (EA) rapeseed (LEAR) oil and high EA rapeseed (HEAR) oil by gavage, and dietary HEAR oil.\(^a,b\).

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Treatment group</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
</tr>
<tr>
<td>Urea</td>
<td>6.20 (0.42)</td>
</tr>
<tr>
<td>CREAT</td>
<td>41.5 (3.3)</td>
</tr>
<tr>
<td>Albumin</td>
<td>31.8 (1.7)</td>
</tr>
<tr>
<td>TP</td>
<td>48.3 (2.2)</td>
</tr>
<tr>
<td>Sodium</td>
<td>145.8 (1.9)</td>
</tr>
<tr>
<td>Potassium</td>
<td>5.00 (0.73)</td>
</tr>
<tr>
<td>Chloride</td>
<td>100.5 (1.0)</td>
</tr>
<tr>
<td>CHOL</td>
<td>1.53 (0.10)</td>
</tr>
<tr>
<td>TRIG</td>
<td>0.63 (0.19)</td>
</tr>
<tr>
<td>NEFA</td>
<td>0.493 (0.081)</td>
</tr>
<tr>
<td>ALT</td>
<td>84.0 (11.4)</td>
</tr>
<tr>
<td>AST</td>
<td>99.8 (19.3)</td>
</tr>
<tr>
<td>CK</td>
<td>1220.8 (422.0)</td>
</tr>
<tr>
<td>LD</td>
<td>3605.3 (1269.8)</td>
</tr>
<tr>
<td>GLD</td>
<td>5.38 (1.07)</td>
</tr>
<tr>
<td>Aldolase</td>
<td>57.8 (11.4)</td>
</tr>
</tbody>
</table>

\(^a\)Values are means, SD in parentheses; n=5 for all groups, except for Groups 1 and 5, where n=4 for all parameters. Group 1 = LEAR oil (1 gavage dose per 24 h), Group 2 = HEAR oil (1 gavage dose per 24 h), Group 3 = HEAR oil (2 gavage doses per 24 h), Group 4 = HEAR oil (3 gavage doses per 24 h), Group 5 = HEAR oil (4 gavage doses per 24 h), Group 6 = HEAR oil (5 gavage doses per 24 h), Group 7 = HEAR oil diet. Further information on regimens of administration is presented in Table 8.1. \(^*\)Significantly different from Group 1, P<0.05; \(^{***}\)P<0.001. \(^b\)Abbreviations and units: as Table 8.6.
Table 8.12. Experiment 2, preliminary 96 h gavage study; serum cardiac troponin I (cTnI) results in individual male Hanover Wistar rats treated with low erucic acid (EA) rapeseed (LEAR) oil and high EA rapeseed (HEAR) oil by gavage, and dietary HEAR oil\textsuperscript{a,b}.

<table>
<thead>
<tr>
<th>Treatment group</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>NS</td>
<td>0.03 {(\cdot)}</td>
<td>&lt;0.03 {(\cdot)}</td>
<td>&lt;0.03 {(\cdot)}</td>
<td>NS</td>
<td>0.04 (0.3)</td>
<td>&lt;0.03 {(\cdot)}</td>
</tr>
<tr>
<td></td>
<td>&lt;0.03</td>
<td>&lt;0.03 {(\cdot)}</td>
<td>0.18 (5.0)</td>
<td>&lt;0.03 {(\cdot)}</td>
<td>NS</td>
<td>&lt;0.03 {(\cdot)}</td>
<td>&lt;0.03 {(\cdot)}</td>
</tr>
<tr>
<td></td>
<td>&lt;0.03</td>
<td>&lt;0.03 {(\cdot)}</td>
<td>&lt;0.03 {(\cdot)}</td>
<td>0.03 {(\cdot)}</td>
<td>0.04 (0.3)</td>
<td>0.15 (4.0)</td>
<td>0.32 (9.7)</td>
</tr>
<tr>
<td></td>
<td>&lt;0.03</td>
<td>&lt;0.03 {(\cdot)}</td>
<td>&lt;0.03 {(\cdot)}</td>
<td>&lt;0.03 {(\cdot)}</td>
<td>0.48 (15.0)</td>
<td>&lt;0.03 {(\cdot)}</td>
<td>0.11 (2.7)</td>
</tr>
<tr>
<td></td>
<td>&lt;0.03</td>
<td>0.03 {(\cdot)}</td>
<td>1.19 (38.7)</td>
<td>&lt;0.03 {(\cdot)}</td>
<td>0.05 (0.7)</td>
<td>0.04 (0.3)</td>
<td>0.49 (17.3)</td>
</tr>
<tr>
<td>Mean</td>
<td>&lt;0.030</td>
<td>0.030 {(\cdot)}\textsuperscript{d}</td>
<td>0.292 [8.7]</td>
<td>0.030 {(\cdot)}</td>
<td>0.190 [5.3]</td>
<td>0.058 [0.9]</td>
<td>0.196 [5.5]</td>
</tr>
<tr>
<td>SD</td>
<td>0.000</td>
<td>0.000</td>
<td>0.506</td>
<td>0.000</td>
<td>0.251</td>
<td>0.052</td>
<td>0.203</td>
</tr>
</tbody>
</table>

\textsuperscript{a}Details of dosing regimens of LEAR oil (Group 1) and HEAR oil (Groups 2 to 6) by gavage and dietary HEAR oil (Group 7) are presented in Table 8.1.

\textsuperscript{b}Values for cTnI are measured in \(\mu\)g/L. NS indicates no sample for analysis.

\textsuperscript{c}{{\(\cdot\)}} indicates the "fold increase" of an individual value over the mean Group 1 (LEAR oil) value.

\textsuperscript{d}{{\[\]}} indicates the "fold increase" of a group mean value over the mean Group 1 (LEAR oil) value.
Table 8.13. Experiment 2, preliminary 96 h gavage study; incidence of microscopic findings in the hearts of male Hanover Wistar rats treated with low erucic acid (EA) rapeseed (LEAR) oil and high EA rapeseed (HEAR) oil by gavage, and dietary HEAR oil*

<table>
<thead>
<tr>
<th>Microscopic finding</th>
<th>Severity grade</th>
<th>Treatment group</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>1</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Myocardial vacuolation</td>
<td>NAD^3</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>Minimal</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Mild</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Moderate</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Marked</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Very marked</td>
<td>-</td>
</tr>
<tr>
<td>Myocardial degeneration/necrosis</td>
<td>NAD</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>Minimal</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Mild</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Moderate</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Marked</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Very marked</td>
<td>-</td>
</tr>
</tbody>
</table>

^3n=5 for animals in all groups. Group 1 = LEAR oil (1 gavage dose per 24 h), Group 2 = HEAR oil (1 gavage dose per 24 h), Group 3 = HEAR oil (2 gavage doses per 24 h), Group 4 = HEAR oil (3 gavage doses per 24 h), Group 5 = HEAR oil (4 gavage doses per 24 h), Group 6 = HEAR oil (5 gavage doses per 24 h), Group 7 = HEAR oil diet. Further information on regimens of administration is presented in Table 8.1.

^3NAD = no abnormalities detected

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**Table 8.14A. Experiment 3, 96 h gavage dosing evaluation study; mean (SD) body weights (g) of male Hanover Wistar rats treated with diets containing sunflower oil or erucic acid (EA), or with EA-containing oils by gavage.**

<table>
<thead>
<tr>
<th>Group</th>
<th>Treatment</th>
<th>Method of administration</th>
<th>Time (h)</th>
<th>-120</th>
<th>-96</th>
<th>-72</th>
<th>0</th>
<th>24</th>
<th>48</th>
<th>72</th>
<th>96</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Sunflower oil diet</td>
<td>Diet</td>
<td></td>
<td>66.2(11.2)</td>
<td>71.4(12.2)</td>
<td>75.0(14.1)</td>
<td>90.0(16.2)</td>
<td>93.8(16.1)</td>
<td>99.8(16.2)</td>
<td>103.4(16.4)</td>
<td>107.4(17.0)</td>
</tr>
<tr>
<td>2</td>
<td>LEAR oil diet</td>
<td>Diet</td>
<td></td>
<td>74.2(6.1)</td>
<td>80.2(6.3)</td>
<td>85.4(7.3)</td>
<td>104.0(7.1)</td>
<td>106.8(7.0)</td>
<td>113.8(8.4)</td>
<td>117.8(9.7)</td>
<td>122.4(8.9)</td>
</tr>
<tr>
<td>3</td>
<td>Spike oil diet</td>
<td>Diet</td>
<td></td>
<td>72.2(7.1)</td>
<td>77.0(7.1)</td>
<td>79.4(7.5)</td>
<td>94.6(6.9)</td>
<td>94.6(8.9)</td>
<td>101.6(7.6)</td>
<td>105.8(9.1)</td>
<td>110.6(7.7)</td>
</tr>
<tr>
<td>4</td>
<td>HEAR oil diet</td>
<td>Diet</td>
<td></td>
<td>73.2(4.3)</td>
<td>77.8(4.4)</td>
<td>81.4(5.4)</td>
<td>97.2(7.7)</td>
<td>97.8(5.0)</td>
<td>104.8(5.0)</td>
<td>112.0(5.4)</td>
<td>115.6(6.2)</td>
</tr>
<tr>
<td>5</td>
<td>Spike oil</td>
<td>Gavage (09.00 h to 17.00 h)</td>
<td></td>
<td>75.4(4.8)</td>
<td>80.8(5.2)</td>
<td>84.4(5.1)</td>
<td>103.6(6.5)</td>
<td>106.4(6.5)</td>
<td>108.6(6.2)</td>
<td>109.2(6.9)</td>
<td>110.6(12.2)</td>
</tr>
<tr>
<td>6</td>
<td>Spike oil</td>
<td>Gavage (09.00 h to 21.00 h)</td>
<td></td>
<td>73.4(3.4)</td>
<td>78.8(3.8)</td>
<td>82.0(4.3)</td>
<td>99.6(6.3)</td>
<td>105.0(4.9)</td>
<td>103.8(4.1)</td>
<td>110.4(3.4)</td>
<td>106.0(10.8)</td>
</tr>
<tr>
<td>7</td>
<td>HEAR oil</td>
<td>Gavage (09.00 h to 17.00 h)</td>
<td></td>
<td>77.6(3.8)</td>
<td>83.0(4.0)</td>
<td>86.6(3.8)</td>
<td>101.8(5.1)</td>
<td>105.0(4.8)</td>
<td>112.4(5.1)</td>
<td>116.6(6.4)</td>
<td>120.6(6.4)</td>
</tr>
<tr>
<td>8</td>
<td>HEAR oil</td>
<td>Gavage (09.00 h to 21.00 h)</td>
<td></td>
<td>70.2(7.9)</td>
<td>74.4(8.5)</td>
<td>78.0(9.2)</td>
<td>93.4(11.4)</td>
<td>99.0(12.1)</td>
<td>104.2(12.9)</td>
<td>110.8(12.6)</td>
<td>115.6(13.3)</td>
</tr>
</tbody>
</table>

*a All 8 treatment groups were maintained on Control ground diet for the 24 h periods from -120 h to 0 h. Animals were fed experimental diets or treated with erucic acid- (EA-) containing oils by gavage during the 24 h periods from 0 h to 96 h. Abbreviations: Spike oil, low EA rapeseed oil mixed with pure EA to give an EA content of 45 %; HEAR oil, high EA rapeseed oil; Sunflower oil diet, sunflower oil mixed with ground diet to give an oil content of 20 % (w/w); LEAR oil diet, low erucic acid (EA) rapeseed (LEAR) oil mixed with ground diet to give an oil content of 20 % (w/w); Spike oil diet, Spike oil mixed with ground diet to give an oil content of 20 % (w/w); HEAR oil diet, HEAR oil mixed with ground diet to give an oil content of 20 % (w/w). Groups 5 and 7 were given a dose volume of oil at 31 ml oil/kg body weight, which was divided into 3 equal aliquots, and administered at 09.00 h, 13.00 h and 17.00 h (“social hours”). For Groups 6 and 8, 3 equal aliquots were administered at 09.00 h, 15.00 h and 21.00 h (“unsocial hours”). The regimens of gavage administration were carried out during every 24 h period for 96 h. There were n=5 rats in each group.
Table 8.14B. Experiment 3, 96 h gavage dosing evaluation study; analysis of body weight data.

<table>
<thead>
<tr>
<th>Group</th>
<th>Treatment</th>
<th>Method of administration</th>
<th>Mean absolute body weight increase from -120 h to 0 h (g)</th>
<th>Mean absolute body weight increase from -120 h to 0 h as % of body weight at -120 h</th>
<th>Mean absolute body weight increase from 0 h to 96 h (g)</th>
<th>Mean absolute body weight increase from 0 h to 96 h as % of body weight at 0 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Sunflower oil</td>
<td>Diet</td>
<td>23.8</td>
<td><strong>36.0</strong></td>
<td>17.4</td>
<td>19.3</td>
</tr>
<tr>
<td>2</td>
<td>LEAR oil</td>
<td>Diet</td>
<td>29.8</td>
<td><strong>40.2</strong></td>
<td>18.4</td>
<td>17.7</td>
</tr>
<tr>
<td>3</td>
<td>Spike oil</td>
<td>Diet</td>
<td>22.4</td>
<td><strong>31.0</strong></td>
<td>16.0</td>
<td>16.9</td>
</tr>
<tr>
<td>4</td>
<td>HEAR oil</td>
<td>Diet</td>
<td>24.0</td>
<td><strong>32.8</strong></td>
<td>18.4</td>
<td>18.9</td>
</tr>
<tr>
<td>5</td>
<td>Spike oil</td>
<td>Gavage</td>
<td>28.2</td>
<td><strong>37.4</strong></td>
<td>7.0</td>
<td>6.8</td>
</tr>
<tr>
<td>6</td>
<td>Spike oil</td>
<td>Gavage</td>
<td>26.2</td>
<td><strong>35.7</strong></td>
<td>6.4</td>
<td>6.4</td>
</tr>
<tr>
<td>7</td>
<td>HEAR oil</td>
<td>Gavage</td>
<td>24.2</td>
<td><strong>31.2</strong></td>
<td>18.8</td>
<td>18.5</td>
</tr>
<tr>
<td>8</td>
<td>HEAR oil</td>
<td>Gavage</td>
<td>23.2</td>
<td><strong>33.0</strong></td>
<td>22.2</td>
<td>23.8</td>
</tr>
</tbody>
</table>

*All other information as Table 8.14A. Data was not analysed statistically.*
Table 8.15A. Experiment 3, 96 h gavage dosing evaluation study; absolute diet consumptions (g diet consumed/rat/24 h) of male Hanover Wistar rats maintained on oil-enriched diets containing sunflower oil or erucic acid (EA)\(^a\).

<table>
<thead>
<tr>
<th>Group</th>
<th>Diet</th>
<th>Time (h)</th>
<th></th>
<th></th>
<th></th>
<th>Mean(^b)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>0 to 24</td>
<td>24 to 48</td>
<td>48 to 72</td>
<td>72 to 96</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>Sunflower oil diet</td>
<td>13.6</td>
<td>11.9</td>
<td>12.1</td>
<td>14.6</td>
<td>13.1</td>
</tr>
<tr>
<td>2</td>
<td>LEAR oil diet</td>
<td>13.2</td>
<td>14.1</td>
<td>13.3</td>
<td>12.9</td>
<td>13.4</td>
</tr>
<tr>
<td>3</td>
<td>Spike oil diet</td>
<td>8.6</td>
<td>11.7</td>
<td>13.2</td>
<td>12.0</td>
<td>11.4</td>
</tr>
<tr>
<td>4</td>
<td>HEAR oil diet</td>
<td>8.2</td>
<td>12.0</td>
<td>15.3</td>
<td>13.0</td>
<td>12.1</td>
</tr>
</tbody>
</table>

\(^a\)Animals were maintained on Control ground diet in the time leading up to the treatment period (i.e. -120 h to 0 h). From 0 h, animals in each dietary group (n=5 rats per cage) were given 150 g of fresh diet every 24 h. Group 1 was fed Sunflower oil diet, Group 2 was fed LEAR oil diet, Group 3 was fed Spike oil diet and Group 4 was fed HEAR oil diet. Further information on treatment regimens may be found in Table 8.2.

\(^b\)The mean of 4 diet consumptions, 0 to 96 h.
Table 8.15B. Experiment 3, 96 h gavage dosing evaluation study; relative diet consumptions (g diet consumed/kg body weight/24 h) of male Hanover Wistar rats maintained on oil-enriched diets containing sunflower oil or erucic acid (EA)\(^a\).

<table>
<thead>
<tr>
<th>Group</th>
<th>Diet</th>
<th>Time (h)</th>
<th></th>
<th></th>
<th></th>
<th>Mean(^b)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>0 to 24</td>
<td>24 to 48</td>
<td>48 to 72</td>
<td>72 to 96</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>Sunflower oil</td>
<td>145.0</td>
<td>119.2</td>
<td>117.0</td>
<td>135.9</td>
<td>129.3</td>
</tr>
<tr>
<td>2</td>
<td>LEAR oil</td>
<td>123.6</td>
<td>123.9</td>
<td>112.9</td>
<td>105.4</td>
<td>116.5</td>
</tr>
<tr>
<td>3</td>
<td>Spike oil</td>
<td>90.9</td>
<td>115.2</td>
<td>124.8</td>
<td>108.5</td>
<td>109.9</td>
</tr>
<tr>
<td>4</td>
<td>HEAR oil</td>
<td>83.8</td>
<td>114.5</td>
<td>136.6</td>
<td>112.5</td>
<td>111.9</td>
</tr>
</tbody>
</table>

\(^a\)Animals were maintained on Control ground diet in the time leading up to the treatment period (i.e. -120 h to 0 h). From 0 h, animals in each dietary group (n=5 rats per cage) were given 150 g of fresh diet every 24 h. Group 1 was fed Sunflower oil diet, Group 2 was fed LEAR oil diet, Group 3 was fed Spike oil diet and Group 4 was fed HEAR oil diet. Further information on treatment regimens may be found in Table 8.2.

\(^b\)The mean of 4 diet consumptions, 0 h to 96 h.
Table 8.15C. Experiment 3, 96 h gavage dosing evaluation study; absolute water consumption (ml water consumed/rat/24 h) of male Hanover Wistar rats treated with diets containing sunflower oil or erucic acid (EA), or with EA-containing oils by gavage.  

<table>
<thead>
<tr>
<th>Group</th>
<th>Treatment</th>
<th>Method of administration</th>
<th>Time (h)</th>
<th>Mean (ml water consumed)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>-120 to -96</td>
<td>0 to 24</td>
</tr>
<tr>
<td>1</td>
<td>Sunflower oil diet</td>
<td>Diet</td>
<td>11.9</td>
<td>11.9</td>
</tr>
<tr>
<td>2</td>
<td>LEAR oil diet</td>
<td>Diet</td>
<td>14.3</td>
<td>15.0</td>
</tr>
<tr>
<td>3</td>
<td>Spike oil diet</td>
<td>Diet</td>
<td>13.2</td>
<td>12.6</td>
</tr>
<tr>
<td>4</td>
<td>HEAR oil diet</td>
<td>Diet</td>
<td>13.2</td>
<td>13.6</td>
</tr>
<tr>
<td>5</td>
<td>Spike oil Gavage</td>
<td>(09.00 h to 17.00 h)</td>
<td>13.1</td>
<td>12.8</td>
</tr>
<tr>
<td>6</td>
<td>Spike oil Gavage</td>
<td>(09.00 h to 21.00 h)</td>
<td>14.0</td>
<td>14.4</td>
</tr>
<tr>
<td>7</td>
<td>HEAR oil Gavage</td>
<td>(09.00 h to 17.00 h)</td>
<td>13.1</td>
<td>12.5</td>
</tr>
<tr>
<td>8</td>
<td>HEAR oil Gavage</td>
<td>(09.00 h to 21.00 h)</td>
<td>10.1</td>
<td>10.2</td>
</tr>
</tbody>
</table>

*Details of treatment regimens are presented in Table 8.2. Other information is provided in Tables 8.14A and 8.14B, and in Tables 8.15A and 8.15B. There were n=5 rats in each treatment group.

*The water consumption for -72 h to 0 h was a 72 h water consumption which has been averaged to provide a 24 h consumption figure.

*The mean of 3 water consumptions, -120 h to 0 h.

*The mean of 4 water consumptions, 0 h to 96 h.
Table 8.16. Experiment 3, 96 h gavage dosing evaluation study; serum clinical chemistry results in male Hanover Wistar rats treated with diets containing sunflower oil or erucic acid (EA), or with EA-containing oils by gavage\(^{a,b,c}\).

<table>
<thead>
<tr>
<th>Treatment group</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
</tr>
</thead>
<tbody>
<tr>
<td>Urea</td>
<td>5.68 (0.52)</td>
<td>5.88 (0.81)</td>
<td>6.95 (0.83)</td>
<td>6.62 (1.73)</td>
<td>7.50 (1.57)</td>
<td>7.68 (1.30)</td>
<td>8.30 (1.59) *</td>
<td>7.34 (0.68)</td>
</tr>
<tr>
<td>CREAT</td>
<td>47.0 (2.9)</td>
<td>48.5 (1.0)</td>
<td>49.3 (1.5)</td>
<td>46.4 (2.5)</td>
<td>47.8 (3.1)</td>
<td>51.2 (7.1)</td>
<td>46.8 (1.5)</td>
<td>47.0 (2.9)</td>
</tr>
<tr>
<td>Albumin</td>
<td>32.4 (2.4)</td>
<td>33.8 (1.0)</td>
<td>33.5 (1.0)</td>
<td>31.4 (2.8)</td>
<td>34.4 (2.5)</td>
<td>34.4 (2.6)</td>
<td>32.8 (1.1)</td>
<td>31.8 (0.8)</td>
</tr>
<tr>
<td>TP</td>
<td>46.8 (3.8)</td>
<td>49.3 (1.3)</td>
<td>49.5 (1.0)</td>
<td>46.0 (4.3)</td>
<td>49.2 (2.2)</td>
<td>51.8 (5.1)</td>
<td>47.6 (1.8)</td>
<td>46.4 (1.1)</td>
</tr>
<tr>
<td>Sodium</td>
<td>145.2 (1.5)</td>
<td>146.5 (2.1)</td>
<td>147.5 (1.0)</td>
<td>145.4 (1.5)</td>
<td>147.0 (2.9)</td>
<td>148.6 (2.7) *</td>
<td>148.6 (0.9) *</td>
<td>146.4 (1.7)</td>
</tr>
<tr>
<td>Potassium</td>
<td>5.10 (0.76)</td>
<td>5.15 (1.10)</td>
<td>5.98 (1.02)</td>
<td>5.24 (0.66)</td>
<td>5.00 (0.66)</td>
<td>4.40 (0.22)</td>
<td>4.58 (0.33)</td>
<td>4.88 (0.30)</td>
</tr>
<tr>
<td>Chloride</td>
<td>100.2 (0.8)</td>
<td>98.5 (0.6)</td>
<td>99.3 (2.2)</td>
<td>100.6 (2.3)</td>
<td>94.6 (5.0) ***</td>
<td>95.8 (0.8) *</td>
<td>97.8 (2.2)</td>
<td>98.6 (1.1)</td>
</tr>
<tr>
<td>CHOL</td>
<td>2.12 (0.30)</td>
<td>2.15 (0.31)</td>
<td>2.30 (0.18)</td>
<td>2.04 (0.40)</td>
<td>2.08 (0.36)</td>
<td>2.14 (0.33)</td>
<td>2.42 (0.41)</td>
<td>2.38 (0.13)</td>
</tr>
<tr>
<td>HDL-CHOL</td>
<td>1.66 (0.19)</td>
<td>1.63 (0.19)</td>
<td>1.53 (0.15)</td>
<td>1.46 (0.27)</td>
<td>1.40 (0.16)</td>
<td>1.58 (0.18)</td>
<td>1.76 (0.32)</td>
<td>1.78 (0.13)</td>
</tr>
<tr>
<td>TRIG</td>
<td>0.60 (0.21)</td>
<td>0.38 (0.10)</td>
<td>0.55 (0.21)</td>
<td>0.32 (0.08)</td>
<td>2.12 (3.57)</td>
<td>0.54 (0.05)</td>
<td>0.64 (0.11)</td>
<td>0.54 (0.16)</td>
</tr>
<tr>
<td>NEFA</td>
<td>0.592 (0.137)</td>
<td>0.430 (0.074)</td>
<td>0.538 (0.086)</td>
<td>0.432 (0.105)</td>
<td>1.518 (2.367)</td>
<td>0.626 (0.147)</td>
<td>0.454 (0.061)</td>
<td>0.450 (0.159)</td>
</tr>
<tr>
<td>ALT</td>
<td>52.8 (7.0)</td>
<td>53.3 (8.0)</td>
<td>90.5 (20.0)</td>
<td>81.4 (10.5)</td>
<td>143.2 (64.0) *</td>
<td>229.2 (100.4) ***</td>
<td>103.0 (10.8)</td>
<td>77.8 (6.2)</td>
</tr>
<tr>
<td>AST</td>
<td>93.0 (23.1)</td>
<td>87.8 (15.9)</td>
<td>101.3 (29.3)</td>
<td>89.8 (12.0)</td>
<td>118.4 (20.9)</td>
<td>140.2 (34.3) *</td>
<td>113.8 (16.5)</td>
<td>103.8 (11.5)</td>
</tr>
<tr>
<td>CK</td>
<td>711.0 (346.3)</td>
<td>681.5 (176.7)</td>
<td>574.5 (220.6)</td>
<td>370.2 (154.2) *</td>
<td>723.2 (160.4)</td>
<td>639.2 (143.8)</td>
<td>368.4 (128.2) *</td>
<td>158.0 (90.9) ***</td>
</tr>
<tr>
<td>LD</td>
<td>2357.8 (1315.8)</td>
<td>2180.0 (814.1)</td>
<td>2207.0 (1468.0)</td>
<td>1454.8 (1204.3)</td>
<td>2830.6 (624.4)</td>
<td>2439.0 (814.1)</td>
<td>2505.2 (926.5)</td>
<td>1987.6 (396.8)</td>
</tr>
<tr>
<td>GLD</td>
<td>4.46 (1.67)</td>
<td>4.85 (0.79)</td>
<td>6.08 (0.56)</td>
<td>7.06 (0.30) ***</td>
<td>6.90 (0.80) ***</td>
<td>8.34 (2.12) ***</td>
<td>6.20 (0.70)</td>
<td>5.50 (0.76)</td>
</tr>
<tr>
<td>Aldolase</td>
<td>69.0 (23.5)</td>
<td>66.5 (13.2)</td>
<td>75.5 (18.8)</td>
<td>69.4 (11.9)</td>
<td>84.6 (11.9)</td>
<td>82.4 (12.5)</td>
<td>81.4 (14.2)</td>
<td>71.0 (5.4)</td>
</tr>
<tr>
<td>cTnl</td>
<td>0.010 (0.000)</td>
<td>0.010 (0.000)</td>
<td>0.018 (0.010)</td>
<td>0.026 (0.030)</td>
<td>0.010 (0.000)</td>
<td>0.010 (0.000)</td>
<td>0.010 (0.000)</td>
<td>0.010 (0.000)</td>
</tr>
</tbody>
</table>

\(^{a}\)Values are means, SD in parentheses; n=5 for all groups, except for Groups 2 and 3, where n=4 for all parameters, and Group 5 for GLD, where n=4. Group 1 = Sunflower oil diet, Group 2 = LEAR oil diet, Group 3 = Spike oil diet, Group 4 = HEAR oil diet, Groups 5 and 6 = Spike oil (3 gavage doses per 24 h period), Groups 7 and 8 = HEAR oil (3 gavage doses per 24 h period). Further information on regimens of administration is presented in Table 8.2 and Tables 8.14A and 8.14B, and in Tables 8.15A, 8.15B and 8.15C.

\(^{b}\)Significantly different from Group 1, P<0.05; ***P<0.001.

\(^{c}\)Abbreviations and units: as Table 8.6, plus HDL-CHOL, high density lipoprotein cholesterol, mmol/L.

\(^{d}\)Serum levels of cTnl were assayed with the Bayer Centaur CP.
Table 8.17. Experiment 3, 96 h gavage dosing evaluation study; incidence of microscopic findings in the hearts of male Hanover Wistar rats treated with diets containing sunflower oil or erucic acid (EA), or with EA-containing oils by gavage\(^a\).

<table>
<thead>
<tr>
<th>Microscopic finding</th>
<th>Severity grade</th>
<th>Treatment group</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>1</td>
</tr>
<tr>
<td>Myocardial vacuolation</td>
<td>NAD(^b)</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>Minimal</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Mild</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Moderate</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Marked</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Very marked</td>
<td>-</td>
</tr>
</tbody>
</table>

\(^a\)Group 1 = Sunflower oil diet, Group 2 = LEAR oil diet, Group 3 = Spike oil diet, Group 4 = HEAR oil diet, Groups 5 and 6 = Spike oil (3 gavage doses per 24 h period), Groups 7 and 8 = HEAR oil (3 gavage doses per 24 h period). Further information on regimens of administration is presented in Table 8.2 and Tables 8.14A and 8.14B, and in Tables 8.15A, 8.15B and 8.15C. There were n=5 rats in each treatment group.

\(^b\)NAD = no abnormalities detected.
Table 8.18. Experiment 4, time course study; mean (SD) body weights (g) of control male Hanover Wistar rats and animals treated with erucic acid by gavage or in the diet.

<table>
<thead>
<tr>
<th>Group</th>
<th>Treatment</th>
<th>Time (h)</th>
<th>Mean absolute body weight increase from 0 h to 96 h (g)</th>
<th>Mean body weight increase from 0 h to 96 h as % of body weight at 0 h</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>-120</td>
<td>-96</td>
<td>-72</td>
</tr>
<tr>
<td>1, 2, 5, 8, 11</td>
<td>Control</td>
<td>70.6 (7.6)</td>
<td>75.8 (8.1)</td>
<td>79.5 (8.6)</td>
</tr>
<tr>
<td></td>
<td>n=</td>
<td>25</td>
<td>25</td>
<td>25</td>
</tr>
<tr>
<td>3, 6, 9, 12</td>
<td>Spike oil by gavage</td>
<td>68.3 (6.9)</td>
<td>73.1 (7.2)</td>
<td>76.8 (7.7)</td>
</tr>
<tr>
<td></td>
<td>n=</td>
<td>20</td>
<td>20</td>
<td>20</td>
</tr>
<tr>
<td>4, 7, 10, 13</td>
<td>HEAR oil diet</td>
<td>69.6 (6.9)</td>
<td>74.1 (7.3)</td>
<td>77.3 (7.8)</td>
</tr>
<tr>
<td></td>
<td>n=</td>
<td>20</td>
<td>20</td>
<td>20</td>
</tr>
</tbody>
</table>

Animals were maintained on extruded Global Rodent Diet in the pre-treatment period from -120 h to 0 h; from 0 h to 96 h, animals were given extruded Global Rodent Diet (control), Spike oil by gavage 3 times per 24 h, or high erucic acid rapeseed (HEAR) oil diet. Values for n are for all animals in each treatment group. Further information on treatment regimens and autopsy time points are presented in Table 8.3. Data was not analysed statistically.
Table 8.19. Experiment 4, time course study; mean absolute water consumptions (ml water consumed/rat/24 h) of control male Hanover Wistar rats and animals treated with erucic acid by gavage or in the diet.

<table>
<thead>
<tr>
<th>Group</th>
<th>Treatment</th>
<th>Time (h)</th>
<th>120 to -96</th>
<th>-96 to -72</th>
<th>-72 to 0</th>
<th>Mean</th>
<th>0 to 24</th>
<th>24 to 48</th>
<th>48 to 72</th>
<th>72 to 96</th>
<th>Mean</th>
</tr>
</thead>
<tbody>
<tr>
<td>1, 2, 5, 8, 11</td>
<td>Control</td>
<td></td>
<td>14.0</td>
<td>12.1</td>
<td>14.3</td>
<td>13.5</td>
<td>16.1</td>
<td>17.2</td>
<td>18.6</td>
<td>18.5</td>
<td>17.6</td>
</tr>
<tr>
<td></td>
<td>n=</td>
<td></td>
<td>25</td>
<td>25</td>
<td>25</td>
<td>-</td>
<td>20</td>
<td>15</td>
<td>10</td>
<td>5</td>
<td>-</td>
</tr>
<tr>
<td>3, 6, 9, 12</td>
<td>Spike oil by gavage</td>
<td></td>
<td>14.0</td>
<td>12.6</td>
<td>13.2</td>
<td>13.3</td>
<td>17.3</td>
<td>18.8</td>
<td>15.9</td>
<td>16.4</td>
<td>17.1</td>
</tr>
<tr>
<td></td>
<td>n=</td>
<td></td>
<td>20</td>
<td>20</td>
<td>20</td>
<td>-</td>
<td>20</td>
<td>15</td>
<td>10</td>
<td>5</td>
<td>-</td>
</tr>
<tr>
<td>4, 7, 10, 13</td>
<td>HEAR oil diet</td>
<td></td>
<td>14.4</td>
<td>11.9</td>
<td>12.7</td>
<td>13.0</td>
<td>13.3</td>
<td>18.0</td>
<td>16.6</td>
<td>16.9</td>
<td>16.2</td>
</tr>
<tr>
<td></td>
<td>n=</td>
<td></td>
<td>20</td>
<td>20</td>
<td>20</td>
<td>-</td>
<td>20</td>
<td>15</td>
<td>10</td>
<td>5</td>
<td>-</td>
</tr>
</tbody>
</table>

*Animals were fed extruded Global Rodent Diet in the pre-treatment period from -120 h to 0 h; from 0 h to 96 h, animals were given extruded Global Rodent Diet (control), Spike oil by gavage 3 times per 24 h, or HEAR oil diet. Values for n are for all animals in each treatment group. Further information on treatment regimens is presented in Table 8.3.

bThe water consumption for -72 h to 0 h was a 72 h water consumption which has been averaged to provide a 24 h consumption figure.

cThe mean of 3 water consumptions, -120 h to 0 h.

dThe mean of 4 water consumptions, 0 h to 96 h.
Table 8.20A. Experiment 4, time course study; serum clinical chemistry results of control male Hanover Wistar rats at the beginning of the treatment period (0 h)\textsuperscript{ab}.

<table>
<thead>
<tr>
<th>Treatment group</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Urea</td>
<td>4.84 (0.49)</td>
</tr>
<tr>
<td>CREAT</td>
<td>43.2 (2.2)</td>
</tr>
<tr>
<td>Albumin</td>
<td>33.4 (1.5)</td>
</tr>
<tr>
<td>TP</td>
<td>48.6 (1.1)</td>
</tr>
<tr>
<td>Sodium</td>
<td>145.4 (1.7)</td>
</tr>
<tr>
<td>Potassium</td>
<td>5.06 (0.51)</td>
</tr>
<tr>
<td>Chloride</td>
<td>101.8 (1.3)</td>
</tr>
<tr>
<td>CHOL</td>
<td>1.82 (0.37)</td>
</tr>
<tr>
<td>TRIG</td>
<td>0.48 (0.20)</td>
</tr>
<tr>
<td>NEFA</td>
<td>0.500 (0.102)</td>
</tr>
<tr>
<td>ALT</td>
<td>54.8 (7.8)</td>
</tr>
<tr>
<td>AST</td>
<td>105.6 (7.5)</td>
</tr>
<tr>
<td>CK</td>
<td>698.6 (120.6)</td>
</tr>
<tr>
<td>LD</td>
<td>2013.2 (466.9)</td>
</tr>
<tr>
<td>GLD</td>
<td>4.44 (1.64)</td>
</tr>
<tr>
<td>Aldolase</td>
<td>62.0 (5.3)</td>
</tr>
<tr>
<td>cTn\textsuperscript{C}</td>
<td>0.010 (0.000)</td>
</tr>
</tbody>
</table>

\textsuperscript{a}Values are means, SD in parentheses; n=5.

\textsuperscript{b}Abbreviations and units: as Table 8.6.

\textsuperscript{c}Serum levels of cTnl were assayed with the Bayer Centaur CP.
Table 8.20B. Experiment 4, time course study; serum clinical chemistry results of control male Hanover Wistar rats and animals treated with erucic acid by gavage or in the diet at 24 h of treatment\textsuperscript{a,b}.

<table>
<thead>
<tr>
<th>Treatment group</th>
<th>Control</th>
<th>Spike oil by gavage</th>
<th>HEAR oil diet</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Urea</strong></td>
<td>5.56 (0.48)</td>
<td>6.90 (0.87) *</td>
<td>6.66 (0.81)</td>
</tr>
<tr>
<td><strong>CREAT</strong></td>
<td>46.6 (1.8)</td>
<td>46.0 (1.0)</td>
<td>47.0 (2.9)</td>
</tr>
<tr>
<td><strong>Albumin</strong></td>
<td>33.6 (0.9)</td>
<td>34.0 (1.2)</td>
<td>33.4 (1.1)</td>
</tr>
<tr>
<td><strong>TP</strong></td>
<td>49.0 (1.6)</td>
<td>49.6 (1.9)</td>
<td>48.2 (1.5)</td>
</tr>
<tr>
<td><strong>Sodium</strong></td>
<td>145.4 (0.5)</td>
<td>147.2 (0.8) *</td>
<td>145.8 (1.1) *</td>
</tr>
<tr>
<td><strong>Potassium</strong></td>
<td>4.70 (0.24)</td>
<td>4.52 (0.45)</td>
<td>4.74 (0.43)</td>
</tr>
<tr>
<td><strong>Chloride</strong></td>
<td>101.8 (0.4)</td>
<td>101.8 (1.3)</td>
<td>101.8 (1.8)</td>
</tr>
<tr>
<td><strong>CHOL</strong></td>
<td>2.02 (0.28)</td>
<td>2.10 (0.16)</td>
<td>1.62 (0.24) *</td>
</tr>
<tr>
<td><strong>TRIG</strong></td>
<td>0.52 (0.13)</td>
<td>0.48 (0.20)</td>
<td>0.50 (0.20)</td>
</tr>
<tr>
<td><strong>NEFA</strong></td>
<td>0.588 (0.123)</td>
<td>0.744 (0.242)</td>
<td>0.756 (0.399)</td>
</tr>
<tr>
<td><strong>ALT</strong></td>
<td>55.6 (5.1)</td>
<td>82.8 (12.9) ***</td>
<td>70.4 (8.0) *</td>
</tr>
<tr>
<td><strong>AST</strong></td>
<td>91.0 (7.6)</td>
<td>87.8 (13.7)</td>
<td>94.0 (8.9)</td>
</tr>
<tr>
<td><strong>CK</strong></td>
<td>558.2 (120.4)</td>
<td>508.6 (108.9)</td>
<td>650.8 (222.7)</td>
</tr>
<tr>
<td><strong>LD</strong></td>
<td>1448.4 (388.6)</td>
<td>1461.2 (446.3)</td>
<td>1594.2 (581.2)</td>
</tr>
<tr>
<td><strong>GLD</strong></td>
<td>4.64 (1.08)</td>
<td>4.90 (0.73)</td>
<td>6.18 (0.61) *</td>
</tr>
<tr>
<td><strong>Aldolase</strong></td>
<td>58.2 (7.7)</td>
<td>71.0 (4.0) *</td>
<td>77.2 (8.6) ***</td>
</tr>
<tr>
<td><strong>cTnl</strong></td>
<td>&lt;0.010 (0.000)</td>
<td>&lt;0.010 (0.000)</td>
<td>0.030 (0.045)</td>
</tr>
</tbody>
</table>

\textsuperscript{a}Values are means, SD in parentheses; n=5 for all groups. Animals were given extruded Global Rodent Diet (control), Spike presented in Table 8.3. *Significantly different from control, P<0.05; ***P<0.001.

\textsuperscript{b}Abbreviations and units: as Table 8.6.
Table 8.20C. Experiment 4, time course study; serum clinical chemistry of control male Hanover Wistar rats and animals treated with erucic acid by gavage or in the diet at 48 h of treatment results$^{a,b}$.

<table>
<thead>
<tr>
<th>Treatment group</th>
<th>Control</th>
<th>Spike oil by gavage</th>
<th>HEAR oil diet</th>
</tr>
</thead>
<tbody>
<tr>
<td>Urea</td>
<td>5.76 (0.50)</td>
<td>5.26 (0.38)</td>
<td>6.18 (0.82)</td>
</tr>
<tr>
<td>CREAT</td>
<td>47.0 (1.4)</td>
<td>44.2 (1.8)</td>
<td>44.6 (2.1)</td>
</tr>
<tr>
<td>Albumin</td>
<td>33.4 (0.9)</td>
<td>31.4 (0.5) $^*$</td>
<td>32.6 (1.3)</td>
</tr>
<tr>
<td>TP</td>
<td>49.0 (2.0)</td>
<td>45.0 (1.0) $^{***}$</td>
<td>47.6 (2.1)</td>
</tr>
<tr>
<td>Sodium</td>
<td>146.0 (1.2)</td>
<td>147.0 (0.7)</td>
<td>146.4 (1.5)</td>
</tr>
<tr>
<td>Potassium</td>
<td>4.86 (0.30)</td>
<td>4.94 (0.27)</td>
<td>4.92 (0.39)</td>
</tr>
<tr>
<td>Chloride</td>
<td>101.6 (1.8)</td>
<td>101.0 (1.6)</td>
<td>101.2 (1.5)</td>
</tr>
<tr>
<td>CHOL</td>
<td>1.94 (0.21)</td>
<td>1.94 (0.18)</td>
<td>1.84 (0.52)</td>
</tr>
<tr>
<td>TRIG</td>
<td>0.66 (0.24)</td>
<td>0.78 (0.38)</td>
<td>0.38 (0.04)</td>
</tr>
<tr>
<td>NEFA</td>
<td>0.790 (0.130)</td>
<td>0.760 (0.129)</td>
<td>0.590 (0.167)</td>
</tr>
<tr>
<td>ALT</td>
<td>76.8 (17.6)</td>
<td>97.4 (11.3)</td>
<td>75.8 (20.2)</td>
</tr>
<tr>
<td>AST</td>
<td>105.0 (15.8)</td>
<td>114.8 (25.6)</td>
<td>99.4 (15.7)</td>
</tr>
<tr>
<td>CK</td>
<td>735.6 (197.1)</td>
<td>926.0 (297.7)</td>
<td>837.6 (150.8)</td>
</tr>
<tr>
<td>LD</td>
<td>2998.4 (1357.2)</td>
<td>2993.2 (988.2)</td>
<td>2302.6 (607.2)</td>
</tr>
<tr>
<td>GLD</td>
<td>5.24 (1.19)</td>
<td>6.02 (1.40)</td>
<td>6.00 (1.03)</td>
</tr>
<tr>
<td>Aldolase</td>
<td>78.2 (16.0)</td>
<td>86.8 (12.7)</td>
<td>78.0 (7.3)</td>
</tr>
<tr>
<td>cTnl</td>
<td>&lt;0.010 (0.000)</td>
<td>0.010 (0.000)</td>
<td>&lt;0.010 (0.000)</td>
</tr>
</tbody>
</table>

$^a$Values are means, SD in parentheses; n=5 for all groups, except control cTnl, where n=4. Animals were given extruded Global Rodent Diet (control), Spike oil by gavage 3 times per 24 h, or HEAR oil diet. Further information on regimens of administration is presented in Table 8.3. *Significantly different from control, P<0.05; $^{***}$P<0.001.

$^b$Abbreviations and units: as Table 8.6.
Table 8.20D. Experiment 4, time course study; serum clinical chemistry results of control male Hanover Wistar rats and animals treated with erucic acid by gavage or in the diet at 72 h of treatment\textsuperscript{a,b}.

<table>
<thead>
<tr>
<th>Treatment group</th>
<th>Control</th>
<th>Spike oil by gavage</th>
<th>HEAR oil diet</th>
</tr>
</thead>
<tbody>
<tr>
<td>Urea</td>
<td>5.92 (0.54)</td>
<td>5.58 (0.92)</td>
<td>5.62 (1.00)</td>
</tr>
<tr>
<td>CREAT</td>
<td>47.4 (1.1)</td>
<td>48.5 (9.1)</td>
<td>47.0 (2.5)</td>
</tr>
<tr>
<td>Albumin</td>
<td>33.4 (0.5)</td>
<td>32.3 (1.0)</td>
<td>33.8 (1.3)</td>
</tr>
<tr>
<td>TP</td>
<td>50.6 (1.1)</td>
<td>47.5 (0.6)\textsuperscript{***}</td>
<td>48.8 (1.9)</td>
</tr>
<tr>
<td>Sodium</td>
<td>145.2 (0.8)</td>
<td>146.8 (1.7)</td>
<td>146.0 (1.0)</td>
</tr>
<tr>
<td>Potassium</td>
<td>4.74 (0.27)</td>
<td>4.73 (0.17)</td>
<td>4.82 (0.40)</td>
</tr>
<tr>
<td>Chloride</td>
<td>99.6 (0.9)</td>
<td>100.0 (1.6)</td>
<td>101.2 (1.9)</td>
</tr>
<tr>
<td>CHOL</td>
<td>2.04 (0.18)</td>
<td>1.98 (0.22)</td>
<td>1.92 (0.29)</td>
</tr>
<tr>
<td>TRIG</td>
<td>0.48 (0.08)</td>
<td>0.65 (0.17)</td>
<td>0.40 (0.07)</td>
</tr>
<tr>
<td>NEFA</td>
<td>0.708 (0.052)</td>
<td>0.963 (0.241)</td>
<td>0.696 (0.139)</td>
</tr>
<tr>
<td>ALT</td>
<td>62.8 (8.3)</td>
<td>70.3 (8.7)</td>
<td>73.0 (12.7)</td>
</tr>
<tr>
<td>AST</td>
<td>96.6 (13.1)</td>
<td>95.3 (16.0)</td>
<td>96.0 (14.4)</td>
</tr>
<tr>
<td>CK</td>
<td>457.6 (88.0)</td>
<td>482.0 (136.5)</td>
<td>425.8 (135.5)</td>
</tr>
<tr>
<td>LD</td>
<td>1508.6 (384.3)</td>
<td>1960.3 (580.3)</td>
<td>1596.0 (439.0)</td>
</tr>
<tr>
<td>GLD</td>
<td>4.96 (0.60)</td>
<td>6.15 (1.56)</td>
<td>5.54 (0.48)</td>
</tr>
<tr>
<td>Aldolase</td>
<td>54.8 (10.4)</td>
<td>71.0 (1.4)\textsuperscript{***}</td>
<td>68.2 (6.8)\textsuperscript{*}</td>
</tr>
<tr>
<td>cTnl</td>
<td>0.010 (0.000)</td>
<td>0.010 (0.000)</td>
<td>0.010 (0.000)</td>
</tr>
</tbody>
</table>

\textsuperscript{a}Values are means, SD in parentheses; n=5 for all groups, except Spike oil gavage, where n=4. Animals were given extruded Global Rodent Diet (control), Spike oil by gavage 3 times per 24 h, or HEAR oil diet. Further information on regimens of administration is presented in Table 8.3. \textsuperscript{b}Significantly different from control, P<0.05; \textsuperscript{***}P<0.001.

\textsuperscript{b}Abbreviations and units: as Table 8.6.
Table 8.20E. Experiment 4, time course study; serum clinical chemistry results of control male Hanover Wistar rats and animals treated with erucic acid by gavage or in the diet at 96 h of treatment\textsuperscript{a,b}.

<table>
<thead>
<tr>
<th>Treatment group</th>
<th>Control</th>
<th>Spike oil by gavage</th>
<th>HEAR oil diet</th>
</tr>
</thead>
<tbody>
<tr>
<td>Urea</td>
<td>6.72 (0.79)</td>
<td>7.52 (2.18)</td>
<td>6.06 (0.84)</td>
</tr>
<tr>
<td>CREAT</td>
<td>47.2 (1.3)</td>
<td>45.2 (2.8)</td>
<td>46.0 (2.0)</td>
</tr>
<tr>
<td>Albumin</td>
<td>34.4 (1.1)</td>
<td>32.0 (1.2)\textsuperscript{*}</td>
<td>33.2 (1.6)</td>
</tr>
<tr>
<td>TP</td>
<td>51.0 (1.7)</td>
<td>46.8 (1.8)\textsuperscript{*}</td>
<td>49.0 (2.5)</td>
</tr>
<tr>
<td>Sodium</td>
<td>147.2 (1.6)</td>
<td>146.2 (0.4)</td>
<td>145.2 (0.8)\textsuperscript{*}</td>
</tr>
<tr>
<td>Potassium</td>
<td>4.82 (0.26)</td>
<td>4.82 (0.19)</td>
<td>4.94 (0.42)</td>
</tr>
<tr>
<td>Chloride</td>
<td>101.0 (1.6)</td>
<td>101.0 (1.6)</td>
<td>99.2 (2.2)</td>
</tr>
<tr>
<td>CHOL</td>
<td>1.80 (0.30)</td>
<td>1.84 (0.17)</td>
<td>1.64 (0.11)</td>
</tr>
<tr>
<td>TRIG</td>
<td>0.42 (0.11)</td>
<td>0.62 (0.18)</td>
<td>0.36 (0.13)</td>
</tr>
<tr>
<td>NEFA</td>
<td>0.594 (0.219)</td>
<td>0.820 (0.242)</td>
<td>0.558 (0.246)</td>
</tr>
<tr>
<td>ALT</td>
<td>56.8 (9.0)</td>
<td>94.8 (13.6)\textsuperscript{***}</td>
<td>74.4 (17.7)</td>
</tr>
<tr>
<td>AST</td>
<td>103.6 (29.4)</td>
<td>113.0 (16.2)</td>
<td>81.2 (7.8)</td>
</tr>
<tr>
<td>CK</td>
<td>647.2 (253.9)</td>
<td>648.4 (143.5)</td>
<td>437.4 (44.4)</td>
</tr>
<tr>
<td>LD</td>
<td>2253.4 (1001.7)</td>
<td>2367.6 (700.2)</td>
<td>1405.8 (337.6)</td>
</tr>
<tr>
<td>GLD</td>
<td>5.22 (0.52)</td>
<td>6.62 (0.63)\textsuperscript{*}</td>
<td>5.92 (0.99)</td>
</tr>
<tr>
<td>Aldolase</td>
<td>61.0 (12.5)</td>
<td>69.4 (9.4)</td>
<td>66.8 (6.5)</td>
</tr>
<tr>
<td>cTnl</td>
<td>0.010 (0.000)</td>
<td>0.010 (0.000)</td>
<td>0.010 (0.000)</td>
</tr>
</tbody>
</table>

Values are means, SD in parentheses; n=5 for all groups. Animals were given extruded Global Rodent Diet (control), Spike oil by gavage 3 times per 24 h, or HEAR oil diet. Further information on regimens of administration is presented in Table 8.3. *Significantly different from control, P<0.05; ***P<0.001.

Abbreviations and units: as Table 8.6.
Table 8.21. Experiment 4, time course study; group mean relative organ weights of control male Hanover Wistar rats and animals treated with erucic acid by gavage or in the diet at 96 h of treatment.

<table>
<thead>
<tr>
<th>Treatment group</th>
<th>Control</th>
<th>Spike oil by gavage</th>
<th>HEAR oil diet</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liver</td>
<td>42.76 (4.91)</td>
<td>42.57 (3.98)</td>
<td>44.56 (5.60)</td>
</tr>
<tr>
<td>Kidneys</td>
<td>8.34 (0.19)</td>
<td>8.17 (0.40)</td>
<td>8.05 (0.61)</td>
</tr>
<tr>
<td>Adrenal glands</td>
<td>0.39 (0.06)</td>
<td>0.43 (0.08)</td>
<td>0.32 (0.03)</td>
</tr>
<tr>
<td>Spleen</td>
<td>3.96 (0.26)</td>
<td>2.95 (0.45) *</td>
<td>4.15 (0.96)</td>
</tr>
<tr>
<td>Thymus</td>
<td>3.97 (0.84)</td>
<td>3.08 (0.41)</td>
<td>3.70 (0.33)</td>
</tr>
<tr>
<td>Testes</td>
<td>11.65 (0.86)</td>
<td>10.89 (2.19)</td>
<td>11.83 (0.99)</td>
</tr>
</tbody>
</table>

*Values are means, SD in parentheses; n=5 for all groups. Relative organ weight values are presented in g/kg. Animals were given extruded Global Rodent Diet (control), Spike oil by gavage 3 times per 24 h, or HEAR oil diet. Further information on regimens of administration is presented in Table 8.3. For paired organs (kidneys, adrenals, testes), the mean weight of both organs was used in the calculations. *Significantly different from control, P<0.05.
Table 8.22. Experiment 4, time course study; incidence of microscopic findings in the hearts of control male Hanover Wistar rats and animals treated with erucic acid by gavage or in the diet at 0, 24, 48, 72 and 96 h of treatment.

<table>
<thead>
<tr>
<th>Microscopic finding</th>
<th>Severity grade</th>
<th>Treatment group</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Number of rats in each group with histological changes</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0 h</td>
<td>24 h</td>
</tr>
<tr>
<td>Myocardial vacuolation</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NAD^b</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>Minimal</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Mild</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Moderate</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Marked</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Very marked</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

^ Animals were given extruded Global Rodent Diet (control; Groups 1, 2, 5, 8 and 11), Spike oil by gavage 3 times per 24 h (Groups 3, 6, 9 and 12), or HEAR oil diet (Groups 4, 7, 10 and 13). Further information on regimens of administration is presented in Table 8.3. There were n=5 rats in each treatment group.

^b NAD = no abnormalities detected.
Table 8.23. Experiment 4, time course study; incidence of microscopic findings in the skeletal muscle (quadriceps femoris) of control male Hanover Wistar rats and animals treated with erucic acid by gavage or in the diet at 96 h of treatment$^a$.

<table>
<thead>
<tr>
<th>Microscopic finding</th>
<th>Severity grade</th>
<th>Treatment group</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Control</td>
</tr>
<tr>
<td>Myofibre vacuolation</td>
<td>NAD$^b$</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Minimal</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>Mild</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Moderate</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Marked</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Very marked</td>
<td>-</td>
</tr>
</tbody>
</table>

$^a$Animals were given extruded Global Rodent Diet (Control), Spike oil by gavage 3 times per 24 h, or HEAR oil diet. Further information on regimens of administration is presented in Table 8.3. There were \( n=5 \) rats in each treatment group.

$^b$NAD= no abnormalities detected.
Table 8.24. Experiment 1, preliminary 96 h feeding study; comparison of individual animal cardiac troponin I (cTnl) levels and the incidence of microscopic findings in the hearts of control male Hanover Wistar rats and animals maintained on diets containing erucic acid (EA)\textsuperscript{a,b}.

<table>
<thead>
<tr>
<th>Group</th>
<th>Rat number</th>
<th>cTnl level (µg/L)</th>
<th>Mean (SD)</th>
<th>Myocardial vacuolation score</th>
<th>Mean (SD)</th>
<th>Myocardial degeneration/necrosis score</th>
<th>Mean (SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1</td>
<td>&lt;0.03</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>&lt;0.03</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>&lt;0.03</td>
<td>&lt;0.030 (0.000)</td>
<td>0</td>
<td>0.0 (0.0)</td>
<td>0</td>
<td>0 (0.0)</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>&lt;0.03</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>&lt;0.03</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>0.06</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>0.03</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>0.03</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>9</td>
<td>0.10</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>0.14</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>11</td>
<td>NS</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>12</td>
<td>0.09</td>
<td>2</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>13</td>
<td>0.07</td>
<td>0.055 (0.030)</td>
<td>2</td>
<td>1.8 (0.4)</td>
<td>0</td>
<td>1.2 (1.1)</td>
</tr>
<tr>
<td></td>
<td>14</td>
<td>0.03</td>
<td>1</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>15</td>
<td>0.03</td>
<td>2</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>16</td>
<td>0.07</td>
<td>2</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>17</td>
<td>0.77</td>
<td>1</td>
<td>3</td>
<td>3</td>
<td>3</td>
<td>1.4 (1.1)</td>
</tr>
<tr>
<td></td>
<td>18</td>
<td>0.08</td>
<td>0.222 (0.308)</td>
<td>3</td>
<td>1.8 (0.8)</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>19</td>
<td>0.13</td>
<td>2</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>0.06</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
</tbody>
</table>

\textsuperscript{a}Group 1, Control ground diet; Group 2, low EA rapeseed (LEAR) oil diet (LEAR oil diet); Group 3, LEAR oil with added EA (Spike) diet (Spike oil diet); Group 4, high EA rapeseed (HEAR) oil diet (HEAR oil diet). n=5 for all groups.

\textsuperscript{b}Values for cTnl are measured in µg/L. NS indicates no sample for analysis. Myocardial vacuolation and degeneration/necrosis scores: 0= no abnormalities detected; 1= minimal; 2= mild; 3= moderate.
Table 8.25. Experiment 2, preliminary 96 h gavage study; comparison of individual animal cardiac troponin I (cTnl) levels and the incidence of microscopic findings in the hearts of control male Hanover Wistar rats treated with low erucic acid (EA) rapeseed (LEAR) oil and high EA rapeseed (HEAR) oil by gavage, and dietary HEAR oil\textsuperscript{a,b}.

<table>
<thead>
<tr>
<th>Group</th>
<th>Rat number</th>
<th>cTnl level (µg/L)</th>
<th>Mean (SD)</th>
<th>Myocardial vacuolation score</th>
<th>Mean (SD)</th>
<th>Myocardial degeneration/necrosis score</th>
<th>Mean (SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>NS</td>
<td>&lt;0.03 (0.000)</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0.2 (0.4)</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>&lt;0.03</td>
<td>0.03</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0.6 (0.9)</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>&lt;0.03</td>
<td>0.292 (0.506)</td>
<td>2</td>
<td>2.2 (0.8)</td>
<td>0</td>
<td>0.6 (0.9)</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>&lt;0.03</td>
<td>0.030 (0.000)</td>
<td>2</td>
<td>1.2 (0.4)</td>
<td>0</td>
<td>0.2 (0.4)</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>NS</td>
<td>0.190 (0.251)</td>
<td>2</td>
<td>1.6 (0.5)</td>
<td>0</td>
<td>1.0 (1.0)</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>&lt;0.03</td>
<td>0.058 (0.052)</td>
<td>2</td>
<td>1.4 (0.5)</td>
<td>0</td>
<td>0.0 (0.0)</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>&lt;0.03</td>
<td>0.196 (0.203)</td>
<td>3</td>
<td>2.4 (0.9)</td>
<td>1</td>
<td>0.8 (0.8)</td>
<td></td>
</tr>
</tbody>
</table>

\textsuperscript{a} n=5 for animals in all groups. Group 1 = LEAR oil (1 gavage dose per 24 h), Group 2 = HEAR oil (1 gavage dose per 24 h), Group 3 = HEAR oil (2 gavage doses per 24 h), Group 4 = HEAR oil (3 gavage doses per 24 h), Group 5 = HAER oil (4 gavage doses per 24 h), Group 6 = HEAR oil (5 gavage doses per 24 h), Group 7 = HEAR oil diet. Further information on regimens of administration is presented in Table 8.1.

\textsuperscript{b} Values for cTnl are measured in µg/L. NS indicates no sample for analysis. Myocardial vacuolation and degeneration/necrosis scores: 0= no abnormalities detected; 1= minimal; 2= mild; 3= moderate.
CHAPTER 9: DISCUSSION

The aim of the project reported in this thesis was to develop models of cardiotoxicity in the Hanover Wistar rat, for the assessment of biomarkers of cardiac injury. In the development of the models, 3 compounds were used: isoproterenol (ISO), allylamine (AA) and erucic acid (EA). The models of cardiotoxicity were then used to evaluate biochemical markers of cardiac injury, including aspartate aminotransferase (AST), creatine kinase (CK) and CK isoenzymes, lactate dehydrogenase (LD) and LD isoenzymes, cardiac troponin I (cTnI), cardiac troponin T (cTnT) and heart fatty acid-binding protein (H-FABP). The assessment of cardiac injury in preclinical animal studies is considered not to be optimised at the present time (Wallace et al., 2004; Walker, 2006; O’Brien, 2006) and improved translational biomarkers for toxic myocardial injury are needed in drug discovery and development. In this way, the present studies attempted to investigate the strengths and weaknesses of the biomarkers described above, and in particular the cardiac troponins (cTns).

Studies on ISO showed that a threshold intraperitoneal (IP) dose of the drug in the female Hanover Wistar rat was 10 µg/kg, and below which biochemical evidence (elevations in serum cTnI levels) and microscopic findings in the heart (myocardial degeneration/necrosis with myocyte loss) were not identified. This dose level was lower than that previously reported to cause microscopic changes in the heart (40 µg/kg; Rona et al., 1959a), but slightly higher than that reported to bring about elevations of cTnI in serum (8 µg/kg; Herman et al., 2006). The measurement of total CK and LD activities were demonstrated to be relatively insensitive for the detection of acute cardiomyocyte injury when compared with cTnI determination. The comparison of the IP and the subcutaneous (SC) dose routes for the administration of 5000 µg/kg ISO to the male Hanover Wistar rat demonstrated some differences in the responses to ISO administration, with the biomarker levels generally being higher using the SC route. It was demonstrated that cTnI was the superior marker in terms of sensitivity and specificity. For example, using the SC route of administration, cTnI results were elevated above the group mean control level at all time points post-dosing (1, 2, 3, 4 and 5 h post-dosing), with the peak cTnI value occurring at 3 h (a 20352.4 % increase). The peak AST levels were also at 3 h, but levels were only 62.3 % greater than the control value. Likewise, the group mean serum total CK peak at 3 h post-dosing was 120.9 %
larger than the control value and the group mean total LD peak at 2 h post-dosing was 192.6 % greater than the group mean control result. A highly reproducible model of cardiac injury was subsequently developed using ISO administration by SC injection at 4000 μg/kg in the male Hanover Wistar rat. Histopathological evidence of myocardial injury was evident at 0.5 h post-dosing, at which time point there were also significant elevations in levels of serum H-FABP (P<0.001), serum cTnI (measured using the ACS: 180S and Beckman Access systems; P<0.001), serum cTnT (measured using the Roche Elecsys 2010; P<0.001) and plasma LD2 (P<0.05). Circulating serum H-FABP levels had returned to base line control values at 12.0 h post-dosing, whereas serum cTnI levels did not return to base line levels until 48.0 h (ACS: 180S) and LD2 levels were at base line values at 72.0 h post-dosing. The group mean serum cTnI (Beckman Access) and cTnT (Roche Elecsys 2010) were still elevated above the concurrent control levels at 72.0 h post-dosing, indicating the long diagnostic time windows of these biochemical parameters. Plasma CK-MB and LD1, LD2 and LD3 activities gave additional confirmation of the site of tissue injury; however serum AST, plasma total CK and plasma LD concentrations did not aid in the diagnosis of cardiac damage. There was a temporal disconnect between maximal cTn results and the histopathological response in this experimental model, as serum cTn levels peaked at 3.0 h post-dosing, whereas the highest histological lesion scores occurred during the phase of declining cTn values.

When the very early time course of ISO-induced cardiomyocyte injury was studied, histopathological evidence of cardiomyocyte damage (myocardial degeneration) was present at 10 min post-dosing, the earliest time point at which light microscopic evidence of ISO-induced cardiac injury has been reported. Serum H-FABP levels were also increased concurrent with microscopic changes in the heart, at 10 min post-dosing; elevations in serum cTnI were first seen at 20 min following ISO administration. Therefore H-FABP was shown to be the earliest marker to be increased in this experimental model; however the H-FABP data was quite variable and the marker is also not specific to the heart. There were some correlations between individual animal H-FABP and cTnI values and the myocardial degeneration severity gradings. In general, animals with higher H-FABP values tended to have higher cTnI values, and animals with higher H-FABP or cTnI values tended to have higher degeneration scores. Furthermore, the fold increases for cTnI results were much greater than those for H-FABP results. It was concluded that both serum H-FABP and cTnI levels were early
indicators of cardiac damage in this model of cardiac injury; however the lack of cardiac specificity of H-FABP may reduce its potential as a marker of cardiomyocyte damage.

In studies with AA it was established that a single gavage dose of 100 mg/kg was sufficient to induce myocardial necrosis and vasculopathy at 48 h post-dosing in the male Hanover Wistar rat; however cTnI elevations were not evident at 100 mg/kg, but at 125 mg/kg. There were some correlations between individual serum cTnI levels, and group mean cTnI levels, and the myocardial degeneration/necrosis severity grades. However, it is suggested that the discrepancies between cTnI elevations and the histopathological evidence of myocardial injury may be related to the 48 h point of autopsy sampling. Serum AST, CK and LD activities were not sensitive enough to detect myocardial injury at AA dose levels up to 350 mg/kg. The time course of AA-induced cardiac injury was studied using a gavage dose level of 300 mg/kg over a 48 h period. Myocardial degeneration/necrosis and vasculopathy were first evident at 12 h post-dosing; however these changes were shown to be independent of one another. Other workers have demonstrated that myocardial degeneration/necrosis precedes histopathological evidence of vascular lesions (Boor et al., 1979; Boor et al., 1980; Boor and Ferrans, 1982). Therefore, the time course of cardiac injury observed in the present work did not agree with that reported in the literature. Serum levels of cTnI were first increased at 8 h post-dosing, before histological evidence of cardiac injury, demonstrating the sensitivity of the marker, although no cTnI elevations were statistically significant. The measurement of serum H-FABP in the time course study gave some additional information for the assessment of cardiomyocyte damage; however results were variable, the increases were small and cTnI levels were evident prior to elevations in serum H-FABP values. cTnI was shown to be an excellent marker of cardiomyocyte necrosis, appearing at an earlier time point than the histological evidence of cardiac necrosis, however further investigation of the behaviour of H-FABP is required.

The gavage or dietary administration of EA as high EA rapeseed oil (HEAR oil) or as Spike oil (pure EA mixed with low EA rapeseed oil) in the male Hanover Wistar rat for periods up to 96 h resulted in myocardial lipidosis in all studies. The administration of EA by gavage to induce myocardial lipidosis has not previously been reported; however the incidence and severity of myocardial lipidosis is more predictable and reproducible using a regimen of dietary EA administration. In rats treated with Spike oil or HEAR oil
by gavage or dietary administration for 96 h, the majority demonstrated myocardial
lipidosis. Myocardial degeneration/necrosis was also present in conjunction with
myocardial lipidosis when Spike oil and HEAR oil were administered in the diet or
HEAR oil was given by gavage (the first 2 studies reported). Levels of cTnl were, in
general, raised in the studies that resulted in myocardial lipidosis and
degeneration/necrosis. There were some correlations between serum cTnl values and
the severity grading of the histopathological lesions, but these are not well defined. For
example, animals with higher cTnl values tended to have higher myocardial vacuolation
scores, and these animals also tended to have positive scores for degeneration/necrosis.
In addition, there was some evidence that degeneration/necrosis may follow the
development of myocardial lipidosis. However, in animals administered low EA
rapeseed oil in the diet, there was some evidence of very slight increases in serum cTnl
levels, with no histopathological cardiac changes. The reasons for these cTnl elevations
are unclear, but it is suggested that some ultrastructural damage, not detectable by light
microscopy, may have occurred. Previous work has reported that the myocardial
lipidosis observed following EA administration is not associated with myocardial
degeneration/necrosis (Chien et al., 1983); rather, the appearance of focal myocardial
necrosis is associated with a longer term feeding schedule (Abdellatif and Vles, 1973;
Charlton et al., 1975). Therefore, the present findings disagree with previously reported
data. Nevertheless, the reasons for the absence of degeneration/necrosis in the 2 later
studies (Experiments 3 and 4) are not understood. The time course of development of
myocardial lipidosis was investigated over a period of 96 h. Lipidosis was present in 4
of 5 rats given Spike oil by gavage or HEAR oil in the diet at 24 h, and in 5 of 5 animals
by 72 h. This finding agrees with Chien et al. (1983), who showed lipidosis after 1 day
of feeding HEAR oil as 20 % by weight of diet. Further studies on EA-induced cardiac
damage are required to fully define the time course of lesion development, to investigate
the alterations in serum biochemistry parameters over a longer period of time than 96 h,
and to study the reversibility of EA-induced cardiac lesions in the post-dosing period.

The studies reported in this thesis therefore indicate that cTnl is a sensitive and specific
marker of cardiac injury, whose release correlates directly with the histopathological
evidence of structural cardiomyocyte damage.

ISO and 2,3,5,6-tetramethyl-p-phenylenediamine (TMPD) were used to investigate H-
FABP as a urinary marker of cardiac and skeletal muscle injury, respectively. H-FABP
was detectable in urine at 24 h post-dosing in both types of muscle injury; however the fold increases in H-FABP following skeletal muscle damage induced by TMPD were much larger. Therefore, these experiments highlight the potential use of H-FABP as a non-invasive marker of cardiac muscle or skeletal muscle injury; however measurement of a tissue-specific marker in the circulation would be necessary for complete differential diagnosis.

The development of models of drug- and chemical-induced cardiotoxicity in the Hanover Wistar rat will allow the further investigation of proposed markers of cardiac injury, and also aid in the search for novel markers. As of today, the behaviour of the cTns have not been thoroughly investigated and mapped with functional changes which may occur in the heart. It may be possible to study functional alterations in the heart (as assessed by techniques such as electrocardiography or echocardiography) and the timings of biomarker release with compounds causing sympathomimetic effects, such as ISO, AA or cocaine. There may be correlations between functional abnormalities and subsequent cTn values when there are structural alterations. For example, when the anticancer drug, doxorubicin (DOX), was given at 2.0 mg/kg IV for 8 weeks to male Wistar rats, there were increases in serum cTnT concentrations at week 10 (2 weeks post-dosing) and at week 12 (4 weeks post-dosing) (Koh et al., 2004). In addition, there was an inverse correlation between serum cTnT levels and the % fractional shortening data, which is a technique for the assessment of left ventricular function. Therefore, studies incorporating functional measurements in the present models of cardiotoxicity may give additional information on the behaviour of the cTns in cardiac damage. In the present studies, where there were elevations in serum cTnI levels with no evidence of myocardial degeneration/necrosis (e.g. with EA), the use of electron microscopy may have provided some indication of reasons for cTnI elevations in the absence of light microscopic changes. Although cTn responses have been investigated using ISO, AA and EA, it is necessary to further validate the marker using a wider range of cardiotoxic agents. For example, serum cTnT levels have been reported to be elevated in acute ethanol intoxication in the rat (Patel et al., 2001). Work was also completed for the present project using repeat dosing schedules of DOX and single and repeat dosing regimens with the antineoplastic drug, cyclophosphamide (CPA); however, due to constraints of thesis length, the work on DOX and CPA is not reported here. Other cardiotoxic compounds of interest for the validation of cTn in the assessment of cardiac injury include hydralazine, a vasodilating antihypertensive which causes focal
subendocardial necrosis in the rat and in the dog (Balazs and Payne, 1971; Balazs et al., 1981); cobalt, which causes diffuse vacuolar cardiomyopathy in man (Balazs and Herman, 1976); sulphonamide drugs, which cause interstitial myocarditis in the mouse (French, 1941); and monensin, an ionophore which causes cardiomyocyte degeneration/necrosis in rodents (Todd et al., 1984).

The behaviour of the cTns in drug-induced cardiac hypertrophy must be also investigated. Compounds resulting in cardiac hypertrophy include repeat dosing schedules of ISO (Kizaki et al., 2005) and thyroxine (Craft-Cormney and Hansen, 1980). In addition, the use of surgical models of ischaemia-reperfusion, such as the coronary artery ligation model and also myocardial stunning, especially as this last experimental procedure results in proteolytic cleavage of the cTnI molecule (Canty and Lee, 2002) may also be of use for the evaluation of the biomarkers described in this project.

The investigation of other emerging biomarkers of cardiac injury could be performed using the models of toxic myocardial injury described here. Reports have highlighted for example the possible utility of serum amyloid A (Kosuge et al., 2007) in the assessment of acute coronary syndromes in man; however, this proposed marker is a marker of inflammation, and therefore may not be of use in the assessment of structural cardiomyocyte damage. In addition, ischaemia-modified albumin has been proposed as a new model of cardiac ischaemia (Falkensammer et al., 2007); however this marker is not specific to the heart, and will be elevated in the bloodstream following skeletal muscle ischaemia. Following the establishment of these models of cardiotoxicity, it may be useful to employ tissue expression profiling techniques, such as microarrays and the reverse transcriptase polymerase chain reaction for changes in gene expression, and proteomics technologies to find potential protein biomarkers of myocardial injury. Toxicoproteomics generally uses 2-dimensional gel electrophoresis followed by mass spectrometry to identify critical proteins in biological systems that are affected by, and respond to, adverse chemical and environmental exposures (Wetmore and Merrick, 2004).
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ABSTRACTS


FULL PAPERS

The Role of a Mouse Aldo-Keto Reductase in Resistance to Toxic Aldehydes and Ketones

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Aldehydes and ketones are widely distributed in nature, some of them are very toxic, and react with cellular macromolecules such as protein and DNA (Wiseman and Halliwell, 1996). Aldo-keto reductase (AKR) are a family of enzymes, which can reduce a wide range of aldehydes and ketones (Ellis and Hayes, 1995). It has been proposed that they play a role as detoxication enzymes. However, the exact physiological role of these enzymes is not clear.

We have established cell lines that stably express one of these AKR members (mouse AKR7A5) (Hinshelwood et al., 2002) to investigate the role of this enzyme in resistance to toxic aldehydes and ketones. The level of expression of the transfected AKR7A5 members was confirmed by western blot and enzyme assays.

A range of assays was applied to observe the effects of toxic aldehydes on control and stably-transfected cell lines. Cytotoxicity was measured using MTT assays, mutagenicity was tested using the HGPRT assay, and apoptosis was assessed using associated-DNA damage as well as levels of caspase activity. MTT assays showed that the IC50 values for trans-2-nonenal increased in cells expressing AKR7A5 (14 μM) compared to control (81 μM). However, AKR7A5 did not appear to increase the IC50 for 4-hydroxynonenal (4-HNE) (18 and 14 μM for control and transfected cells, respectively). Following treatment of cell lines with 4-hydroxynonenal, a 1.6-fold increase in mutation frequency was observed in control V79 (pCI-Neo) cells (9.8 ± 3.2/42 ± 1.7) compared with untreated cells (6.2 ± 1.3/42 ± 7.2). However, there was no increase in mutation frequency in V79 cells transfected with AKR7A5 following 4-HNE treatment, indicating that the cells transfected with AKR7A5 are more resistant to the mutagenicity of 4-HNE. The DeadEnd™ TUNEL System was used to detect the extent of DNA damage caused by 4-HNE. Cells transfected with AKR7A5 suffered less DNA damage caused by 4-HNE than did control cells.

In summary, mouse AKR7A5 can protect against the cytotoxicity and mutagenicity of some aldehydes in vitro. The new cell line created therefore has the potential for toxicity screening and drug-metabolism studies.

Keywords: Aldo-keto reductase; Apoptosis; 4-Hydroxynonenal; Mutagenicity

References
Results are means ± S.D (N = 3–6).
* P < 0.05.
** P < 0.01.
*** P < 0.001.

group) were autopsied 1 day after the last dose. Blood, marrow flushes and serum samples were prepared, and tissues (heart, spleen, thymus, sternum) placed in formalin. Blood, marrow and serum enzymes were assayed using conventional techniques; serum cTnI was measured using the ACS 180 kit (Bayer).

Over the 12 weeks, DOX reduced body weight increases in a dose-related fashion. At autopsy, there was some evidence of peritonitis at higher DOX levels, at later stages of the study. There were no clear/consistent effects on heart relative weight (RW), but spleen and thymus RW decreased in relation to time and dose. Haematology showed time and dose-related changes: in general terms, DOX caused significant increases in blood neutrophils, platelets, reticulocytes, and MCV/MCH, and significant decreases in RBC/Hb, lymphocytes and marrow cellularity. Serum clinical chemistry results demonstrated, in overall terms, increases in aldolase (ALD), AST, TCK and LDH, and decreases in ALT. These changes generally reflected the dose of DOX administered. Changes in ALD and ALT were particularly consistent (Table 1).

cTnI assays showed (scheduled autopsies) that DOX administration (at all levels) caused no increase in cTnI values (over the control level of <0.03 μg/l) at any time, indicating absence of cardiac injury. Histology (at scheduled autopsies) generally demonstrated lesions of increasing significance with time and dose: lymphoid depletion in the thymus cortex/medulla and in the splenic white pulp, and reduction in sternal marrow cellularity. There was no clear evidence of DOX-induced cardiac lesions.

We conclude that DOX administration by ip injection at 0.5–2.0 mg/kg, twice weekly, for 6–12 weeks, to female rats, induces toxicity to haematopoietic and lymphoid tissues, but not to the heart, when animals are autopsied at 1 day after the last dose of the drug.

Keywords: Doxorubicin; Rat; Toxicity; Myelotoxicity; Lymphoid toxicity

Reference

Bromodichloromethane-Induced Renal Tubule Cell Proliferation: Possible Link to Formic Acid Excretion in Rats and Mice
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Cardiotoxicity of isoproterenol and levels of serum cardiac troponin I in the Han Wistar rat: a threshold dose response study

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Isoproterenol (isoprenaline; ISO) is a synthetic catecholamine and a potent beta adrenergic agonist. However, administration of ISO at high doses in the rat induces severe cardiac necrosis (Rona et al., 1959). The aim of the present study was to assess the cardiotoxicity of ISO to identify the threshold dose causing an elevation in serum cardiac troponin I (cTnl), a marker of cardiac injury. We also wished to study drug-induced effects on serum enzyme markers of cardiac injury and metabolic parameters.

Table 1

<table>
<thead>
<tr>
<th>Dose (mg/kg)</th>
<th>cTnl (μg/L) 2h</th>
<th>cTnl (μg/L) 24h</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>&lt;0.03 (0.00)</td>
<td>&lt;0.03 (0.00)</td>
</tr>
<tr>
<td>0.001</td>
<td>&lt;0.03 (0.00)</td>
<td>&lt;0.03 (0.00)</td>
</tr>
<tr>
<td>0.005</td>
<td>&lt;0.03 (0.00)</td>
<td>&lt;0.03 (0.00)</td>
</tr>
<tr>
<td>0.01</td>
<td>0.09 (0.08)</td>
<td>&lt;0.03 (0.00)</td>
</tr>
<tr>
<td>0.05</td>
<td>0.28 (0.34)</td>
<td>&lt;0.03 (0.00)</td>
</tr>
<tr>
<td>0.1</td>
<td>1.25 (0.33)**</td>
<td>&lt;0.03 (0.00)</td>
</tr>
<tr>
<td>0.5</td>
<td>2.20 (2.07)*</td>
<td>0.05 (0.04)</td>
</tr>
<tr>
<td>1.0</td>
<td>1.04 (1.37)</td>
<td>&lt;0.03 (0.00)</td>
</tr>
<tr>
<td>5.0</td>
<td>0.74 (1.59)</td>
<td>&lt;0.03 (0.00)</td>
</tr>
<tr>
<td>10.0</td>
<td>4.82 (2.65)**</td>
<td>0.09 (0.09)</td>
</tr>
<tr>
<td>25.0</td>
<td>2.99 (2.15)**</td>
<td>0.11 (0.14)</td>
</tr>
</tbody>
</table>

Results are means (S.D.); n = 5. Significantly different from control: *p < 0.05, **p < 0.01, ***p < 0.001.

ISO generally increased mean cTnl levels in a dose-related fashion, but the correlation was not clearly consistent. At the 2 h time point, cTnl levels were raised above baseline controls (<0.03 μg/L) at all doses above 0.01 mg/kg, although not all animals were affected. By 24 h, levels of cTnl were undetectable, except at the highest doses (Table). This indicates that ISO may induce cardiotoxicity at doses of 0.01 mg/kg and above in the Han Wistar rat. Increases were observed in levels of CK and LDH at the higher doses (10, 25 mg/kg), possibly suggesting cardiom yocyte damage. However, this also demonstrates the relative insensitivity of these markers in comparison to measurement of cTnl. Increases in NEFA showed a drug-induced change at the higher dose levels (10, 25 mg/kg), reflecting increased lipolysis and probably due to the increased circulating catecholamine levels.

In conclusion, when ISO was administered as a single IP injection at doses of 0.01 mg/kg and above, increases in serum cTnl levels were observed. Measurement of total CK and LDH activities were demonstrated to be relatively insensitive in comparison to cTnl determination. These patterns of response are indicative of ISO-induced acute cardiom yocyte injury.

Reference


ISO

Female Han Wistar rats (n = 130; mean weight 151 g; Harlan) were given a single intraperitoneal (IP) injection of ISO (Sigma) at 0 (vehicle control), 25, 10, 5, 1, 0.5, 0.1, 0.05, 0.01, 0.005 and 0.001 mg/kg. At 2 and 24 h post dosing, animals were autopsied (n = 5/group). Blood was taken for serum clinical chemistry and hearts placed in formalin. Serum enzymes (creatine kinase, CK; lactate dehydrogenase, LDH; alanine aminotransferase; aspartate aminotransferase; glutamate dehydrogenase; aldolase) and metabolic parameters (lactate; glucose; non-esterified fatty acids [NEFA]; potassium) were assayed using conventional techniques; cTnl was measured by automated immunochemiluminescence using the ACS: 180 kit (Bayer Diagnostics, Newbury).
Repeat dose study to investigate the toxicity of doxorubicin in the female Han Wistar rat

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The anthracycline doxorubicin (DOX) is active against a variety of tumours, including leukaemias and lymphomas, breast and lung cancers. However, drug use is limited by adverse effects, particularly myelotoxicity and cardiotoxicity. DOX toxicity may be delayed and may occur when treatment has ceased. The aim of the present study was to investigate DOX toxicity in a repeat dose study in the female Han Wistar rat, focusing especially on changes occurring in the post-dosing period.

Female Han Wistar rats (n = 139; mean weight 74 g; Harlan), were given DOX (Pharmacia) by intraperitoneal (IP) injection, twice weekly for up to 12 weeks, at 0 (control), 0.5, and 0.85 mg/kg. At 0, 2, 4, 8 and 12 weeks during the dosing period, rats (n = 5-6/group) were autopsied 1 day after their final dose. In addition, rats (n = 4-6/group) were autopsied during the non-dosing "recovery period", at week 13, 14, 15, 16, 17 and 18. Blood, marrow flushes and serum samples were prepared, and tissues (heart, spleen, thymus, sternum, lungs, liver, kidneys, small intestine, ovaries) placed in formalin. Blood and marrow cell counts, and clinical chemistry profiles were assayed using conventional techniques. Serum cardiac troponin I (cTnI) and serum cardiac troponin T (cTnT) were measured by automated immunochemiluminescence, using the ACS: 180 (Bayer Diagnostics, Newbury, UK) and the Elecsys 2010 (Roche Diagnostics, Lewes) respectively.

DOX treatment produced a dose-related reduction in body weight gain over the 12-week dosing period. Clinical evidence of drug toxicity was seen, but mainly in the post-dosing period. Upon autopsy, macroscopic and microscopic changes consistent with DOX-induced localised peritonitis were observed in a proportion of treated animals. Macroscopic changes included the accumulation of abdominal fluid, mesenteric thickening and intra-abdominal adhesions. Macroscopic changes affected a variety of abdominal viscera and included peritoneal/capsular fibrosis and inflammatory cell infiltration. The incidence and severity generally increased with dose and time, particularly in the post-dosing period. More significantly affected animals also showed splenic, thymic and renal changes in addition to changes in clinical chemistry and haematological measurements. These changes are considered to reflect the localised tissue response to DOX at the site of administration. There were no clear dose-related microscopic changes consistent with DOX-induced cardiotoxicity. Ventricular myocardial vacuolation, graded as minimal, which is consistent with DOX-induced cardiotoxicity, was observed in only two treated females (0.5 and 0.85 mg/kg) killed at week 16/17 (recovery period). However, this change did not clearly correlate with increases in serum cTnI concentration. In addition, focal myocardial degeneration/necrosis, graded as mild, was observed in two treated females (0.85 mg/kg), also in the recovery period. This change was associated with increases in cTnI concentration in these animals and is considered to contribute to a statistically significant increase in group mean in cTnI (0.26 µg/L versus 0.03 µg/L, p < 0.05) present at week 16. Reduced sternal bone marrow cellularity, graded as minimal to mild was observed in a small proportion of treated animals, consistent with the reduction in lymphocyte and marrow nucleated cell counts observed during the course of the study and is an established effect of DOX administration. Measurement of cTnT offered no additional diagnostic significance in the detection of cardiotoxicity in this model.

We conclude that the IP administration of DOX at 0.5 and 0.85 mg/kg for 12 weeks induces a range of pathological changes during and after the period of drug administration. However, there was no microscopic evidence of DOX-induced dose related cardiotoxicity which clearly correlated with significant increases in serum cTnI levels. The lack of cardiotoxicity in association with the clear changes in the abdominal cavity indicates that this is not an appropriate route of DOX administration in this experimental model.
Table 1
Cytotoxicity of Cu-Ad: % cell death

<table>
<thead>
<tr>
<th>Cu-Ad (μM)</th>
<th>0</th>
<th>34.2</th>
<th>62.5</th>
<th>125</th>
<th>187.5</th>
<th>250</th>
</tr>
</thead>
<tbody>
<tr>
<td>HepG2</td>
<td>12</td>
<td>24</td>
<td>32</td>
<td>61</td>
<td>80</td>
<td>97</td>
</tr>
<tr>
<td>C2C12</td>
<td>5</td>
<td>14</td>
<td>24</td>
<td>42</td>
<td>65</td>
<td>85</td>
</tr>
<tr>
<td>A549</td>
<td>4</td>
<td>4</td>
<td>6</td>
<td>8</td>
<td>12.9</td>
<td>29</td>
</tr>
<tr>
<td>NIH3T3</td>
<td>13</td>
<td>25</td>
<td>30</td>
<td>34</td>
<td>39</td>
<td>74</td>
</tr>
<tr>
<td>MCF7</td>
<td>5</td>
<td>7</td>
<td>10</td>
<td>17</td>
<td>32</td>
<td>69</td>
</tr>
<tr>
<td>MCF7/Bl2</td>
<td>5</td>
<td>7</td>
<td>9</td>
<td>15</td>
<td>28</td>
<td>61</td>
</tr>
</tbody>
</table>

and nickel were developed and tested. The ligands used are pyrimidine and purine compounds such as fluorocytosine, azapurines, 9-ethyl adenine and 6-mercaptopurine (Liu et al., 2004). The cell killing effects of these complexes were attributed to the formation of stable bifunctional inter/intra strand adduct with DNA blocking thus replication.

A new copper–adenine complex (Cu-Ad) was synthesized and characterized by elemental analysis, infrared spectroscopy, UV–vis spectroscopy, mass spectrometry and thermal analysis. The aim of the study is to screen in vitro biological effect of Cu-Ad on: cell toxicity, mitochondrial bioenergetics, differentiation, replication and transcription.

Different cell lines were cultured (50,000) and treated for 24 h with Cu-Ad (0–250 μM) following which the viability of cells was estimated using the trypan blue exclusion test. Table 1 shows more cell death in HepG2 and C2C12 cells following Cu-Ad treatment with IC50 of 97 and 146 μM, respectively.

The addition of Cu-Ad (1 nmole) to a PCR reaction decreased the amplification of TBX-20 gene and Exon-11 of the WND gene by 68 and 54%, respectively suggesting a possible effect on replication. Similarity Cu-Ad (1 nmole) inhibited significantly the binding of GATA proteins to DNA indicating an effect on transcription.

C2C12 differentiation was induced by Switching C2C12 cells from DMEM media supplemented with 20–0.5% FBS. Cu-Ad at concentrations greater than 2.5 μM was found to inhibit C2C12 (70%) myoblast differentiation into myotubes but had no effect at lower concentration (0.5–1 μM).

Cu-Ad (2–9 nM) inhibited in a concentration dependent manner the activity of NADH oxidase in isolated rat liver sub-mitochondrial particles with an IC50 of 4.5 nM. Whereas Cu-Ad had no effect on succinate dehydrogenase or on F3F1 ATPase, the inhibition of NADH oxidation would reduce significantly ATP level; and may possibly be one of the causes underlying the cell death induced by Cu-Ad complex.

In conclusion, we have identified a new class of compounds with diversified potent biochemical effects with the possible use as anti-cancer agents. Cu-Ad complex induced cell death in HepG2 and C2C12 cells; inhibited replication, transcription, differentiation and mitochondrial NADH oxidation. Structural analogues of Cu-Ad are currently being designed that will allow us to evaluate the structure function relation ship to specific cellular target.

References
doi:10.1016/j.tox.2006.05.075

Cardiotoxicity of allylamine and levels of serum cardiac troponin I in the Han Wistar rat

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Allylamine (AA) is an unsaturated primary amine used in the production of polymers and the synthesis of pharmaceutical agents. In addition to being a severe respiratory, eye and skin irritant, AA is cardiotoxic causing myocardial and vascular lesions in several animal species (Guzman et al., 1961). The main aim of the present study was to assess the cardiotoxicity of AA in the rat by histological changes and the measurement of serum cardiac troponin I (cTnI), a proposed marker of cardiac injury. We also wished to evaluate the usefulness of cTnl measurements versus traditional serum markers of cardiac injury: creatine kinase (CK) and lactate dehydrogenase (LD). Previous studies with isoprenaline in our laboratory have shown that serum cTnI levels in the rat peak 2–3 h post dosing, and may still be detectable at 24 h. However, this diagnostic time window may vary according to the mechanism of cardiomyocyte damage induced by other cardiotoxicants.

Male Han Wistar rats (n = 45; mean weight 182 g; Harlan) were dosed once with AA by gavage at 0, 25, 50, 75, 100, 125, 150, 175 and 200 mg/kg. At 48 h post dosing, animals were autopsied (n = 5/group). Blood was taken for serum clinical chemistry and hearts placed in
Table 1: Effect of allylamine on serum biochemical markers and cardiac pathology at 48 h post dosing

<table>
<thead>
<tr>
<th>Dose (mg/kg)</th>
<th>Serum cTnl (µg/L)</th>
<th>Serum total CK (U/L)</th>
<th>Serum total LD (U/L)</th>
<th>Incidence of degeneration/necrosis</th>
<th>Incidence of cTnl positivity (&gt;0.1 µg/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0.034 (0.009)</td>
<td>596.0 (100.0)</td>
<td>1450.4 (284.8)</td>
<td>0/5</td>
<td>0/5</td>
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<tr>
<td>25</td>
<td>0.030 (0.000)</td>
<td>358.5 (116.8)</td>
<td>651.5 (241.2)</td>
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<td>5/5</td>
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<td>1.146 (0.681)***</td>
<td>635.6 (200.8)</td>
<td>1662.4 (600.1)</td>
<td>5/5</td>
<td>4/5</td>
</tr>
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</table>

Results are means (S.D.); n = 5. **p < 0.001 using one-way ANOVA and Dunnett's post test.

formalin for histology. Serum enzymes (total CK, total LD) were measured by conventional techniques, and cTnl was measured by automated immunochromiluminescence (ACS: 180 kit; Bayer Diagnostics, Newbury).

AA increased serum levels of cTnl in a dose-related manner (Table 1). Levels of cTnl were raised above the control value (0.03 µg/L) at dose levels of 125 mg/kg and above, with statistical significance at 200 mg/kg (p < 0.001). In contrast, CK and LD levels showed no clear evidence of cardiac injury. Myocardial degeneration and necrosis, graded minimal to marked, was present in a proportion of animals dosed at 100 mg/kg and above (Table 1). Lesions increased in incidence and severity with increasing dose, and were located predominantly in the apex and interventricular septum. Changes were characterised by intense eosinophilia, vacuolation and fragmentation of muscle fibres, inflammatory cell infiltration, interstitial oedema, and spindle cell (probably fibroblast) activation.

In conclusion, AA administration caused a dose-related increase in serum cTnl at levels of 125 mg/kg and above. Histological examination of hearts showed evidence of drug-induced acute myocardial injury at a dose of 100 mg/kg and above. The slight discrepancy between cTnl values and histopathological evidence of myocardial injury may relate to sampling time (48 h). Measurement of total CK and total LD activities were shown to be insensitive in the assessment of cardiac injury.

Reference


doi:10.1016/j.tox.2006.05.076

Persistent gene expression changes induced by diethylstilbestrol in the neonatal mouse uterus: Role of epigenetics in carcinogenesis

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Xenoestrogen-induced uterine carcinogenesis is associated with persistent changes in gene expression (Li et al., 2003). An epigenetic mechanism, whereby DNA methylation of affected genes is perturbed, has been proposed as the basis for this persistent deregulation.

To explore this hypothesis, we used a neonatal mouse model to measure uterine gene expression changes induced by diethylstilbestrol (DES). From postnatal Day (PND) 1–5, CD1 mice received daily subcutaneous doses of DES at 1 or 1000 µg/kg/day that are known to result in high incidence of uterine tumours (31 and 90%, respectively) 18 months after exposure (Newbold et al., 1990, 2001). Control animals received vehicle (corn oil) alone. Animals were sacrificed at PND 6, 8, 12, 17, 30 and 50. The uterus was weighed, one horn was fixed and processed for histopathological evaluation, and the other was flash frozen for transcript profiling using Affymetrix Mouse Genome 430.2 GeneChips. Results from histopathological evaluation, together with oestrous cycle staging performed by microscopic assess-

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Preliminary Studies On Erucic Acid-Induced Cardiac Lipidosis In The Male Wistar Rat

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Erucic acid (EA) is a long chain fatty acid (22:1) occurring at high concentrations in some rapeseed oils. Short-term feeding of high EA rapeseed (HEAR) oil in the rat has been associated with cardiac lipidosis (Abdellatif and Vles 1973). The aims of the present study were to investigate the cardiotoxic effects of HEAR oil and pure EA mixed with low EA rapeseed (LEAR) oil (“Spike oil”), administered in the diet for 96 h. We also wished to evaluate possible changes in serum levels of cardiac troponin I (cTnl) and other clinical chemistry parameters and relate changes to the type of cardiac injury. Previous work with acute dosing of isoproterenol in our laboratory has shown that serum cTnl levels in the rat appear at 1 h post-dosing, and may still be detectable at 24 h (York et al. 2007). However this diagnostic time window may vary according to the mechanism of cardiomyocyte damage.

Male Hanover Wistar rats (n=20; mean weight 100 g; Harlan) were fed ground diet (Control) (Harlan), LEAR oil diet, Spike oil diet and HEAR oil diet (n=5/group) for a period of 96 h. Oils were mixed with ground diet to give an oil content of 20 % (w/w). At 96 h, animals were autopsied and blood taken for serum clinical chemistry and hearts placed in formalin for histology. Serum parameters were measured by conventional techniques, and cTnl was measured by automated immunochemiluminescence (ACS: 180; Bayer Diagnostics, Newbury).

The feeding of high levels of EA (Spike oil diet, HEAR oil diet) increased serum levels of alanine aminotransferase (ALT), aspartate aminotransferase (AST) and cTnl (Table 1). At autopsy, the hearts of animals fed Spike oil diet and HEAR oil diet were noticeably pale, with prominent coronary vessels. There were significant increases in relative heart weights of high EA-treated animals (Table 1). A diffuse microvesicular myocardial vacuolation (graded as minimal to moderate) was present in all animals given high levels of EA; Oil Red O staining demonstrated the vacuoles contained neutral lipid. An unexpected myocardial degeneration/necrosis, graded minimal to moderate, was present in 4 of 5 animals given Spike oil diet and 5 of 5 animals given HEAR oil diet. There was no clear correlation between the magnitude of individual animal cTnl values and histopathological severity grades for myofibre vacuolation or degeneration/necrosis. The reasons for the increases in serum cTnl levels in the LEAR oil group are unclear, particularly as no cardiac lesions were identified.

Table 1: Effects of erucic acid on serum biochemical markers and relative heart weights.

<table>
<thead>
<tr>
<th></th>
<th>Control diet</th>
<th>LEAR oil diet</th>
<th>Spike oil diet</th>
<th>HEAR oil diet</th>
</tr>
</thead>
<tbody>
<tr>
<td>ALT (U/L)</td>
<td>51.6 (8.3)</td>
<td>64.6 (11.0)</td>
<td>77.2 (9.5) *</td>
<td>84.2 (19.7) ***</td>
</tr>
<tr>
<td>AST (U/L)</td>
<td>93.2 (9.9)</td>
<td>89.0 (6.7)</td>
<td>107.8 (24.8)</td>
<td>128.6 (14.7) ***</td>
</tr>
<tr>
<td>cTnl (µg/L)</td>
<td>&lt;0.030 (0.000)</td>
<td>0.078 (0.043)</td>
<td>0.055 (0.030)</td>
<td>0.222 (0.308)</td>
</tr>
<tr>
<td>Relative heart weight (g/kg)</td>
<td>4.59 (0.21)</td>
<td>5.14 (0.56)</td>
<td>5.32 (0.39) *</td>
<td>5.62 (0.39) ***</td>
</tr>
</tbody>
</table>

Results are means (SD); n=5. *p<0.05, ***p<0.001 using one-way ANOVA and Dunnett’s post test.

In conclusion, the dietary administration of Spike oil and HEAR oil caused alterations in serum levels of cTnl. Histological examination of hearts showed evidence of EA-induced myocardial injury, characterised by myocardial lipidosis and also degeneration/necrosis. Further investigations are required to define the time course of cardiac injury and identify other possible target organs of toxicity (e.g. liver), as suggested by the minimal increases observed in serum ALT and AST activity.

References
Characterization of Troponin Responses in Isoproterenol-Induced Cardiac Injury in the Hanover Wistar Rat

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ABSTRACT

The investigations aimed to evaluate the usefulness of cardiac troponins as biomarkers of acute myocardial injury in the rat. Serum from female Hanover Wistar rats treated with a single intraperitoneal (IP) injection of isoproterenol (ISO) was assayed for cardiac troponin I (cTnI) (ACS: 180SE, Bayer), cTnT (Immulite 2000, Diagnostic Products Corporation) and cardiac troponin T (cTnT) (Elecsys 2010, Roche). In a time-course study (0.25 to 20.0 mg/kg ISO), serum cTnI (ACS: 180SE) and cTnT increased above control levels at 1 hour postdosing, peaking at 2 hours (cTnI, 4.30 µg/L; cTnT, 1.79 µg/L), and declined to baseline by 48 hours, with histologic cardiac lesions first seen at 4 hours postdosing. The Immulite 2000 assay gave minimal cTnI signals, indicating poor immunoreactivity towards rat cTnI. In a dose-response study (0.25 to 20.0 mg/kg ISO), there was a trend for increasing cTnI (ACS: 180SE) values with increasing ISO dose levels at 2 hours postdosing. By 24 hours, cTnI levels returned to baseline although chronic cardiac myodegeneration was present. We conclude that serum cTnI and cTnT levels are sensitive and specific biomarkers for detecting ISO induced myocardial injury in the rat. Serum troponin values reflect the development of histopathologic lesions; however peak troponin levels precede maximal lesion severity.

Keywords. Cardiac troponin I; cardiotoxicity; isoproterenol; rat; toxicity; troponin.

INTRODUCTION

In man, serum levels of the cardiac troponins (cTn), particularly troponin I (cTnI) and troponin T (cTnT), are widely used in the detection of acute myocardial infarction and a range of other cardiac conditions (Mair, 1997; Christenson and Azzazy, 1998; Panteghini, 2004; Adamcova et al., 2005; Apple et al., 2005; Dybdahl et al., 2005). However, the use of serum cTnI and cTnT in the detection of cardiac injury in laboratory animal species is not as widespread (Herman et al., 1998; Bertisch et al., 1999; Bertinchant et al., 2000; Adamcova et al., 2005; Walker, 2006). Wallace et al. (2004) reviewed the usefulness of serum troponins as biomarkers of drug-induced cardiac toxicity in preclinical safety assessment studies as part of the role of the Expert Working Group (EWG) of the Nonclinical Studies Subcommittee (NCSS), which had been established by the US Food and Drug Ad-

ministration, and which reported to the Advisory Committee for Pharmaceutical Sciences (ACPS).

In considering the case for advocating the increased use of cTnI, the NCSS wished to improve the accuracy by which preclinical studies predict a clinical outcome with respect to potential adverse drug reactions, and also propose new biomarkers that would strengthen the interface between preclinical laboratory studies and clinical trials (Wallace et al., 2004). In addition, the EWG also set out the desired characteristics of the ideal serum/plasma biomarker for the detection of myocardial injury, and suggested that these were: specificity, sensitivity, predictability and robustness (Wallace et al., 2004). Nevertheless, the EWG also concluded that additional work was required for the further validation of the utility of cTnI, and put forward a series of future goals, which included:

1. Evaluation of the kinetics of release and return to baseline (i.e. the diagnostic window) of cTnI, and the correlation with histopathology, following the administration of compounds that cause distinct forms of cardiomyocyte injury.
2. Determination of whether there is a threshold for the increase in serum cTnI, and below which there is no substantial or sustained cardiomyocyte injury.
3. Establish whether there is a diagnostic advantage of measuring serum cTnI, or cTnT, or cTnI and cTnT, in the various forms of cardiomyocyte injury.
4. Establish whether the variation in the cTn assay platforms will influence diagnostic sensitivity within and across laboratory animal species.
With the above proposals in mind, we have carried out a series of studies involving isoproterenol (ISO) induced cardiac injury in the female Hanover Wistar rat. ISO was the first pure β-adrenoceptor agonist to be synthesised (Sears and Løtvall, 2005). The drug was developed in the 1940s and quickly became widely used for the relief of the symptoms of asthma (Waldeck, 2002). However, although ISO is a very potent β agonist, with almost no action on α-adrenoceptors, the drug does not distinguish between β₁ and β₂ receptors. As a result, ISO has significant extrapulmonary side effects, such as tachycardia, arrhythmias and palpitations, because the drug stimulates β₁ receptors in the heart. ISO is a synthetic catecholamine and undergoes rapid metabolism, resulting in a very short duration of action (Dollery, 1998).

The drug is no longer used in the UK and USA for the relief of asthma symptoms (BNF, 2006). However, ISO has become widely used in toxicological studies as a model drug to induce cardiac muscle injury with myocardial ischemia and the formation of infarct-like lesions (Rona et al., 1959; Handforth, 1962; Judd and Wexler, 1974; Bleuel et al., 1995); the mechanism of toxicity is therefore closely related to the pharmacological action of the drug. In the rat, at high doses, ISO quickly stimulates β₁ and β₂ receptors in the heart, inducing an abnormally rapid heart rate (β₁ activity) and a fall in blood pressure (β₂ activity), resulting in cardiac tissue anoxia/hypoxia due to elevated oxygen demand and bringing about severe myocardial necrosis (Rona et al., 1959). These changes are associated with significant increases in both serum cTnI and cTnT (Bleuel et al., 1995; Bertinchant et al., 2000; Wallace et al., 2004). To induce cardiac lesions in the rat, ISO is generally administered by the subcutaneous, intraperitoneal or intravenous routes (Wexler and Handforth, 1962; Judd and Wexler, 1974; Bleuel et al., 1995); and the formation of infarct-like lesions (Rona et al., 1959; Gryglewski et al., 1971; Benjamin et al., 1989; Bleuel et al., 1995; Mohan and Bloom, 1999; Inamoto et al., 2000; O'Brien et al., 2006) and elevations in the levels of serum cTn are generally evident within 4 hours of drug administration; the increases in serum cTn levels are closely correlated with the severity of myocardial injury (Wallace et al., 2004; Walker, 2006).

The purposes of the present investigations were: (1) to assess the linearity and precision of the cTnI assay using the ACS: 180SE (Bayer), together with the storage stability of the rat-specific immunoreactive cTnI signal; (2) to identify the onset of the release of cTn and correlate this timepoint with the nature and severity of the histopathologic lesion in the heart from 1 to 48 hours after the administration of ISO; (3) to identify the magnitude of the cTn signal with increasing dose levels of ISO over the dose range 0.25 to 20.0 mg/kg; (4) to correlate the magnitude and duration of the cTn signal (ACS: 180SE; Bayer Healthcare Diagnostics) with the cTnT signal (Elecsys 2010; Roche Diagnostics) and also relate these 2 signals to the cTnI signal obtained with the DPC Immulite 2000 (Diagnostic Products Corporation); and, (5) to correlate the magnitude and duration of the cTn signal with the more traditional biomarkers of cardiomyocyte injury, and in particular with both creatine kinase (CK) and lactate dehydrogenase (LD) isoenzymes. Preliminary reports of these findings have been published in abstract form (Chen et al., 2004; Brady et al., 2005).
Additional assays of serum cTnl concentrations in selected experiments were also performed by automated immunochemiluminescence on the Immulite 2000 (Diagnostic Products Corporation, Llanberis, UK). This assay used a mouse monoclonal capture antibody and an alkaline phosphatase-conjugated polyclonal detection antibody, both directed towards epitopes between amino acid residues 33–110 of cTnl (Scharnhorst et al., 2002).

Total creatine kinase (CK), total lactate dehydrogenase (LD), alanine aminotransferase (ALT), aspartate aminotransferase (AST), glutamate dehydrogenase (GLD) and aldolase activities in serum were measured by automated spectrophotometric methods on the Advia 1650 (Bayer Healthcare Diagnostics, Newbury, UK) using commercial diagnostic kits. Electrophoresis of CK and LD isoenzymes was performed on the REP automated electrophoresis system (Helena Laboratories Corporation, Gateshead, UK).

Histopathological Examination

At autopsy, the heart was removed, weighed and immediately immersed in 10.5% phosphate-buffered formalin fixative. Following fixation, the hearts were trimmed using a standard pattern (Isaacs, 1998) to provide a transverse section through the middle of the ventricles and longitudinal sections of the apex and base of the heart. Tissues were processed routinely into paraffin and 3 μm sections cut and stained with hematoxylin and eosin (H&E). A single standard section from each animal was examined (blind review) microscopically by one operator and lesions graded as minimal (grade 1) (occasional individual myofibre injury), mild (grade 2) (multiple individual myofibre injury), moderate (grade 3) (larger focal to locally extensive areas of myofibre injury), or moderately severe (grade 4) (the majority of fibres in the myocardium affected).

Statistical Analysis and Data Presentation

ISO treated and control groups were compared using an unpaired 2-tailed Student's t-test (Microsoft Excel; Microsoft Corporation). Data is presented as results from individual animals, or as means (SD) for groups of animals; the number of animals in a group is shown as n. In certain instances, the difference in a parameter between a vehicle treated (control) group and an ISO treated group is expressed as a "fold increase"; here, the increase in the mean value of a parameter in the ISO treated group, or, the increase in the value of a parameter in an ISO treated individual animal, is compared with the mean value of the parameter in the concurrent control group of animals. Where histopathologic changes in the heart are quantified, the method of quantification is described in the relevant table of results.

Experimental Design

Experiment 1: Characterization of the cTnl assay using the ACS: 180SE (Bayer)

Experiment 1a: Immunoreactivity and Linearity of the cTnl Response: Immunoreactivity and linearity of the cTnl response within the working range (0–50 μg/L) was assessed using (i) varying dilutions of rat cardiac homogenate prepared freshly in PBS; (ii) an experimentally-induced in vivo generated rat-specific cTnl in serum; (iii) purified commercial calibration materials (Hytest, Turku, Finland) prepared from rat cardiac homogenates. Dilutions of the material were prepared in species-specific cTnl negative serum.

Experiment 1b: Study on the Stability of cTnl in Serum: Female Hanover Wistar rats (n = 27; mean body weight 172.7 g; SD, 8.7 g; B and K Universal) were randomized into 2 groups; 22 animals were treated with ISO at 50.0 mg/kg by IP injection, and 5 (controls) were dosed with PBS (vehicle) by the same route. At 2 hours postdosing, rats were sacrificed by the IP injection of pentobarbitone sodium and blood removed from the abdominal aorta and collected into serum separator tubes that were maintained at room temperature (RT). At 2 hours after the autopsy, serum was prepared and assayed for cTnl. The mean level of cTnl, measured on the ACS: 180SE, for the ISO treated animals (n = 22) was 25.30 (SD: 13.28) μg/L, and for the control animals (n = 5), <0.03 (SD: 0.00) μg/L.

Additional measurement of cTnl was also performed to provide correlation data (reported later). The serum samples were then pooled to provide cTnl positive material; the level of cTnl in the pooled sample was 27.50 μg/L. The pooled sample was then divided into aliquots to investigate the immunoreactive cTnl stability in serum at RT, 4°C, −20°C, and −80°C.

Experiment 2: Time-Course Study (1 to 48 hours postdosing): Female Hanover Wistar rats (n = 32; mean body weight 111.2 g; SD, 6.8 g; B and K Universal) were randomized into 8 groups of 4 animals each. ISO was given as a single IP injection at 50.0 mg/kg to 7 groups of animals, and one group (controls) received PBS (vehicle). Animals were autopsied at 0 hours (vehicle treated control), and at 1, 2, 4, 6, 12, 24, and 48 hours postdosing. Blood was removed for serum preparation and hearts were taken for histopathologic investigation. Serum cTnl levels were measured using the ACS: 180SE (Bayer) and with the Immulite 2000 (Diagnostic Products); cTnT was assayed using the Elecsys 2010 (Roche).

Experiment 3: Dose-Response Study (8.0 to 40.0 mg/kg Isoproterenol): Female Hanover Wistar rats (n = 35; mean body weight 116.4 g; SD, 10.0 g; B and K Universal) were randomized into 7 groups of 5 animals each. ISO was administered by IP injection at 8.0, 16.0, 24.0, 32.0, 40.0 and 48.0 mg/kg; control animals were dosed with PBS (vehicle). Animals were autopsied at 5 hours postdosing. Blood was removed for the preparation of serum and hearts were taken for histlogic study. As in Experiment 2, serum cTnl levels were assayed with the ACS: 180SE and with the DPC Immulite 2000; values for cTnT were determined using the Elecsys 2010.

Experiment 4: Dose-Response Study (0.25 to 20.0 mg/kg Isoproterenol): Female Hanover Wistar rats (n = 108; mean body weight 124.1 g; SD, 15.4 g; Harlan UK) were randomized into 12 dose level groups of 9 animals each. ISO was administered by IP injection at 0.25, 0.5 and 1.0 mg/kg (part 1 dose level grouping); 2.0, 4.0 and 6.0 mg/kg (part 2 dose level grouping); and 8.0, 10.0 and 20.0 mg/kg (part 3 dose level grouping); each grouping of 3 ISO dose levels had a separate control group which was treated with PBS (vehicle) by the IP route. Of the 9 rats in each of the 12 part 1, 2, and 3 groupings, 5 were autopsied at 2 hours postdosing, and the remaining 4 animals were autopsied at 24 hours postdosing. At autopsy, blood was taken for the preparation of serum and hearts were placed in fixative for histologic study.
Serum was assayed for cTnI (ACS: 180SE) and for the levels of several serum enzymes conventionally used to assess cardiac toxicity, including LD (total activity) and LD isoenzymes (LD1, 2, 3, 4, and 5), CK (total activity) and CK isoenzymes (CKMB, CKBB and CKMM); also, ALT, AST, GLD and aldolase activities were measured. Histologic assessment of cardiac histopathology was carried out and involved the quantification of lesions on a scale of 0 to 4, and the mean lesion score was expressed as the myodegeneration score.

**RESULTS**

**Experiment 1a: Immunoreactivity and Linearity of the cTnI Response:** Reproducible linear responses were found with all the immunoreactive cTnI materials tested over the dynamic range of the assay (0.03 to 50 μg/L). Serum cTnI levels in control/untreated rats were found to be below the lower limit of quantitation (<0.03 μg/L). Interassay coefficients of variation (CV: n = 50) were 11.1% (0.27 μg/L) and 6.3% (13.3, 33.2 μg/L).

**Experiment 1b: Stability:** Stability studies demonstrated that immunoreactive serum cTnI was stable for 24 hours (RT), 14 days (4°C) and up to 3 months (−20°C and −80°C). Additionally, no significant loss of immunoreactivity was observed following multiple freeze-thaw cycles (up to n = 5 cycles).

**Experiment 2: Time-Course Study (1 to 48 Hours Dosing):** Levels of cTnI and cTnT; and the results from the histopathologic assessment of cardiac tissues, are presented in Table 1. The level of serum cTnI measured with the ACS: 180SE was stable for 24 hours (RT), 14 days (4°C) and up to 3 months (−20°C and −80°C). Additionally, no significant loss of immunoreactivity was observed following multiple freeze-thaw cycles (up to n = 5 cycles). Levels of serum cTnI measured with the ACS: 180SE ranged from 2.25 to 5.20 (mean 4.30, SD 1.37 μg/L). Serum cTnI levels in control/untreated rats were found to be below the lower limit of quantitation (<0.03 μg/L). ISO treated vehicle treated (control) rats was <0.03 μg/L. ISO treated animals showed a positive response at 1 hour postdosing (0.48 μg/L), but this level represented a 142-fold increase above the mean control value of <0.03 μg/L; also, clear evidence of cTnI positivity was demonstrated from 1 to 6 hours postdosing.

Levels of serum cTnI measured with the Elecsys 2010 showed a similarity in the pattern of responses to the results obtained for cTnI with the ACS: 180SE (Table 1); however, the fold responses for cTnT were generally greater than the fold increases for cTnI. Levels for cTnT were first positive at 1 hour postdosing with ISO; here, all 4 animals examined were positive, with values ranging from 0.13 to 2.86 (mean 1.54, SD 1.37 μg/L) (mean control baseline levels were <0.01, SD 0.00 μg/L). As with cTnI levels, at 2 hours after ISO administration values of cTnT assayed with the Elecsys 2010 were higher than at 1 hour, the mean result being 1.79 μg/L (p < 0.001).

As with cTnI, levels of cTnT then showed a general gradual decline from 4 to 24 hours postdosing, and at the 24- and 48-hour time points, only 4 of the 8 animals investigated showed a positive response above the baseline value of <0.01 μg/L. The release of cTnT paralleled the findings for cTnI, with cTnT values peaking at 2 hours postdosing; the mean level of cTnT at this time (1.79 μg/L) representing a 179-fold increase over the baseline control value. Similarly, as with cTnI, clear evidence of cTnT positivity was evident from 1 to 6 hours post-ISO dosing.

When serum cTnI concentrations were measured using the Immulite 2000 platform (Table 1), in contrast with the above findings for cTnI and cTnT, peak mean values were evident at 1 hour postdosing (0.48 μg/L), but this level represented

<table>
<thead>
<tr>
<th>Time postdosing (h)</th>
<th>cTnI: ACS: 180SE (μg/L)</th>
<th>cTnI: DPC Immulite (μg/L)</th>
<th>cTnI: Elecsys 2010 (μg/L)</th>
<th>Histopathology (number of animals)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 (Control)</td>
<td>&lt;0.030 (0.000)</td>
<td>0.368 (0.038)</td>
<td>&lt;0.010 (0.000)</td>
<td>NAD</td>
</tr>
<tr>
<td>1</td>
<td>4.265 (3.857)</td>
<td>0.480 (0.101)</td>
<td>1.535 (1.366)</td>
<td>Myofibre dystrophy</td>
</tr>
<tr>
<td>2</td>
<td>4.295 (1.376)**</td>
<td>0.373 (0.096)</td>
<td>1.790 (0.415)**</td>
<td>Acute myodegeneration</td>
</tr>
<tr>
<td>4</td>
<td>2.033 (1.386)*</td>
<td>0.358 (0.057)</td>
<td>0.756 (0.537)*</td>
<td>Chronic myodegeneration</td>
</tr>
<tr>
<td></td>
<td>[65.8]</td>
<td></td>
<td>[74.6]</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>2.713 (1.443)**</td>
<td>0.373 (0.051)</td>
<td>1.033 (0.317)**</td>
<td></td>
</tr>
<tr>
<td>12</td>
<td>0.508 (0.569)</td>
<td>0.338 (0.079)</td>
<td>0.197 (0.194)</td>
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</tr>
<tr>
<td>24</td>
<td>0.110 (0.47)</td>
<td>0.285 (0.045)*</td>
<td>0.069 (0.089)</td>
<td></td>
</tr>
<tr>
<td>48</td>
<td>&lt;0.030 (0.000)</td>
<td>0.295 (0.142)</td>
<td>0.058 (0.060)</td>
<td></td>
</tr>
</tbody>
</table>

*ISO was administered at 50 mg/kg by IP injection; there were n = 4 animals at each time point; cTnI was assayed with the ACS: 180SE (Bayer) and with the DPC Immulite (Diagnostic Products); cTnT was assayed with the Elecsys 2010 (Roche); [ ] indicates the "fold increase" of the mean value over the control mean value; NAD = no abnormalities detected; *significantly different to the control animals. p < 0.05; ** p < 0.01; *** p < 0.001.
Figure 1.—H & E stained sections of progressive changes in cardiac myofibres in the left ventricular inner myocardium following the administration of isoproterenol (ISO), at 50.0 mg/kg (intraperitoneal injection) to female Hanover Wistar rats. (A) Control rat showing the normal appearance of cardiac myofibres; original magnification (OM) ×400. (B) Early changes in the myocardium at 4 hours post-ISO dosing showing increased myofibre swelling and eosinophilia (OM ×400). (C) At 6 hours post-ISO administration there is acute myodegeneration with swollen eosinophilic myofibres and a neutrophil infiltrate (OM ×400). (D) Chronic myodegeneration at 24 hours after the administration of ISO with the loss of myofibres and a mononuclear cell infiltration (graded mild to moderate) (OM ×400).

less than a 1-fold increase over the control baseline value (0.37 μg/L). At other time points the results also indicated poor immunoreactivity and sensitivity (Table 1) with this assay.

Histopathologic examination of hearts from control and ISO treated rats showed that the earliest changes in the myocardium were evident at 4 hours after the administration of ISO (Figure 1) when a single animal of the 4 studied showed minimal (grade 1) myofibre eosinophilia (characterized by swelling and increased eosinophilia of cardiac myofibres). At 6 hours, 2 rats of the 4 examined demonstrated similar minimal to mild changes, with a third animal showing acute myodegeneration (characterized by swollen eosinophilic myofibres with a neutrophil infiltrate). At 12 hours dosing, 3 of the 4 rats investigated had minimal to mild acute degeneration, and at 24 and 48 hours, all animals showed mild to moderate chronic myodegeneration (characterized by a loss of cardiac myofibers and a mononuclear cell infiltration, graded as mild to moderate).

These results are presented in Table 1 and the relationship between the onset of histopathologic lesions and serum levels of cTnI and cTnT are illustrated in Figure 2. The first appearance of cardiac lesions, at 4 to 6 hours postdosing, is seen to be clearly demarcated from the times of onset of cTnI and cTnT positivity in the serum at 1 and 2 hours after the administration of ISO.

Experiment 3: Dose-Response Study (8.0 to 48.0 mg/kg Isoproterenol): Results are presented in Table 2. It is seen that for cTnI levels assayed with the ACS: 180SE, from 8.0 to 48.0 mg/kg ISO, the mean fold increases of the 6 dose level groups ranged from 45.1 (at 16.0 mg/kg ISO) to 111.9
The histologic assessment of hearts taken from ISO-treated rats at the autopsy at 5 hours postdosing (Table 2) demonstrated evidence of myofibre eosinophilia in a proportion of the animals from each ISO dose level group. This was graded as minimal (grade 1) in animals treated at 8.0 and 16.0 mg/kg ISO. The incidence of the lesions was increased at the higher ISO dose levels reaching a maximum in animals treated with 24.0 mg/kg. The increased incidence at >24.0 mg/kg was accompanied by an increase in severity grade in some animals (to mild myofibre eosinophilia) with a single animal (rat number 29) treated with 40.0 mg/kg ISO showing progression of the lesion type to mild acute myodegeneration. The lesions were predominantly localized to the left ventricular inner myocardium particularly affecting the apex of the heart and the papillary muscles. There appeared to be a trend for the magnitude of the histologic lesions to be associated with rats having higher CtnI values.

**Experiment 4: Dose-Response Study (0.25 to 20.0 mg/kg Isoproterenol):** In this study, rats were dosed with ISO at lower dose levels than in Experiments 2 and 3. Selected serum clinical biochemistry results and histologic findings are presented in Table 3 (2 hours postdosing) and Table 4 (24 hours postdosing).
ISO dose (mg/kg)  cTnl (µg/L)  LD (U/L)  LD1 (%)  LD2 (%)  CK (U/L)  CKMB (%)  Myodegeneration score
0 (Control)  0.0309 (0.005)  2234.5 (656.1)  1.90 (0.22)  2.08 (0.43)  849.2 (255.2)  14.20 (2.59)  0 (0)
0.25  0.0379 (0.024)  1803.6 (385.6)  3.86 (1.05)  4.02 (1.55)  671.2 (145.9)  9.98 (0.90)  0 (0)
0.5  0.790 (1.124)  1993.3 (311.3)  3.11 (1.14)  3.18 (1.00)  619.8 (94.5)  11.42 (4.71)  0 (0)
1.0  0.790 (1.124)  1993.3 (311.3)  3.24 (0.59)  2.76 (0.95)  669.0 (120.0)  9.06 (2.87)  0 (0)
0 (Control)  0.0609 (0.042)  2302.3 (384.7)  1.82 (0.43)  1.82 (0.33)  871.8 (161.2)  9.86 (1.15)  0 (0)
2.0  8.494 (4.629)  1670.0 (439.4)  5.08 (1.86)  6.76 (3.33)  568.8 (107.6)  15.40 (2.82)  0 (0)
4.0  15.722 (10.080)  1551.2 (380.6)  7.82 (4.04)  10.26 (4.41)  523.6 (151.4)  15.80 (1.33)  0 (0)
6.0  15.642 (10.166)  1551.2 (380.6)  7.82 (4.04)  10.26 (4.41)  523.6 (151.4)  15.80 (1.33)  0 (0)
8.0  15.642 (10.166)  1551.2 (380.6)  7.82 (4.04)  10.26 (4.41)  523.6 (151.4)  15.80 (1.33)  0 (0)
10.0  15.642 (10.166)  1551.2 (380.6)  7.82 (4.04)  10.26 (4.41)  523.6 (151.4)  15.80 (1.33)  0 (0)

*a All other information as Table 3, except there were 4 animals in each ISO dose level group.
of 15.72 15.63, and 14.80 μg/L respectively, giving 261-fold, 259-fold, and 493-fold increases above the concurrent control values of 0.06, 0.06, and 0.03 μg/L, respectively. However, mean levels of cTnI at 10.0 and 20.0 mg/kg ISO were, at 2 hours postdosing, lower than at 4.0 and 6.0 mg/kg ISO, with mean values of 7.73 and 3.20 μg/L, respectively; the reasons for this result are unclear. Another feature of the cTnI values at these high dose levels of ISO was the variability of the response. For example, at 10.0 and 20.0 mg/kg ISO, 1 of the 5 animals at each dose level gave a result of <0.03 μg/L, the baseline (control) value. Indeed, baseline values were evident in 6 of the 45 animals treated with ISO at the 2 hour postdosing autopsy.

At 24 hours after the administration of ISO (Table 4), cTnI levels, in general, had fallen to baseline values at dose levels of 0.25 to 4.0 mg/kg ISO. However, at dose levels of 6.0, 8.0, and 10.0 mg/kg ISO there was still some evidence of mild positivity at 24 hours postdosing in some individual animals, with 5 out of a total of 12 animals treated at these ISO dose levels showing values of cTnI above baseline control values (<0.03 μg/L); this involved 2 out of 4 animals at 6.0 mg/kg ISO, 2 of 4 animals at 8.0 mg/kg, and 1 of 4 animals at 10.0 mg/kg. Nevertheless, at 20.0 mg/kg ISO at 24 hours postdosing, troponin positivity was still clearly evident in 3 animals treated at this dose level where cTnI values of 2.92, 6.33, and 15.43 μg/L were observed. Nonetheless, this mean value of 6.20 μg/L, although not statistically significant, represented a 206-fold increase above the control baseline value of <0.03 μg/L. This finding of cTnI positivity at 24 hours postdosing at the high ISO dose level of 20.0 mg/kg was of interest, demonstrating that cTnI positivity and the magnitude of response was maintained at this time point.

Measurement of enzymes that have been conventionally used to assess injury in the heart, were examined at 2 hours post ISO dosing (Table 3). LD1 and LD2 isoenzymes were studied and results for both isoenzymes are given separately. These data are expressed as percentages of the total LD values, which are also presented (LD U/L) in Table 3. It is seen that statistically significant increased activities for LD1 and LD2 were evident at 0.25 mg/kg ISO. Indeed, the activity of LD1 was significantly increased at all dose levels of ISO at 2 hours postdosing (except at 0.5 mg/kg ISO), and activities of LD2 were significantly raised at dose levels of 2.0 mg/kg ISO and above. The maximum increase of LD1 was at 4.0 mg/kg ISO; here the activity was 7.8%, a 3.3-fold increase above the baseline control value of 1.8%.

For LD2, the maximum increase was also at 4.0 mg/kg ISO, and here there was a 4.6-fold increase above the baseline value. It is of interest to note that the results for LD1 and LD2, which show a pattern of response with a maximum peak at 4.0 mg/kg ISO, are, in general terms, similar to the pattern of response for cTnI, which also demonstrated a maximal peak at 4.0 mg/kg ISO. However, the fold increases for LD1 (3.3) and LD2 (4.6) at 4.0 mg/kg are considerably less than the fold increase (261) for cTnI at 2 hours postdosing with ISO.

It is also of interest to note that the mean results for LD2 (as % of the total LD values), at 2 hours after dosing with ISO, are equal to or above the mean results for LD1 (%) at all ISO dose levels except at 1.0 mg/kg ISO (Table 3). This pattern of change is also evident in the fold increases of LD1 and LD2: the fold increases for LD2 above baseline values at dose levels of 2.0 mg/kg ISO and above, are all higher that the fold increases for LD1; this appears to be a consistent observation.

At 24 hours post ISO dosing (Table 4), the mean activities of LD1 and LD2 had, in overall terms, returned to approximately normal levels; however, at 20.0 mg/kg ISO the levels of LD1 and LD2 remained above the baseline control values showing 2.3-fold increases in each case (p < 0.05 for LD1, NS for LD2). An examination of the activities of LD1 and LD2 in individual animals at 24 hours post ISO dosing indicated that in rats where serum levels were increased for these parameters above baseline control values, these individual animals also had cTnI levels that remained high at this time point.

Another point of interest to emerge from the examination of the LD isoenzyme data at 24 hours post-ISO dosing (Table 4), is that while mean LD1 activities did show levels (as % or as fold increases) above baseline control values (NS or p < 0.05), these levels were, in general, often higher that the levels for LD2. This finding would suggest that serum levels of LD2 return to baseline more rapidly than levels of LD1. This effect was seen for example at 20.0 mg/kg ISO at 24 hours, where LD1 remained significantly increased above LD2. This effect, in the presence of cTnI positivity, where LD2 was raised above LD1 at 2 hours, to be followed at 24 hours where LD2 activities are reduced below those of LD1, is sometimes referred to as the “LD1-LD2 flip,” and relates to the different half-lives of the 2 isoenzymes in serum (LD1 having a longer half-life than LD2).

For the isoenzyme CKMB at 2 hours post ISO dosing, the peak activity (as %) was at 4.0 mg/kg (Table 3), which again paralleled the findings for cTnI, LD1, and LD2; indeed, the increase for CKMB above control levels at 2.0 mg/kg ISO was 0.6-fold (p < 0.01), and at 4.0 mg/kg ISO there was also a 0.6-fold (p < 0.001) increase. However, in many instances, at a particular ISO dose level, the levels of CKMB showed considerable variability, and the individual results in ISO-treated animals were often directly comparable to baseline control values, even when levels of cTnI were greatly increased. At 24 hours postdosing, the mean activities of CKMB had fallen below baseline values at all ISO dose levels, except at 2.0, 4.0, and 6.0 mg/kg (Table 4).

At 2 hours postdosing with ISO, levels of the serum enzymes ALT, AST, GLD, LD, CK, and aldolase showed, in general terms, no consistent, or dose-related increases (data not shown). However, in the case of AST, there was some evidence of small increases in activity in individual animals at the higher ISO dose levels. These small increases for AST were, for example, to 174, 195, 207, and 244 U/L and in these instances the levels of cTnI in these individual animals were also very high, being 15.07, 41.32, 17.40, and 28.42 μg/L, respectively. The mean concurrent control activity of AST was 119.0 U/L. For CK, at some higher ISO dose levels (2.0, 4.0 and 6.0 mg/kg) there was evidence of small but statistically significant decreases in the mean levels of activity at 2 hours postdosing (p < 0.01 at each ISO dose level). Total LD activity similarly showed little demonstration of useful diagnostic change in ISO-treated rats; indeed, there were statistically significant decreases in mean levels of activity at 2.0 and 4.0 mg/kg ISO (p < 0.05 at each dose level). Levels of
ALT, GLD, and aldolase showed little evidence of meaningful change in ISO treated animals. At 24 hours postdosing with ISO, mean levels of ALT, AST, GLD, LD, CK, and aldolase showed no evidence of significant, or dose-related changes, and therefore the levels of these enzymes gave no indication of having any diagnostic value.

The histopathologic assessment of cardiac lesions at 2 hours postdosing is set out in Table 3, with the changes quantified by severity grade on a scale of 0 to 4, and the mean (SD) of the grades expressed as the myodegeneration score. At 2 hours postdosing, 3 animals, 1 control and 1 each treated with 8.0 and 10.0 mg/kg ISO, showed minimal (grade 1) lesions characterized as chronic myodegeneration. Given the results from the time course study (Experiment 2), these observable lesions were considered to predate the start of the study and therefore represent background pathology. However, it is of interest to note that the 2 ISO treated individual animals had extremely high cTnl levels, 28.42 and 18.92 μg/L, respectively.

At 24 hours, post ISO dosing, all lesions seen in the hearts examined were characterized as chronic myodegeneration and the mean myodegeneration score showed a trend towards increasing severity with increasing dose levels of ISO (Table 4). At dose levels of 0.25 to 4.0 mg/kg ISO, the mean myodegeneration scores ranged from 1.0 to 2.3 (with p values from <0.05 to <0.01). In the case of the animals treated with ISO at 6.0, 8.0, 10.0, and 20.0 mg/kg, the mean myodegeneration scores were from 2.8 (p < 0.001; 8.0 mg/kg), to 3.3 at 10.0 and 20.0 mg/kg ISO (p < 0.001 at both dose levels). In addition, there was a trend for animals with the more severe forms of chronic myodegeneration to have higher serum levels of cTnl; for example at 20.0 mg/kg ISO, the myodegeneration scores of the 4 rats in the group were 2, 3, 4 and 4, and the cTnl values of these animals were 0.12, 15.43, 2.92, and 6.33 μg/L, respectively.

**Discussion**

In Experiment 1, the assay for cTnl using the Bayer ACS: 180SE platform was found to be immunoreactive and provides a linear response to rat-specific cardiac homogenate, purified standards and in vivo generated cTnl in rat serum over a dynamic range of 0.03 to 50 μg/L. Between batch precision of the assay (11.3%) was demonstrated to concentrations of 0.3 μg/L. Additionally, the robustness of the assay was highlighted by exceptionally good stability of the immunoreactive cTnl signal when stored at 4°C or when frozen (−20°C and −80°C), and when samples were subjected to repetitive freeze-thaw cycles. Our findings in identifying the suitability of this assay to assess cTnl responses in the rat are characterized as chronic myodegeneration. Given the results from the time course study (Experiment 2), these observable lesions were considered to predate the start of the study and therefore represent background pathology. However, it is of interest to note that the 2 ISO treated individual animals had extremely high cTnl levels, 28.42 and 18.92 μg/L, respectively.

The increased relative activity of the isoenzymes LD1 and LD2 following cardiac injury, and the variation in the half-life of the 2 isoenzymes, has been discussed in several reports (Wolf et al., 1986; Preus et al., 1989; Ladi et al., 1990; Bertinchant et al., 2000; Walker, 2006). LD1 has been found to have the longest circulating half-life of the LD isoenzymes and relative shifts in the serum LD1/LD2 ratio have been shown to correlate with the severity and duration of cardiac injury (Wolf et al., 1986; Preus et al., 1988). At 2 hours postdosing in Experiment 4 (Table 3), activities of LD2 in ISO treated rats were generally equal to or higher than the activities of LD1. However, at 24 hours postdosing, LD1 activity was greater than LD2 (Table 4). These alterations in LD1 and LD2 isoenzyme levels over a 24-hour period have been referred to as the "LD1-LD2 flip," and it has been suggested that these changes can be accounted for by LD1 having a longer half-life than LD2 (Walker, 2006).

It is seen (Table 1 and 2) that the magnitude of the increase in cTnT in the serum of ISO treated rats, above the baseline control values, is slightly greater than the magnitude of change in serum cTnl above baseline control values. For example, in Table 1, the maximum mean fold increase at 2 hours postdosing was 142.2 for cTnl and 178.0 for cTnT; in Table 2, the maximum mean fold increase at 5 hours postdosing was 111.9 for cTnl and 172.0 for cTnT with maximum fold increases for individual animals (data not shown) of 314 for cTnl and 474 for cTnT. However, in overall terms it is considered that the sensitivity of both biomarkers appears to be approximately similar in this particular experimental model. Furthermore, when serum samples from individual animals in Experiments 1, 2, and 3 were assayed for both cTnl and cTnT (n = 83), and the data re-analyzed and plotted with the line of best fit (Figure 3), cTnl and cTnT levels were shown to be closely correlated (R² = 0.9202). This result would therefore indicate that either serum cTnl, or serum cTnT can be used as a suitable biomarker of ISO induced cardiac injury in the rat. However at cTnl levels below 0.3 μg/L with the ACS: 180SE, the precision of the assay was considered less...
sensitive compared to the cTnT assay (Table 1; Figure 3) but it is considered that interpretation of the experimental data was not compromised.

Nevertheless, these findings with cTnI measured with the ACS: 180SE (Bayer) and with cTnT measured with the Elecsys 2010 (Roche) are in clear contrast to the data on cTnI levels measured with the DPC Immulite (Diagnostic Products). Here the signal for cTnI was minimal; in Table 1 (Experiment 2) it is seen that the maximal mean fold increase in cTnI was 0.3 (at 1 hour postdosing), and in Experiment 3 the maximal fold increase for an individual animal was 1.5 (48.0 mg/kg ISO). These findings are supported by a recent report (O’Brien et al., 2006) where the dynamic range of the DPC Immulite assay was found to be approximately 1% of the Bayer Centaur assay in the detection of ISO induced cTnI release. These very low maximal fold increases determined with the DPC Immulite platform are considered to predominantly reflect differences in immunoreactivity and sensitivity, indicating the varying antibody epitope selectivities employed in the respective Bayer and DPC cTnI assays.

In the present investigations, serum levels of cTnI measured with the ACS: 180SE correlated closely with the onset of histopathologic findings (obtained from routine H&E stained sections) which were consistent with changes previously reported for this class of compounds (Van Vleet et al., 2000). Clear evidence of myofibre eosinophilia was seen in 1 of 4 animals (Experiment 2) at 4 hours post-ISO dosing (Table 1; Figure 1); acute myodegeneration in 1 of 4 rats was evident at 6 hours postdosing, and chronic myodegeneration in each of 4 animals was observed at 24 hours postdosing (Table 1; Figure 1).

In Experiment 3, minimal/mild myofibre eosinophilia was observed at 3 hours post ISO dosing, and in 1 animal (of a total of 30 treated with ISO) mild acute myodegeneration was also evident at this time point (Table 2). In Experiment 4 (Table 4), at 24 hours post ISO administration, chronic myodegeneration was seen in all drug treated animals, and there appeared to be evidence of increasing severity of the lesions with increasing dose level of ISO. These observations of histologic change at 24 hours were in concordance with the magnitude of troponin changes at 2 hours. It is considered that morphologic changes associated with cardiac injury could possibly be identified earlier than at 4–5 hours postdrug treatment with the use of transmission electron microscopy, or with special stains, or immunoenzyme histochemistry.

A close examination of the data from the several experiments reported here shows that within a particular time-point group (Table 1), or ISO dose level group (Tables 2, 3, 4), serum cTnI (and cTnT) levels appear in certain cases to show considerable within-group variability, often in conjunction with a relatively large SD. However, this variability of cTnI/cTnT levels is also evident on the close examination of the published results of other workers (Bertinchant et al., 2000; O’Brien et al., 2006). The basis of this variability is unclear but it is probably multifactorial in origin. However, it is considered that the route of administration of ISO may be involved (i.e., the intraperitoneal route in the present studies), with resulting effects on the rate of drug absorption. Indeed, in more recent studies in our laboratories, the drug has been given by subcutaneous administration and this appears to have reduced, to a degree, the amount of within-group variability. Nevertheless it is considered that the peak duration of cTnI release, or diagnostic window, following acute ISO induced cardiac injury was identified in these experiments and appropriate sampling for cTnI measurement with this particular mechanism of injury should be between 1-6 hours following dosing.

Another difference between the present investigations, and those recently reported by other workers, is that female rats were used. However, although there appears to be a current trend towards the use of male rats in studies on ISO induced cardiac toxicity (Ferrans et al., 1969; Lutmer and Wexler, 1971; Bleuel et al., 1995; Nakatsui et al., 1997; Inamoto et al., 2000; Prabhoo et al., 2006), nevertheless there still remain numerous examples of reports involving the use of female animals (Wexler et al., 1963; Kahn et al., 1969; Tang and Taylor, 1984; Hrdina and Kvetina, 1990; Bertsch et al., 1997; O’Brien et al., 2006).

At the present time, as the measurement of serum cTn in cardiac injury is a relatively new technique in toxicologic investigations, the place of the assay in the process of preclinical drug safety evaluation is unclear. It has been suggested that the measurement of cTn should be recommended for the identification of ongoing cardiomyocyte injury. However, it has also been proposed that cTn assays should not be advocated for inclusion in routine preclinical studies, but rather that the test would best be employed when further "follow-up" investigative examinations are required. Possibly, that is, when it has been established that some form of cardiac injury is present, or when it is known that acute tachycardia/arrhythmias have been induced. This would therefore suggest that the cTn assay may be used, not as a routine procedure, but rather as part of "reflective analysis" and a "case-by-case approach," particularly if the time of onset of myocardial injury can be clearly established. However, the excellent sensitivity and specificity of cTn as a biomarker of cardiac injury will certainly encourage the consideration of the inclusion of cTn within the core screen, particularly with increased investigative use of this marker in sub-acute/chronic and chronic studies where the potential mode of cardiac injury may be quite different to the experimental model used in the present investigations.
A recent search of the literature has demonstrated that in studies involving cTn, the compound of frequent choice for the induction of cardiac lesions is ISO; also it would appear that ISO-induced changes in the heart are beginning to be characterized and reported in the literature, and related to serum levels of cTn. Nevertheless, this highlights the situation that in other types of cardiac injury, induced with other model compounds, cTn responses have not been well described and may have prolonged diagnostic windows for optimal measurement in comparison with ISO induced cardiac injury. This has become clear in studies on cardiac biomarker evaluation in our own laboratories where attention has now turned to examine different models of experimental injury with a series of other cardiotoxic agents.

In conclusion, and in agreement with the reports of Wallace et al. (2004), O’Brien et al. (2006) and Walker (2006), it is considered that in the present studies, cTn was confirmed as being a specific and sensitive biomarker for the detection of myocardial damage in the rat. On injury to the heart, cTn is rapidly released, and in serum, cTn is a robust marker and shows good stability in storage at 4°C, −20°C and −80°C. The duration of release of cTn in the blood is also adequately long in acute injury. Increased levels of serum cTn show an association with the development of cardiac lesions and precede maximal lesion severity in acute models of cardiac injury. Assays of cTn in serum are rapid, specific, simple, accurate, relatively inexpensive, and easy to perform, although there is some evidence that technical improvements in the assay are required at low serum concentrations. The usefulness of cTn as a biomarker is enhanced as it is not expressed in nontarget tissues and also because cTn bridges between preclinical and clinical studies.

ACKNOWLEDGMENTS
We gratefully acknowledge the assistance of the technical staff at the School of Pharmacy for care of the animals and the technical support of the Clinical Pathology and Histology groups of GlaxoSmithKline, UK and the Clinical Pathology Laboratories of AstraZeneca, UK. SB acknowledges the support of GlaxoSmithKline, UK, and the School of Pharmacy.


