CYTOCHROME P450 AND THE IMMUNE RESPONSE TO PROLONGED ADMINISTRATION OF ISONIAZID, NEVIRAPINE AND PARACETAMOL IN A RAT MODEL

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ABSTRACT

Drug-induced liver injury is a major adverse drug reaction that presents during isoniazid (INH) and nevirapine (NVP) treatment, and after paracetamol (PAR) overdose. It was postulated that after these drugs are metabolised, reactive metabolites are formed which attack cellular proteins and result in the formation of antigenic metabolite-protein adducts. Subsequently, the immune system starts to eliminate hepatocytes expressing these adducts, and this leads to the development of overt drug-induced liver injury. As such, the effect of prolonged administration of INH, NVP and PAR on the cytochrome P450 (CYP450) and immune response was investigated here.

A high performance liquid chromatography (HPLC) method for the simultaneous determination of INH, NVP and PAR in plasma was developed. Sample preparation involved protein precipitation with zinc sulphate and methanol, followed by solid phase extraction. The mobile phase was 0.06% trifluoroacetic acid (A) and acetonitrile (B) and run by a gradient programmer over a C_{18} (4.60 x 250 mm) 5 µ analytical column at 1 ml/min, while the eluent was detected by UV at 260 nm. INH, PAR, internal standard and NVP eluted at retention times of 3.1, 9.9, 10.6 and 11.6 minutes, respectively. The average 5 day calibration curves of INH, NVP and PAR were linear with regression equations and correlation coefficients of $y = 0.029x + 0.025$ ($r^2 = 0.9954$), $y = 0.043x + 0.127$ ($r^2 = 0.9968$) and $y = 0.097x + 0.070$ ($r^2 = 0.9997$), respectively. The method was used successfully to monitor INH, NVP and PAR in rat plasma.

The CYP450 and immune response to prolonged administration of INH, NVP and PAR were investigated using an SPD rat model. For each drug, the animal experiment was divided into three phases. In phase I, rats were orally administered saline solution (S), INH (20 mg/kg), NVP (200 mg/kg) or PAR (500 mg/kg), while for phase II, rats received S, INH, NVP or PAR in combination with an immune stimulant, levamisole (LMS; 2.5 mg/kg), and lastly, during phase III, rats received S, INH, NVP or PAR along with a CYP450 inducer, carbamazepine (CBZ; 60 mg/kg). In each phase, five rats per group were sacrificed after 2, 7, 14, 28 and 42 days. Blood

was analysed for full blood count, CD4 and CD8 counts, liver function, renal function, IL-2, IL-10, IgG, IgM and drug concentrations. A piece of liver was sent for histopathology testing, and the activity of rat CYP1A2, CYP2E1 and CYP3A2 were analysed.

During administration of the test drugs alone, both INH and NVP triggered an early Th1 immune response that was associated with liver injury, and counteracted by a later Th2 immune response which was associated with healing. Overall, the liver injury correlated with low concentrations of NVP, but high INH concentrations. That said, INH increased CYP2E1 activity, while NVP increased that of CYP3A2.

When LMS (immune stimulant) was co-administered, INH liver injury was exacerbated, while for NVP it was the same. Again, INH and NVP provoked a Th1 response (injury) that was counteracted by a Th2 response (healing). Here, the liver injury was also associated with low NVP concentrations, and high INH concentrations.

During co-administration of CBZ (CYP450 inducer), INH and NVP caused the same immune response, and resulted in improvement of the liver injury. Again, the liver injury was associated with low NVP concentrations, and high INH concentrations. Also, INH increased CYP2E1 activity and NVP increased CYP3A2 activity, but not to the same extent as the test drugs alone.

PAR did not exhibit a distinct pattern of immune response by which to associate it with the liver injury, most probably because the concentrations were too low for generation of toxic metabolites.

In conclusion, the pattern of immune response to prolonged administration of INH and NVP shows that the immune system is involved in the drug-induced liver injury, probably as a protective buffer to prevent further drug toxicity.

DECLARATION OF INDEPENDENT WORK

1. I, Zanelle Bekker, declare that the doctoral research thesis that I herewith submit at the University of the Free State, is my independent work and that I have not previously submitted it for a qualification at another institution of higher education.
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PROMOTER'S DECLARATION

I, Professor A. Walubo, the promoter of the doctoral research thesis entitled: Cytochrome P450 and the immune response to prolonged administration of isoniazid, nevirapine and paracetamol in a rat model, hereby certify that the work in this project was done by Zanelle Bekker at the Department of Pharmacology, University of the Free State.

I hereby approve submission of this thesis and also affirm that this has not been submitted previously, either in part or in its entirety, to the assessors, neither to this or any other institution for admission to a degree or any other qualification.

_____ _____

Signature Date

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"For out of His fullness we have all received one grace after another and spiritual blessing upon spiritual blessing and even favour upon favour and gift upon gift." John 1:16 (AMP)

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ABBREVIATIONS

1-OH-MDZ	1-hydroxymidazolam
6-OH-CZN	6-hydroxychlorzoxazone
Abs	absorption
Acc	accuracy
AIDS	acquired immune deficiency syndrome
ALP	alkaline phosphatase
ALT	alanine aminotransferase
AST	aspartate aminotransferase
Bas	basophils
BSA	bovine serum albumin
BUN	blood urea nitrogen
Cal	calibration
CBZ	carbamazepine
CC	correlation coefficient
CD	cluster of differentiation
CNS	central nervous system
Conc	concentration
Cr	creatinine
CV	coefficient of variation
CYP450	cytochrome P450
D	days
DNA	deoxyribonucleic acid
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme-linked immunosorbent assay
Eos	eosinophils
GC	gas chromatography
GIT	gastrointestinal tract
H	hours
Hb	haemoglobin
HBV	hepatitis B virus
Hct	haematocrit

HCV	hepatitis C virus
HEPES	hydroxyethyl piperazineethanesulphonic acid
HIV	human immunodeficiency virus
HIV/TB	human immunodeficiency virus/tuberculosis
HPLC	high performance liquid chromatography
IFN	interferon
Ig	immunoglobulin
IL	interleukin
INH	isonicotinic acid hydrazine/isoniazid
IS	internal standard
LC-MS	liquid chromatography tandem mass spectrometry
LFT	liver function test
LMS	levamisole
LPS	lipopolysaccharide
Ly	lymphocytes
MCH	mean corpuscular haemoglobin
MCHC	mean corpuscular haemoglobin concentration
MCV	mean corpuscular volume
Mo	monocytes
NAC	*N*-acetylcysteine
NADP	β-nicotinamide adenine dinucleotide phosphate
NAPQI	*N*-acetyl-*p*-benzoquinone-imine
NAT2	*N*-acetyltransferase 2
Neu	neutrophils
NK	natural killer cell
NKT	natural killer T cell
NNRTI	non-nucleoside reverse transcriptase inhibitor
NVP	nevirapine
PAR	paracetamol
pKa	acid dissociation constant
Plt	platelets
RCC	red cell count
RF	resorufin
RFT	renal function test

RNA	ribonucleic acid
RR	reaction rate
S	saline solution
SD	standard deviation
SP	sulphapyridine
SPD	Sprague-Dawley
SPE	solid phase extraction
Stab	stability
TEAP	tetraethylammoniumphosphate
Temp	temperature
TGF	tumour growth factor
TLC	thin layer chromatography
TNF	tumour necrosis factor
UnRx	untreated
UV	ultra violet
WCC	white cell count

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CHAPTER 1

INTRODUCTION

Drug-induced liver injury is responsible for at least 10% of adverse drug reactions. Of concern is that it has hampered the use of essential drugs such as isoniazid and nevirapine in some patients, and is the most feared adverse effect after paracetamol overdose (Jaeshke *et al*., 2006). Whereas several mechanisms have been postulated, the immune system has been implicated as a mediator and determinant for progression of the liver injury (Holt and Ju, 2006).

It was observed that metabolic activation of a drug leads to formation of reactive metabolites, which attack proteins, thus forming metabolite-protein adducts. Some of these adducts are antigenic, hence, stimulate the immune system which starts a process to eliminate cells (including hepatocytes) expressing these adducts (Liu and Kaplowitz, 2007). It was then explained that most people do not develop liver injury because the body is able to efficiently eliminate the antigenic adducts (by metabolism of antioxidant mechanism) and/or to counter the pro-inflammatory response. However, in few subjects, there is failure to counter the inflammatory response, which leads to progressive destruction of the liver cells, and overt drug-induced liver injury presents (Walgren *et al*., 2005).

Supporting evidence has been found in animal studies (mice) on paracetamol where liver injury/toxicity was attenuated by administration of specific immune suppressants, while prolonged administration was associated with increased tolerance to high doses of paracetamol without liver injury (Liu and Kaplowitz, 2007). Recently, it was observed that prolonged administration of nevirapine in rats was associated with progressive stimulation of interleukin-2 and improvement in liver histology, and lipopolysaccharide administration protected against nevirapine-induced liver injury (Bekker *et al.*, 2012). Also, activation of the immune system is associated with isoniazid-induced liver injury (Tafazoli *et al*., 2008). Of note, despite several reports demonstrating the role of different immune markers, there has been little or no information on the correlation of these markers with each other and the drug-induced liver injury. Specifically, the immune system is in a dynamic state

during which some immune markers increase, while others decrease (James *et al*., 2003). Therefore, a time profile response of the immune system is necessary to determine when the respective markers would be of use in predicting the extent of liver injury. As such, a study on the cytochrome P450 and immune response to prolonged administration of isoniazid, nevirapine and paracetamol was undertaken in the hope that it would shed light on the mechanism(s) and possible modes of therapy for drug-induced liver injury.

CHAPTER 2

LITERATURE REVIEW

2.1 AN OVERVIEW ON THE PHARMACOLOGY OF ISONIAZID, NEVIRAPINE, AND PARACETAMOL

2.1.1 Isoniazid

Isoniazid, or isonicotinic acid hydrazide (INH), is a first-line drug used for treatment of tuberculosis (Clarke, 2004; Brenner and Stevens, 2006 b). It is a synthetic analogue of pyridoxine (Figure 2.1) with chemical formula $C_6H_7N_3O$, molecular weight of 137.1 g/mol, and pKa of 1.8, 3.5, and 10.8 at 20℃ (Clarke, 2004; Howland and Mycek, 2006 b).

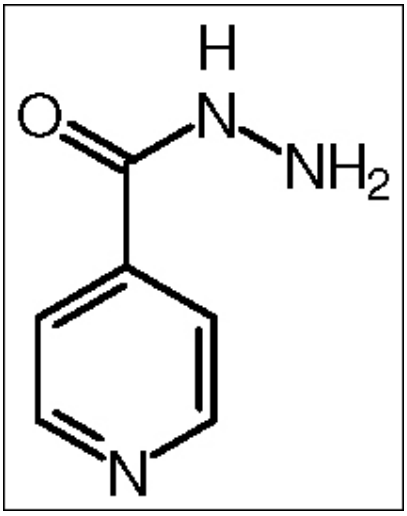

Figure 2.1: The chemical structure of isoniazid (From: http://content.answcdn.com/main/content/img/oxford/oxfordBiochemistry/0198529171.isoniazid.1.jpg)

Isoniazid is activated by mycobacterial catalase-peroxidase, an enzyme encoded by the KatG gene (Brenner and Stevens, 2006 b). The mechanism of action of isoniazid is by covalent binding of the activated drug to two enzymes, inhibin A and β-ketoacyl acyl carrier protein synthase A, subsequently inhibiting the enzymes (Howland and Mycek, 2006 b). These enzymes play an essential role in the synthesis of mycolic acid, a unique very-long-chain β-hydroxylated fatty acid found in the mycobacterial cell wall. Decreased mycolic acid synthesis leads to a loss of acid-fastness of the mycobacterial cell wall (Howland and Mycek, 2006 b).

Isoniazid is readily absorbed after oral administration, however, absorption is impaired when the drug is taken with food such as carbohydrates and aluminium-containing antacids (Brenner and Stevens, 2006 b; Howland and Mycek, 2006 b). Isoniazid distributes widely to tissues and reaches sufficient intracellular concentrations to eradicate organisms inside cells and caseous material. The drug is extensively metabolised by conjugation with acetyl coenzyme A, which is catalysed by *N*-acetyltransferase 2 (NAT2), resulting in the formation of the primary metabolite, acetylisoniazid (Brenner and Stevens, 2006 b). This *N*-acetylation is genetically determined, with the fast acetylator trait being autosomally dominant (Howland and Mycek, 2006 b). Because of the different rates of isoniazid acetylation, individuals with the fast phenotype have lower plasma concentrations of isoniazid than individuals with the slow phenotype (Brenner and Stevens, 2006 b). Therefore, in fast acetylators the half-life of isoniazid is approximately 1 hour, while in slow acetylators it is 3 – 5 hours (Clarke, 2004). Isoniazid is not significantly protein bound, and is excreted by glomerular filtration in the urine (Clarke, 2004; Howland and Mycek, 2006 b).

Unfortunately, the use of isoniazid is associated with a variety of side-effects, of which the development of severe liver injury/toxicity is one of the most cumbersome (Shen *et al*., 2006). Other isoniazid-associated adverse effects include neurotoxic effects, gastrointestinal symptoms, and hypersensitivity reactions (Tostmann *et al.*, 2008).

2.1.2 Nevirapine

Nevirapine (NVP) is a potent non-nucleoside reverse transcriptase inhibitor (NNRTI) used for treatment of human immunodeficiency virus-1 (HIV-1) infection, where it is used in combination with other anti-retroviral agents (Cheeseman *et al.,* 1993). It is also used as monotherapy for the prevention of mother-to-child HIV-1 transmission (Mirochnick *et al.,* 2000). Nevirapine is a benzodiazepine derivative (Figure 2.2) with chemical formula $C_{15}H_{14}N_4O$, and molecular weight of 266.3 g/mol (Mirochnick *et al*., 2000; Clarke, 2004). It is a weak base with a pKa of 2.8 and is highly lipophilic.

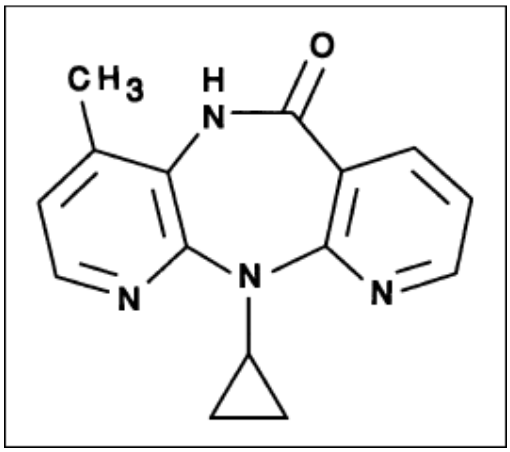

Figure 2.2: The chemical structure of nevirapine (From: Cheeseman *et al*., 1993)

The mechanism of action of nevirapine is by highly selective, non-competitive inhibition of the HIV reverse transcriptase enzyme. It binds to a site adjacent to the active site of reverse transcriptase, leading to a conformational change of the enzyme. Consequently, HIV ribonucleic acid (RNA) cannot be transcribed into complimentary viral deoxyribonucleic acid (DNA), hence, cannot be incorporated into the host genome (Howland and Mycek, 2006 c).

Nevirapine is well absorbed after oral administration and absorption is not affected by food or antacids. Since the drug is highly lipophilic, it enters the foetus and mother's breast milk with ease. It is also widely distributed in tissues, including the central nervous system (CNS). Nevirapine is metabolised in the liver by cytochrome P450 (CYP450) isoforms, CYP3A4 and CYP2B6 (Howland and Mycek, 2006 c). Both CYP3A4 and CYP2B6 are also induced by nevirapine, thereby leading to autoinduction of these enzymes. This autoinduction results in a decrease in the half-life of nevirapine from 45 hours, after a single dose, to 25 – 30 hours after repeated dosing (Boehringer Ingelheim Pharmaceuticals, 2011). Nevirapine is 60% protein bound and very little is excreted unchanged in the urine (Clarke, 2004; Howland and Mycek, 2006 c).

Nevirapine is associated with severe liver damage and -failure, which has hampered its use (Haehl, 2000). Other adverse effects caused by nevirapine treatment include: skin and hypersensitivity reactions, granulocytopenia, lymphadenopathy and renal dysfunction (Boehringer Ingelheim Pharmaceuticals, 2011).

2.1.3 Paracetamol

Paracetamol or acetaminophen (PAR) is an analgesic and antipyretic drug used for the treatment of moderate pain and fever. It is a *p*-aminophenol derivative (Figure 2.3) with chemical formula $C_8H_9NO_2$, and molecular weight of 151.2 g/mol (Clarke, 2004; Brenner and Stevens, 2006 a). Paracetamol is a weak acid with a pKa of 9.5 at 25℃.

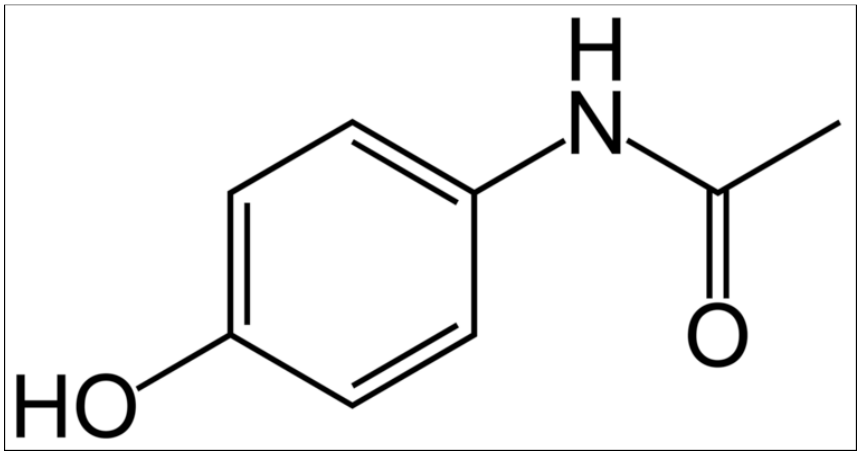

Figure 2.3: The chemical structure of paracetamol (From: http://en.wikipedia.org/wiki/File:Paracetamol-skeletal.svg)

A major hypothesis for the mechanism of action of paracetamol is that the drug is hydrolysed to *p*-aminophenol which reaches the brain through the general circulation, where it is converted to arachidonic acid amide, which is a substrate for a cannabinoid receptor (Toussaint *et al.*, 2010). It has weak anti-inflammatory activity, partly due to sensitivity to peroxides present in inflamed tissues (Brenner and Stevens, 2006 a). Furthermore, paracetamol does not affect platelet function or increase blood clotting time, and is the analgesic/antipyretic of choice in children with viral infections as it does not increase the occurrence of Reye syndrome as is the case with aspirin (Howland and Mycek, 2006 a).

The drug is rapidly absorbed from the gastrointestinal tract (GIT), exhibits minimal plasma protein binding, and is well distributed to peripheral tissues and the CNS (Brenner and Stevens, 2006 a). Significant first-pass metabolism occurs in the luminal cells of the GIT, as well as in hepatocytes. The largest portion of paracetamol is conjugated in the liver to form inactive glucuronidated or sulphated

metabolites. A smaller portion is hydroxylated by the CYP450 system to form *N*-acetyl-*p*-benzoquinone-imine (NAPQI), a highly reactive and potentially dangerous metabolite associated with hepatotoxicity during paracetamol overdose. Under normal circumstances, this dangerous metabolite is rapidly inactivated by conjugation with glutathione. Paracetamol exhibits a half-life (at therapeutic doses) of 1 – 3 hours in adults, and 5 hours in neonates (Clarke, 2004). The drug and its metabolites are excreted in the urine (Brenner and Stevens, 2006 a; Howland and Mycek, 2006 a).

The most fearsome side-effect after paracetamol overdose is hepatotoxicity which is potentially fatal (James *et al*., 2003). Other paracetamol-associated adverse reactions include asthma and hypertension (Toussaint *et al.*, 2010), as well as drowsiness and euphoria (Bertolini *et al.*, 2006).

2.2 THE ROLE OF THE IMMUNE SYSTEM IN DRUG-INDUCED LIVER INJURY

The immune system is a collection of barriers, such as physical, *i.e.*, skin, ciliated epithelia, mucous membranes, and chemical, *i.e.*, enzymes, stomach acid, as well as responses, namely the innate and adaptive (or acquired) immune responses (Moser and Leo, 2010; Figure 2.4), which interact to produce a collection of mechanisms to protect the body against disease (Sherwood, 2004).

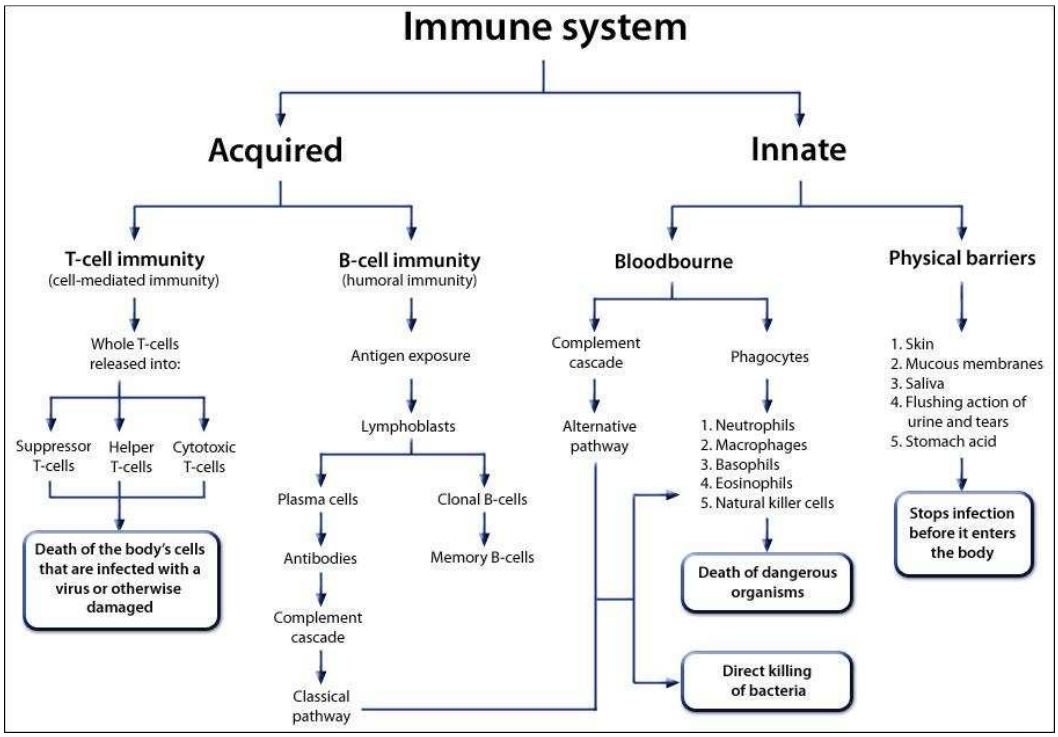

Figure 2.4: Schematic illustration of the components of the human immune system (From: http://www.myvmc.com/uploads/VMC/Anatomy/Immune_system_300.jpg)

2.2.1 Innate immunity

Innate immunity is the body's first line of defence against any foreign invader, providing almost immediate protection (Moser and Leo, 2010). It is a non-specific response (Sherwood, 2004), and often described as "generic" (Alberts *et al.*, 2002) as it does not confer long-lasting immunity, although it is the most dominant system of host defence in most organisms (Litman *et al.,* 2005).

The innate immune response is characterised by inflammation, one of the first responses to infection (Kawai and Akira, 2006), brought on and regulated by cells of the innate immune system. These cells are of haematopoietic origin, and include resident tissue cells, *i.e.*, macrophages and dendritic cells, as well as cells of movement, *i.e.*, neutrophils, eosinophils and monocytes, which migrate throughout the body by means of the blood and lymph circulation (Moser and Leo, 2010). Furthermore, it also includes proteins and molecules, present in biological fluids or released from cells upon activation, such as cytokines and chemokines, as well as membrane-bound receptors to bind with invading pathogens (Chaplin, 2010).

Components of the innate immune response contribute to activation of antigen-specific cells of the adaptive immune response, and, in addition, the antigen-specific cells amplify their own responses by enhancing innate effector mechanisms in order to control invading pathogens completely (Chaplin, 2010).

2.2.2 Adaptive immunity

The adaptive immune response is preceded and activated by the innate immune response, and serves as a second line of host defence against infection (Moser and Leo, 2010). This response is activated as soon as a pathogen enters the body, but it takes time to react since the adaptive immune system is composed of a small number of cells with specificity for any pathogen, which must first proliferate in order to produce sufficient numbers to create an effective response against the invader (Chaplin, 2010; Moser and Leo, 2010). The adaptive response produces cells with a long lifespan which enter a dormant state but are able to react rapidly after a second encounter with their specific antigen, thereby establishing immune memory (Chaplin, 2010). The adaptive immune system can be divided into two responses, namely the cell-mediated immune response and the humoral immune response (Figure 2.5).

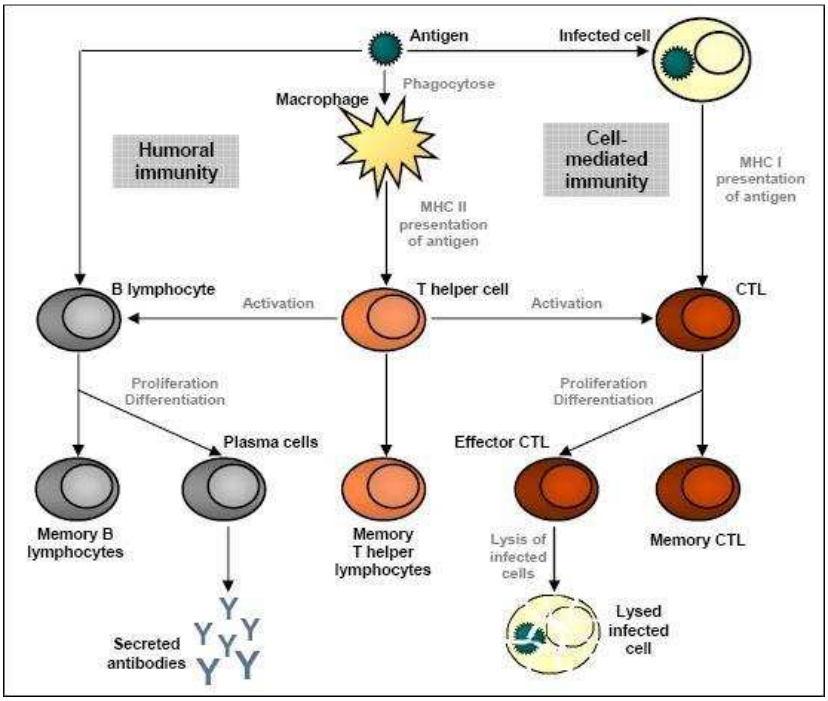

Figure 2.5: An illustration of the two responses of the adaptive immune system, humoral immune response and cell-mediated immune response (From: Kroeber, 2007)

2.2.2.1 Cell-mediated immunity

(a) Cells of the cell-mediated immune response

i. T cells

T cells, or T lymphocytes, a type of white blood cell, are derived from pluripotential cells, and migrate to the thymus for maturation (Chaplin, 2010). T cells are recognised by the presence of T cell receptors (TCR) on their surfaces (Chaplin, 2010) that determine major histocompatibility complex (MHC) restriction and peptide specificity of an individual T cell (Zajac and Harrington, 2008). There are several subsets of T cells, each with a unique function.

ii. Helper T cells

Helper T (Th), or cluster of differentiation (CD) 4 cells, interact with antigen-MHC class II complexes that are mostly expressed by immune cells (Moser and Leo, 2010). They may have cytotoxic effects, but show no phagocytic activity, nor can they destroy an infected host cell or pathogens. Rather, Th cells provide help to B cells and cytotoxic T cells for the sake of antibody production, cytotoxic T cell activity and memory development (Zajac and Harrington, 2008). Also, they secrete important cytokines to either support or inhibit certain innate immune cells (Zajac and Harrington, 2008; Moser and Leo, 2010).

Naïve Th cells further differentiate into selected helper/effector Th cells, namely T helper 1 (Th1), T helper 2 (Th2), follicular T helper (fTh) or T helper 17 (Th17) cells, under the control of cytokines produced during antigen-specific stimulation (Figure 2.6; Moser and Leo, 2010).

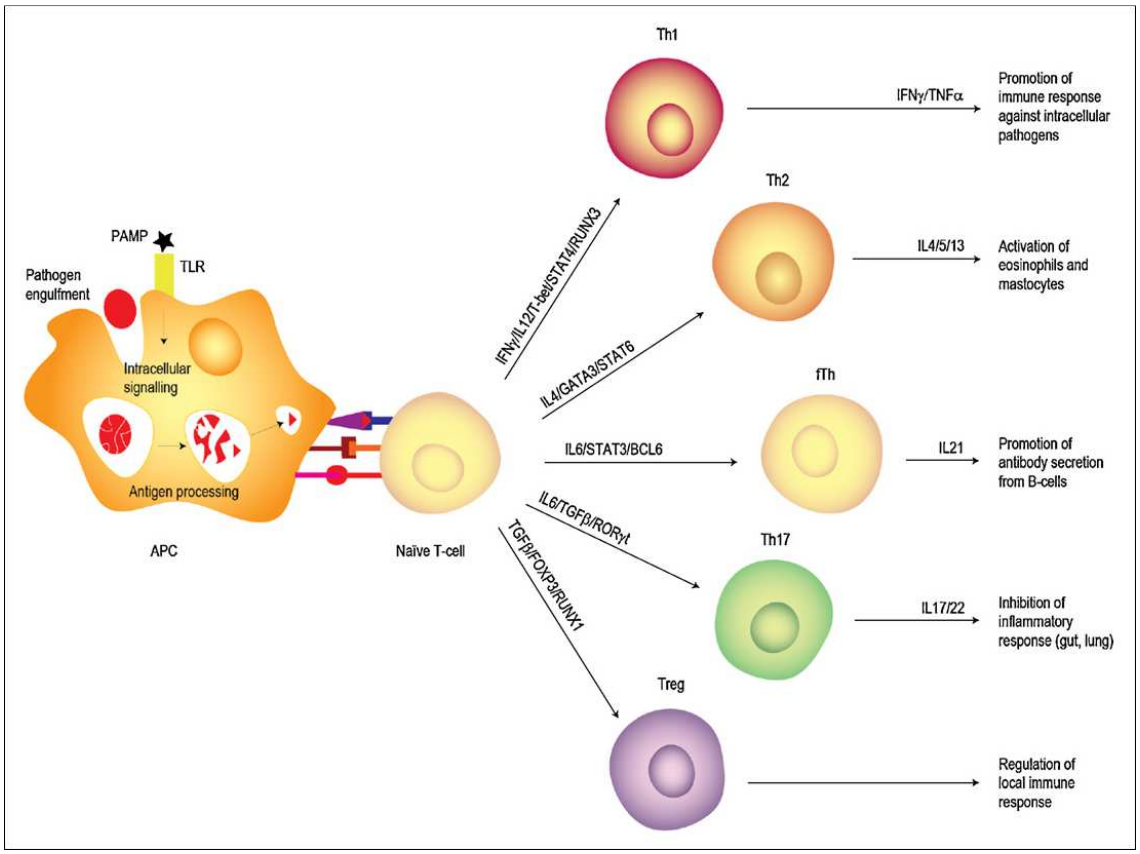

Figure 2.6: The differentiation of naïve helper T cells into Th1, Th2, fTh, and Th17 cells (From: Moser and Leo, 2010)

- Th1 cells: Th1 cells (effector cells) primarily secrete the cytokine interferon-gamma, which is known to increase MHC expression, display potent anti-viral effects, promote differentiation and activity of CD8 cells, and are critical for immune response against intracellular pathogens (Zajac and Harrington, 2008; Moser and Leo, 2010).
- Th2 cells: Th2 cells (effector cells) predominantly secrete cytokines interleukin-4, -5, and -13, activate eosinophils and mastocytes to eradicate large extracellular parasites, and produce the antibody, IgE, associated with humoral immune responses (Zajac and Harrington, 2008; Moser and Leo, 2010).
- fTh cells: fTh cells promote high levels of antibody secretion from antigen-specific B cells, and produce interleukin-21 which regulates humoral responses *in vivo* (Moser and Leo, 2010).
- Th17 cells: Th17 cells secrete interleukin-17 and -22, which play a role in the regulation of the local immune response to gut and lung pathogens (Moser and Leo, 2010).

iii. Cytotoxic T cells

Cytotoxic T (Tc), or CD8 cells interact with a specific antigen-MHC class I complex in order to kill virally infected target cells. They secrete a pore-forming protein, perforin, and subsequently granzymes that initiate an apoptotic response leading to the rapid cell death of the infected cell (Moser and Leo, 2010). In addition to their direct killing functions, Tc cells also secrete interferon-gamma and tumour necrosis factor-alpha, which inhibit intracellular pathogen replication while preserving the integrity of the infected cells (Zajac and Harrington, 2008; Moser and Leo, 2010).

iv. Regulatory T cells

Regulatory T (Treg) cells, or suppressor T cells, are characterised by the expression of the CD4 and CD25 markers, and the forkhead box protein transcription factor (Chaplin, 2010; Moser and Leo, 2010). These cells play an important regulatory role that acts to down modulate immune responses by the production of the suppressive cytokines, interleukin-10 and transforming growth factor-beta (Zajac and Harrington, 2008; Chaplin, 2010). Treg cells are thus beneficial to the host by limiting chronic immune responses which can cause extensive tissue damage if uncontrolled (Zajac and Harrington, 2008; Moser and Leo, 2010).

v. Natural killer T cells

Natural killer T (NKT) cells are a group of T lymphocytes that recognise glycolipid antigens presented by CD1, a non-classical MHC class I-like molecule (Gao and Radaeva, 2013). They are subdivided into three subsets based on the expressions of CD4 and CD8, namely: CD4+, CD8+, and CD4/CD8 double-negative subsets (Kee *et al.*, 2012). Once activated, these subsets rapidly produce Th1 cytokines such as interferon-gamma and tumour necrosis factor-alpha; Th2 cytokines, including interleukin-4 and -13; Th17 cytokines, interleukin-17 and -22; and fTh cell cytokine, interleukin-21 (Kee *et al.*, 2012; Gao and Radaeva, 2013; Van Kaer *et al.*, 2013). Activated NKT cells also produce cytotoxic mediators, such as perforin, to kill target cells (Gao and Radaeva, 2013). Furthermore, NKT cells cannot develop immunological memory, but interact with cells of the innate and adaptive immune systems and thus participate in a variety of immune responses (Van Kaer *et al.*, 2013).

vi. Gamma-delta T cells

Gamma-delta (γδ) T cells are a minor T cell population that express TCRs which consist of γ and δ heterodimers (Direskeneli, 2013). These cells lack surface expression of CD4 or CD8 and recognise antigens by a non-MHC-restricted method (Knight *et al.*, 2012). Activated γδ T cells secrete Th1 cytokines, interferon-gamma and tumour necrosis factor-alpha, and display cytolytic activity against virus-infected and tumour cells by combining conventional adaptive features with innate-like responses (Knight *et al.*, 2012; Direskeneli, 2013).

(b) Cytokines

Cytokines are a group of low molecular weight proteins that function as signalling compounds and chemical mediators (Isomäki and Punnonen, 1997). They are produced and secreted on demand (Kress, 2010) by many types of cells of the immune system in tissues undergoing defence, growth and repair, and play a particularly prominent role in the innate and adaptive immune responses (Hopkins, 2003). Cytokines have multiple activities on different cell types, also known as pleiotropy, and the effects of two cytokines may be overlapping, creating a sense of redundancy of the cytokine network (Isomäki and Punnonen, 1997; Kress, 2010).

Based on functional or structural similarities, cytokines can be divided into interleukins (IL), tumour necrosis factors (TNF), interferons (IFN), transforming growth factors (TGF) and chemokines (Isomäki and Punnonen, 1997; Kidd, 2003). They are further divided into pro-inflammatory cytokines, for the support of Th1 immune responses, and anti-inflammatory cytokines, for the support of Th2 responses, respectively (Sult, 2003; Priimägi *et al.*, 2005).

As briefly described in Section 2.2.2.1 a) ii, Th1 and Th2 cells originate from naïve T cells, but are produced under the influence of antigen presenting cells (APCs; Kidd, 2003). Initially, a naïve T cell has to make contact with an APC, and depending on the type of antigen to which it is exposed, will differentiate into either Th1 or Th2 cells. Should the antigen be of intracellular nature, the outcome of differentiation will likely be that of Th1 cells. Here, APCs secrete the cytokine IL-12, which stimulates the production of Th1 cells, as well as NK cells. In turn, NK cells produce IFN-γ, which further promotes IL-12 secretion from the APCs, and a subsequent strong Th1

cell lineage (Elenkov and Chrousos, 1999; Kidd, 2003). For a Th2 outcome, differentiation is initiated by IL-6 produced by APCs associated with extracellular antigen, as well as IL-4, released by NK cells, mast cells, and eosinophils. Th2 cell maturation further promotes IL-4 secretion, which, in combination with the other participating cell types, leads to the production of more Th2 cells (Bergmann and Van Hemmen, 2001; Kidd, 2003). Th1 and Th2 responses are mutually inhibitory, meaning that IL-12 and IFN-γ will inhibit a Th2 response, and IL-4 and IL-10 will inhibit a Th1 response (Elenkov and Chrousos, 1999).

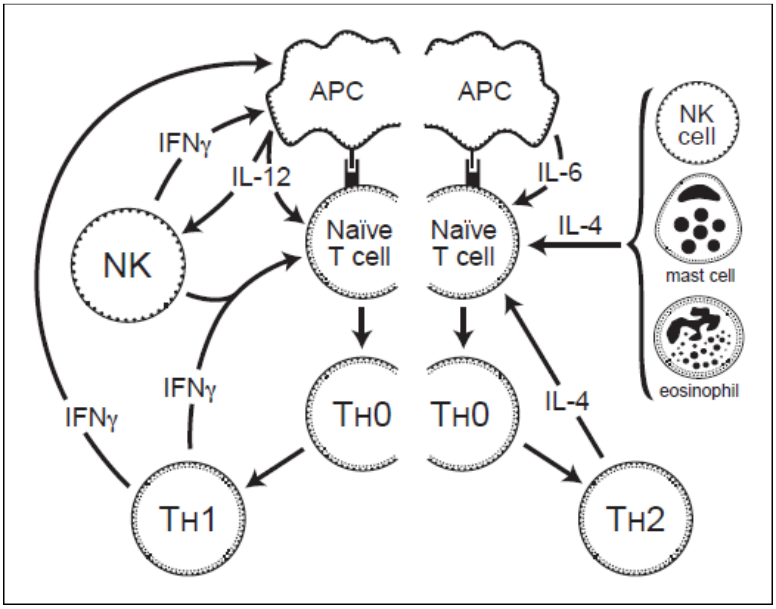

Figure 2.7: Cytokine-directed differentiation of Th1 and Th2 cells from naïve T cells (From: Kidd, 2003)

A Th1 response is associated with pro-inflammatory actions (Isomäki and Punnonen, 1997). It provides defence against intracellular pathogens and cell injury, by orchestrating cell-mediated immunity (Bergmann and Van Hemmen, 2001; Sult, 2003). A Th2 response is responsible for anti-inflammatory actions (Isomäki and Punnonen, 1997), such as antibody secretion, and complement, mast cell, and eosinophil activation. This type of immune response directs humoral immunity, and subsequent resistance to extracellular pathogens (Bergmann and Van Hemmen, 2001; Sult, 2003). Important pro-inflammatory/Th1 cytokines include IL-2, IFN-γ and TNF-α, while anti-inflammatory/Th2 cytokines are IL-4, IL-5, IL-6, IL-9, IL-10 and IL-13 (Sult, 2003). For purposes if this study, only IL-2 and IL-10 will be briefly discussed.

i. Interleukin-2

IL-2 is a small, pro-inflammatory cytokine produced primarily by activated T cells, *i.e.*, Th1 and Tc cells, some B cells and dendritic cells (Malek, 2003; Gaffen and Liu, 2004). It is a known “T cell growth factor“ and therefore targets activated T cells, in order to stimulate growth and differentiation of the T cell response; B cells, to promote antibody secretion; NK cells, for the production of TNF-α and IFN-γ; as well as macrophages (Gaffen and Liu, 2004).

IL-2 production, in short, relies on antigen recognition by TCRs. Upon antigen-TCR binding, the activated T cell rapidly secretes IL-2, which is swiftly followed by the expression of a high affinity IL-2 receptor (IL-2R; Gaffen and Liu, 2004). This IL-2/IL-2R interaction permits rapid and selective expansion of effector T cell populations, such as antigen-specific Tc cells, contributing to the development of T cell immunologic memory (Gaffen and Liu, 2004). Furthermore, IL-2 plays an important role in the development and expansion of Treg cells, and thereby controls the immune response in order to prevent autoimmunity (Thornton *et al.*, 2004). This is considered to be the main non-redundant function of IL-2 (Malek, 2003).

Lastly, since IL-2 is regarded as a T cell growth factor, it indirectly contributes to the production of most T cell-derived cytokines. The cytokine shows a wide array of effects on the immune system, and is of utmost importance in the regulation of immune activation and homeostasis (Gaffen and Liu, 2004).

ii. Interleukin-10

IL-10 is well characterised as one of the most crucial anti-inflammatory cytokines in the immune response (Opal and DePalo, 2000). It is mainly synthesised by monocytes/macrophages, and after activation, by Th, Tc, Treg and B cells (Isomäki and Punnonen, 1997; Mege *et al.*, 2006). Furthermore, IL-10 has the ability to promote its own production through Treg cells (Pierson and Liston, 2010).

IL-10 is a potent inhibitor of pro-inflammatory cytokines, IL-2 and IFN-γ (Opal and DePalo, 2000), which directly inhibits the proliferation of T cells (Isomäki and Punnonen, 1997). It also indirectly inhibits T cells by the down regulation of antigen presenting capacity of monocytes (Isomäki and Punnonen, 1997). Here, IL-10

deactivates monocyte/macrophage pro-inflammatory cytokine synthesis, *i.e.*, TNF-α, IL-1, IL-6, IL-8, IL-12 and granulocyte-macrophage colony-stimulating factor (Opal and DePalo, 2000). Furthermore, IL-10 enhances MHC class II expression on B cells, consequently leading to increased immunoglobulin production (Pierson and Liston, 2010). Lastly, IL-10 is a potent stimulator of NK and Tc cells (Mege *et al.*, 2006).

IL-10 thus plays a very important role in regulating both the innate and adaptive immune responses, and generally protects the host from chronic inflammation by preventing the development of immunopathological lesions (Opal and DePalo, 1999; Mege *et al.*, 2006).

2.2.2.2 Humoral immunity

(a) B cell development

B cells (or lymphocytes) are immune cells which express cell surface immunoglobulin (Ig) receptors for the recognition of specific antigens (LeBien and Tedder, 2008). These cells originate from haematopoietic stem cells in the bone marrow (Chaplin, 2010), start their development in primary lymphoid tissue, *i.e.*, foetal liver and foetal/adult marrow, and finally mature in secondary lymphoid tissue, such as lymph nodes and the spleen (LeBien and Tedder, 2008). The endpoint and functional goal of B cell development and differentiation, is antibody production (LeBien and Tedder, 2008).

(b) Immunoglobulins

Immunoglobulins, or antibodies, can both recognise and eliminate antigens or pathogens from the body. These molecules are roughly Y-shaped and made up of two identical heavy chains (IgH) and two identical light chains (IgL). There are five types of heavy chains, *i.e.*, α, γ, δ, ε and μ, which can be associated with the two types of light chains, namely, κ and λ. The type of heavy chain determines the isotype of the immunoglobulin molecule, namely, IgA (α), IgD (δ), IgE (ε), IgG (γ) and IgM (μ; Moser and Leo, 2010). For each antibody isotype, the amino terminal portions of the heavy and light chains vary in amino acid sequence (Chaplin, 2010). Each component chain (H or L) contains one variable (V) domain and one or more constant (C) domains (Schroeder and Cavacini, 2010). V domains are designated

VH (for H chains) and Vκ or Vλ (for L chains), respectively. The position of one VH segment and one Vκ or Vλ segment creates the antigen-binding portion of the immunoglobulin, therefore each immunoglobulin has two identical antigen-binding sites (Figure 2.8; Chaplin, 2010).

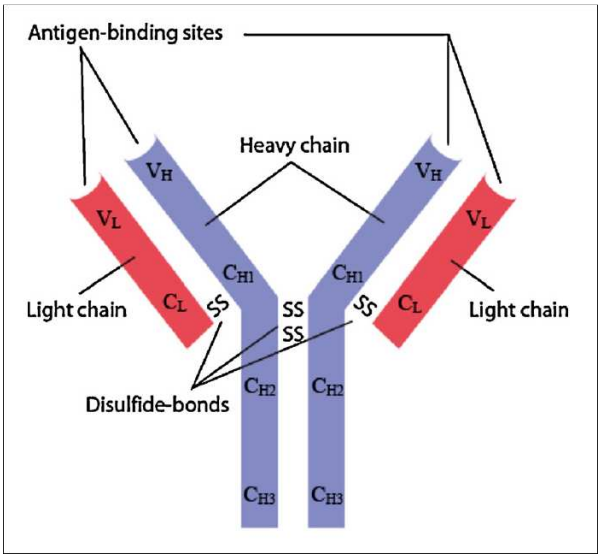

Figure 2.8: The structure and components of immunoglobulins (From: Moser and Leo, 2010)

In the early stages of B cell development, V domains are rearranged and expressed in association with the μ H chain to produce IgM, and later IgD by alternative splicing of the IgM molecule. Later on in the development process, and in response to antigenic stimulation, the V domains can associate with the other isotypes, IgA, IgE and IgG (Schroeder and Cavacini, 2010). This is termed isotype switching, and is regulated by the type of antigen and cytokines (Chaplin, 2010; Schroeder and Cavacini, 2010). IL-10 causes switching to IgG, IL-4 and IL-13 cause switching to IgE, and TGF-β causes switching to IgA (Chaplin, 2010). Immunoglobulin isotype is important as it determines the effector mechanism of a given antibody (Moser and Leo, 2010). For purposes of this study, only IgM and IgG will be briefly discussed.

i. Immunoglobulin M

IgM is the first immunoglobulin expressed during B cell development, and functions by opsonising, or coating, antigen for destruction. It is strongly associated with a primary immune response and is used for the diagnosis of acute pathogen exposure. IgM-bearing B cells have the ability to respond quickly to antigens, and are also known as natural antibodies (Schroeder and Cavacini, 2010).

ii. Immunoglobulin G

As the predominant immunoglobulin isotype, IgG is further divided into four subclasses, namely IgG1, IgG2, IgG3, and IgG4, which exhibit different functions (Schroeder and Cavacini, 2010). IgG is secreted during the secondary response, and are involved in the neutralisation of toxins and viruses (Moser and Leo, 2010; Schroeder and Cavacini, 2010).

In summary, the immune system uses cytokines as signals of communication between cells in order to generate an immune response. Since cytokines have the ability to intensify and suppress immune responses, a normal immune system is both dynamic and balanced between Th1 and Th2 activities, and can switch back and forth between responses as needed (Sult, 2003). Moreover, it is of utmost importance to acknowledge that the immune response is far more complex than depicted in this review, and this should be appreciated when analysing the data.

A. Drug-induced liver injury associated with immune stimulation

Drug-induced liver injury is considered a very serious adverse drug reaction. Of concern is that it has restricted the use of efficacious drugs such as isoniazid and nevirapine in some patients, and is the most feared adverse effect after paracetamol overdose (Jaeshke *et al*., 2006). Whereas several mechanisms have been postulated, the immune system has been implicated as a mediator and determinant for progression of the liver injury, since the liver contains a unique composition of innate and adaptive immune cells (Holt and Ju, 2006).

As such, several drugs have been shown to induce liver injury by activation of the immune system, *i.e.*, paracetamol (Jaeshke *et al*., 2006; Liu and Kaplowitz, 2006 and 2007), ranitidine (Luyendyk *et al*., 2003), and trovafloxacin (Shaw *et al*., 2007).

2.2.3 Immune response during isoniazid-induced liver injury

2.2.3.1 Isoniazid-induced liver injury

The use of isoniazid for the treatment of *Mycobacterium tuberculosis* infection poses risk for the development of severe liver injury/toxicity in 1 – 2% of patients, of which 20% of these patients present with elevated liver enzyme levels (Shen *et al*., 2006).

The toxicity presents approximately three months after the start of treatment, and the risk of developing the adverse reaction is age related, *i.e.*, increases with increasing age. Daily consumption of alcohol, chronic liver disease and injection drug use are other factors which further increase the risk of hepatitis (Tostmann *et al.*, 2008). Patients may experience any of the following symptoms: nausea, vomiting, rash, fever, ataxia, slurring of speech, peripheral neuritis, dizziness and stupor (Romero and Kuczler, 1998).

2.2.3.2 Immune-mediated mechanism of isoniazid-induced liver injury

Involvement of the immune system in isoniazid-induced liver injury has been implicated, however, the root thereof lies in the metabolism of isoniazid. Isoniazid is mainly metabolised via acetylation by NAT2, a hepatic enzyme, which results in the production of acetylisoniazid. Thereafter, acetylisoniazid is hydrolysed into acetylhydrazine and isonicotinic acid. Lastly, a small portion of isoniazid is directly hydrolysed into isonicotinic acid and hydrazine. Initial isoniazid studies demonstrated acetylhydrazine as the toxic metabolite to initiate liver injury by its ability to covalently bind to liver proteins *in vivo* (Yue *et al*., 2009). However, more recently it was reported that hydrazine and not acetylhydrazine is the causative hepatotoxin (Tayal *et al*., 2007; Tostmann *et al*., 2008).

Activation of the immune system has been demonstrated in the pathogenesis of tuberculosis, as seen with the tuberculin skin test by which cell-mediated immunity is measured (Mack *et al*., 2009). Here immune markers such as neutrophils, Th cells, Tc cells, IFN-γ and TNF-α are implicated (Mack *et al*., 2009). A report by Tafazoli and co-workers (2008) showed that a hepatocyte inflammation model increased isoniazid cytotoxicity twofold, while it increased hydrazine cytotoxicity sixteenfold. Hence, it can be assumed that tuberculosis itself may be an underlying factor of isoniazid-induced liver injury.

Further evidence in humans was observed with a positive lymphocyte transformation test in which lymphocytes from patients with a history of isoniazid-induced liver injury recognised isoniazid-modified proteins (Warrington *et al.*, 1978). More recently, antibodies against these isoniazid-modified proteins in patients with isoniazid-

induced liver failure could be detected, and even an immune response in those with only mild isoniazid-induced liver injury (Metushi *et al.*, 2014 a and b).

2.2.3.3 Other isoniazid-associated adverse reactions

The most common side effects associated with isoniazid use are skin reactions, and gastrointestinal and neurological disorders (Tostmann *et al.*, 2008). During isoniazid toxicity recurrent seizures, profound metabolic acidosis, coma and even death may occur. Laboratory tests may show an elevated anion gap and metabolic acidosis, hyperglycemia, hypokalemia, glucosuria and ketonuria (Romero and Kuczler, 1998).

2.2.4 Immune response during nevirapine-induced liver injury

2.2.4.1 Nevirapine-induced liver injury

Symptomatic hepatic events (regardless of severity) occur in 4% of patients on nevirapine treatment (Boehringer Ingelheim Pharmaceuticals, 2011). This adverse event usually emerges within the first six weeks of treatment and can lead to severe liver damage and/or liver failure (Haehl, 2000). Other hepatic events include fulminant and cholestatic hepatitis, as well as hepatic necrosis. Patients may experience non-specific prodromal symptoms such as fatigue, malaise, anorexia, nausea, jaundice, and hepatomegaly (Boehringer Ingelheim Pharmaceuticals, 2011).

2.2.4.2 Immune-mediated mechanism of nevirapine-induced liver injury

To date, the exact mechanism of nevirapine-induced liver injury is unknown. In general, the injury/toxicity occurs within the first six weeks of treatment in HIV+ female patients with a CD4 count >250 cells/mm^3, and HIV+ male patients with a CD4 count >400 cells/mm^3 (Boehringer Ingelheim Pharmaceuticals, 2011). Interestingly, toxicity was also observed in non-HIV-infected individuals after using nevirapine as post exposure prophylaxis (Patel *et al*., 2004). Thus, increased CD4 count is considered as a predisposing factor of nevirapine-induced liver injury. Also, HIV+ patients co-infected with the hepatitis B virus (HBV) and HCV are at greater risk of developing nevirapine-induced liver injury, as the progression of liver disease is accelerated in these patients (Dieterich *et al*., 2004).

In a recent departmental study, it was observed that prolonged administration of nevirapine in rats was associated with progressive stimulation of IL-2 and,

surprisingly, improvement in liver histology. In addition, nevirapine selectively induced lymphocytosis, thereby implying that nevirapine is a slow onset immune stimulant (Bekker *et al.*, 2012).

Other recent animal studies have indicated that there is a link between nevirapine toxicity and the immune system, especially regarding skin reactions (Popovic *et al.,* 2006). In one study, there was a prominent increase in total lymphocyte and macrophage cell counts in the nevirapine treated rats when compared to the control rats (Popovic *et al.,* 2006). In another study, Shenton and co-workers (2005) observed more evidence of the immune-mediated mechanism. It was found that there was a delay between the initiation of nevirapine treatment and the onset of skin rash, the presence of perivascular mononuclear cell infiltrates in the dermis of rash patients, and a decrease in time to onset, as well as an increase in the severity of rash on nevirapine rechallenges. Most importantly, susceptibility could be transferred to naïve mice with CD4 cells, and the depletion of CD4 cells was protective (Shenton *et al.*, 2005).

2.2.4.3 Other nevirapine-associated adverse reactions

Nevirapine-induced skin reactions occur in 16% of patients, and vary from mild and maculopapular to life threatening rashes (Shenton *et al.*, 2007). The more severe and life threatening skin reactions, such as Stevens-Johnson syndrome and toxic epidermal necrolysis, are associated with symptoms such as rash (grade 3 and 4), constitutional findings, organ dysfunction and rhabdomyolysis (Boehringer Ingelheim Pharmaceuticals, 2011). The most common hypersensitivity reactions associated with nevirapine use are: severe rash, rash accompanied by fever, malaise, fatigue, muscle and/or joint pain, blisters, oral lesions, conjunctivitis, facial oedema, eosinophilia, granulocytopenia, lymphadenopathy, or renal dysfunction. Other concerns include fat redistribution and the immune reconstitution syndrome (Boehringer Ingelheim Pharmaceuticals, 2011).

2.2.5 Immune response during paracetamol-induced liver injury

2.2.5.1 Paracetamol-induced liver injury

Paracetamol-induced liver injury is a dose-dependent and rather predictable adverse drug reaction (Liu and Kaplowitz, 2007), *i.e.*, not idiosyncratic. It occurs after acute

overdose, with 7.5 g in adults, and 150 mg/kg in children being the lowest dose capable of causing toxicity (Bertolini *et al*., 2006). Paracetamol causes a centrilobular hepatic necrosis which is potentially fatal (James *et al*., 2003).

During the first 24 hours after paracetamol overdose patients may present with symptoms such as nausea and vomiting (Walubo *et al.*, 2004). After the initial 24 hours up to 72 hours represents the onset of liver injury, where symptoms which mimic infectious hepatitis present. Here, elevated aspartate aminotransferase levels precede other signs of actual liver dysfunction, hypoglycaemia and metabolic acidosis. Maximal injury/toxicity occurs 72 – 96 hours after ingestion. Death may result from multiorgan failure, haemorrhage, acute respiratory distress syndrome, sepsis and cerebral oedema (Bertolini *et al*., 2006). Should the patient survive the initial insult, resolution of liver function and complete recovery will occur (Rowden *et al*., 2005). Fortunately, paracetamol-induced liver injury can be resolved by *N*-acetylcysteine (NAC). It is an effective antidote when administered within 8 hours after paracetamol overdose (Rowden *et al*., 2005).

2.2.5.2 Immune-mediated mechanism of paracetamol-induced liver injury

Paracetamol-induced liver injury is initiated by NAPQI, a product of CYP450 metabolism. Under normal circumstances NAPQI is detoxified by hepatic glutathione. However, after paracetamol overdose and subsequent overproduction of NAPQI, glutathione becomes depleted, and the excess NAQPI binds to hepatic proteins leading to the formation of NAPQI-protein adducts. These adducts have the ability to cause oxidative stress, and finally hepatocyte death (Liu and Kaplowitz, 2007).

Involvement of the immune system, and more specifically the innate immune system, has been implicated in paracetamol-induced liver injury (James *et al*., 2003). After paracetamol overdose various cytokines and chemokines are formed, which attract neutrophils and monocytes into the liver. Unfortunately, the available evidence is not convincing enough to support that these pro-inflammatory mediators cam lead to direct cell death. Nevertheless, inflammatory cytokines are capable of modulating intracellular events within hepatocytes, which can alter toxicity (Jaeschke *et al.*, 2012).

A recent animal study has indicated that IFN-γ, a pro-inflammatory cytokine, plays a pivotal role in the progression of liver injury. Here, IFN-γ as well as IL-6 null mice were less susceptible to paracetamol-induced liver injury, while IL-10 initiated anti-inflammatory responses in the liver (Liu and Kaplowitz, 2007). It was later confirmed that these observation were due to dimethyl sulphoxide, a solvent used in the study (Masson *et al.*, 2008). Despite the limitations, ultimately the initial study demonstrated that IFN-γ has the potential to modulate paracetamol-induced toxicity (Jaeschke *et al.*, 2012).

Furthermore, the involvement of the innate immune system in paracetamol-induced liver injury shows to be more of protective nature, and therefore plays a bigger role in the repair of the injury rather than in the initiation of the lesion.

While involvement of the immune system in paracetamol-induced liver injury is appreciated, it remains controversial and the characteristics and mechanisms of the liver injury are different from that seen with isoniazid and nevirapine.

2.2.5.3 Other paracetamol-associated adverse reactions

Paracetamol use is relatively safe, but has been associated with adverse reactions such as asthma and hypertension (Toussaint *et al.*, 2010), as well as drowsiness and euphoria (Bertolini *et al.*, 2006).

2.3 THE ROLE OF METABOLIC ACTIVATION IN DRUG-INDUCED LIVER INJURY

The superfamily of CYP450 consists of heme-containing enzymes which function primarily as mono-oxygenases (Delgoda and Westlake, 2004). CYP450 enzymes are primarily located in the endoplasmic reticulum of hepatocytes and can also be found in the intestinal mucosa (Belitsky and Yakubovskaya, 2008). These enzymes are responsible for the phase I biotransformation of drugs with a wide variety of chemical structures. The most common reactions catalysed by CYP450 enzymes include: aliphatic hydroxylation, aromatic hydroxylation, epoxidation, *N*-dealkylation, *O*-dealkylation and heteroatom oxidation (Saxena *et al.*, 2008). In the human body, CYP450 enzymes play a major role in the production of cholesterol, corticosteroids

and fatty acids, as well as in the metabolism of many exogenous and endogenous compounds, and conventional medicines (Delgoda and Westlake, 2004).

A. Drug-induced liver injury associated with metabolic activation

Drugs are mostly metabolised in the liver by biotransformation reactions, and as such the liver is very susceptible to drug-induced injury. It was observed that after phase I biotransformation of a drug by CYP450 oxidation, reactive metabolites were formed (Liu and Kaplowitz, 2007). Formation of these reactive metabolites occurs due to the metabolite's ability to escape phase II biotransformation, or detoxification, and therefore cannot be successfully eliminated from the body (Park *et al*., 2005). Furthermore, reactive metabolites have the ability to attack and irreversibly bind to proteins, leading to the formation of metabolite-protein adducts (Liu and Kaplowitz, 2007). Some of these adducts are antigenic, hence, are presented to T cells, thereby stimulating the immune system which starts a process to eliminate cells (including hepatocytes) expressing these adducts. Subsequently, hepatotoxicity develops due to liver injury (Walgren *et al*., 2005).

Several drugs, such as paracetamol, halothane, diclofenac and thiazolidinedione antidiabetics (Park *et al*., 2005) have shown to induce liver injury by a metabolic activation pathway.

2.3.1 Metabolism of isoniazid

2.3.1.1 Acetylation of isoniazid

The major metabolic pathway of isoniazid is acetylation by the hepatic enzyme, NAT2. Isoniazid is acetylated into acetylisoniazid, after which it is hydrolysed to form acetylhydrazine and isonicotinic acid (Figure 2.9). Furthermore, a small portion of isoniazid is hydrolysed into isonicotinic acid and hydrazine (Tostmann *et al*., 2008).

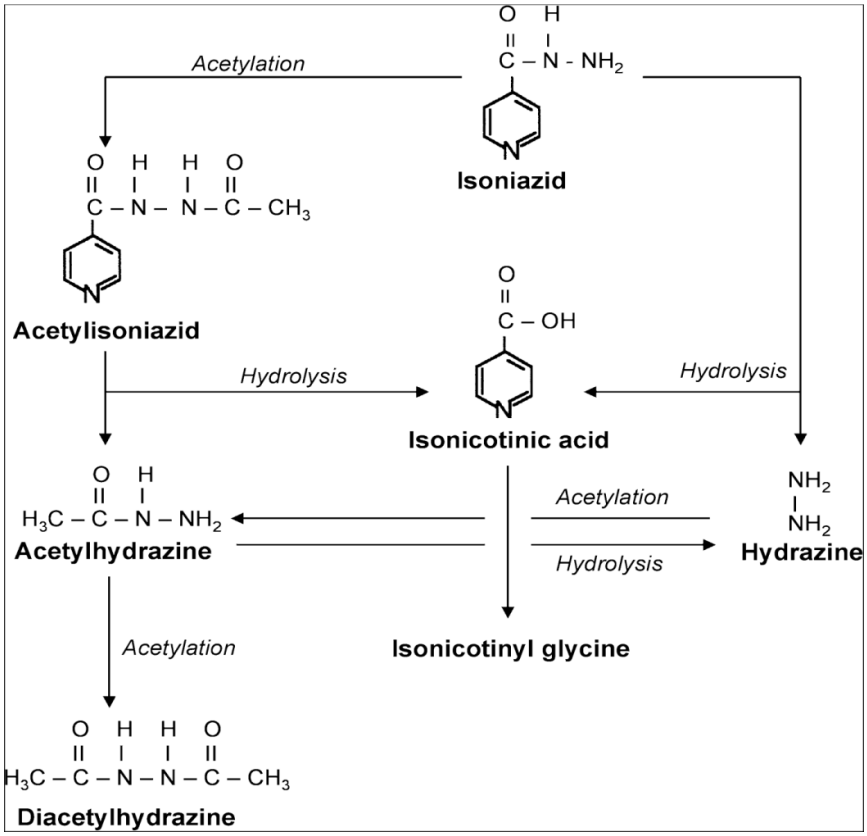

Figure 2.9: The metabolic pathway of isoniazid (From: Tostmann *et al*., 2008)

Acetylation is genetically determined, with the fast acetylator trait being autosomally dominant (Howland and Mycek, 2006 b). Therefore, individuals with the fast phenotype have lower plasma concentrations of isoniazid than individuals with the slow phenotype (Brenner and Stevens, 2006 b).

2.3.1.2 Metabolic pathway of isoniazid-induced liver injury

Previous research postulated that the isoniazid metabolite, acetylhydrazine, was associated with isoniazid-induced liver injury. In contrast, recent research has indicated that hydrazine is in fact the toxic metabolite responsible for the undesired adverse reaction (Tostmann *et al*., 2008).

Hydrazine is metabolised by oxidation, leading to the formation of nitrogen and diimide, as well as nitrogen centred radicals. These radicals most likely contribute to the toxic process (Tostmann *et al*., 2008). Therefore, it was also concluded that slow acetylators are at greater risk of developing isoniazid-induced liver injury, as more

isoniazid remains in the body to be directly hydrolysed into hydrazine. Of note, acetylhydrazine can also be converted into hydrazine (Tostmann *et al*., 2008).

The CYP450 isoform, CYP2E1 has also been implicated in isoniazid-induced liver injury. It is well known that a major role of CYP2E1 is the production of free radicals (Poloyac *et al*., 2001). Previous animal studies have revealed that CYP2E1 is induced by isoniazid, and its toxic metabolite, hydrazine. Therefore, the administration of isoniazid resulted in increased plasma concentrations of hydrazine and CYP2E1 activity, which lead to the development of liver injury (Yue *et al*., 2004; Tostmann *et al*., 2008). These results correlate with the hypothesis that oxidative stress due to reactive oxygen species produced by CYP2E1, may contribute to isoniazid-induced liver injury as well (Shen *et al*., 2006; Tostmann *et al*., 2008). More confirmation of this was reported, after depleted glutathione levels in association with increased reactive oxygen species after isoniazid administration were reversed with NAC (Tostmann *et al*., 2008). A hepatoprotective effect was also observed with tocopherol administration, during which this potent antioxidant scavenged free radicals during isoniazid treatment (Tayal *et al*., 2007).

2.3.2 Metabolism of nevirapine

2.3.2.1 Cytochrome P450 metabolism of nevirapine

Nevirapine undergoes significant hepatic metabolism by 2-, 3-, 8- and 12-hydroxylation, followed by glucuronidation of these metabolites. CYP3A4 is responsible for the formation of 2- and 12-hydroxynevirapine, while CYP2B6 is involved in the formation of 3- and 8-hydroxynevirapine (Wen *et al*., 2009).

12-Hydroxynevirapine has the potential to be sulphated followed by loss of sulphate which results in a reactive quinone methidide. In addition, 4-carboxynevirapine is formed after further oxidation of 12-hydroxynevirapine (Wen *et al*., 2009; Figure 2.10). Of note, both CYP3A4 and CYP2B6 are induced by nevirapine, thereby leading to autoinduction of these enzymes (Boehringer Ingelheim Pharmaceuticals, 2011).

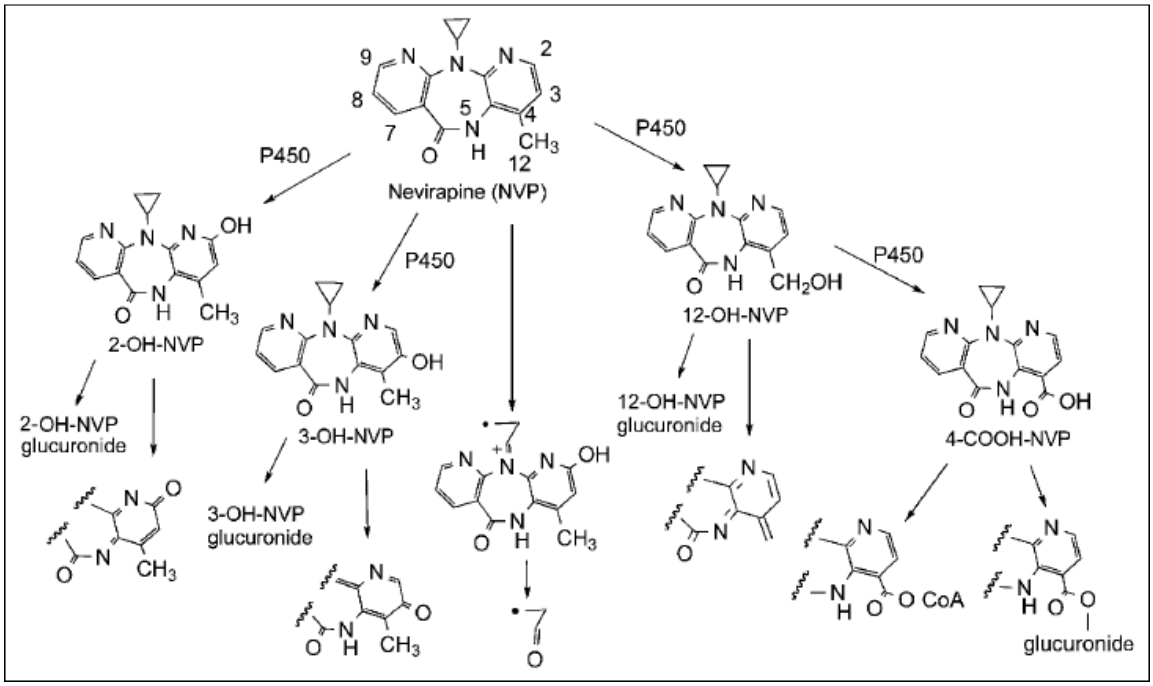

Figure 2.10: The CYP450 metabolism of nevirapine (From: Chen *et al*., 2008)

2.3.2.2 Metabolic pathway of nevirapine-induced liver injury

Wen and co-workers (2009) reported that 12-hydroxynevirapine is the suspected culprit involved in nevirapine-induced liver injury. As mentioned, 12-hydroxynevirapine is converted to a reactive quinone methidide after a sulphation reaction, as well as 4-carboxynevirapine after further oxidation. 4-Carboxynevirapine has the ability to generate acyl glucuronide, which has the potential to bind to cellular proteins (Wen *et al*., 2009). It was also reported by Chen and colleagues (2008) that 12-hydroxynevirapine may be responsible for nevirapine-induced skin rash.

In a recent departmental study, it was observed that after prolonged nevirapine administration, nevirapine concentrations were lower in the nevirapine only group as opposed to higher nevirapine concentrations in the group to which lipopolysaccharide (LPS) was co-administered. These results imply that more nevirapine was metabolised to immunogenic metabolites leading to increased IL-2 and IFN-γ levels (immune stimulation), and liver injury in the nevirapine only group. Conversely, the higher nevirapine concentrations in the LPS co-administered group indicated that less nevirapine was metabolised to immunogenic metabolites, hence little or no liver injury occurred (Bekker *et al.*, 2012).

This observation conforms to a previous report in which nevirapine toxicity was associated with CYP3A induction (Walubo *et al*., 2006). It means that increased metabolism of nevirapine due to auto-enzyme induction within the first two weeks of therapy could be responsible for perpetuating this immune toxicity and/or liver injury.

2.3.3 Metabolism of paracetamol

2.3.3.1 Glucuronidation, sulphation and cytochrome P450 metabolism of paracetamol

The majority of paracetamol is metabolised by conjugation, while a smaller portion is either combined with sulphate or cysteine (Bertolini *et al*., 2006). The smallest portion of paracetamol is oxidised by CYP450 metabolism (Figure 2.11). More specifically, CYP1A2, CYP2E1 and CYP3A4 metabolise paracetamol into its reactive metabolite, NAPQI (James *et al*., 2003). NAPQI is swiftly detoxified by glutathione, converting it into non-toxic cysteine or mercapturate conjugates, which are eliminated in the urine (James *et al*., 2003; Bertolini *et al*., 2006).

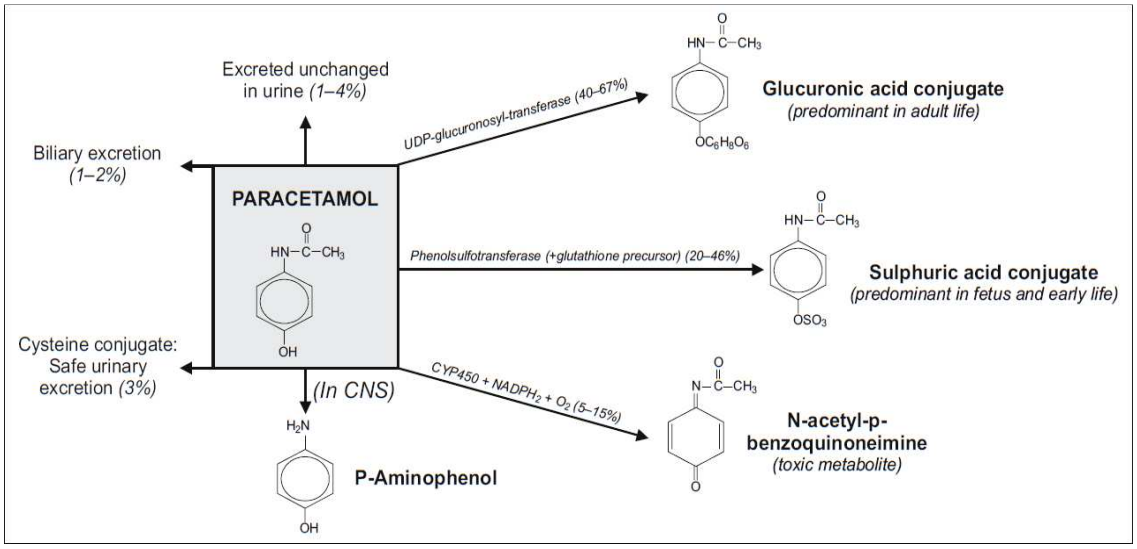

Figure 2.11: The first-pass and CYP450 metabolism of paracetamol (From: Bertolini *et al*., 2006)

2.3.3.2 Metabolic pathway of paracetamol-induced liver injury

The reactive metabolite of paracetamol, NAPQI has been extensively researched and declared as the ultimate determinant in paracetamol-induced liver injury after paracetamol overdose (James *et al*., 2003; Rowden *et al*., 2005; Bertolini *et al*., 2006). At therapeutic doses NAPQI is rapidly detoxified by glutathione to non-toxic

metabolites. However, after paracetamol overdose conjugation pathways are overwhelmed, CYP450 metabolism is in overdrive which results in increased NAPQI formation, and finally depletion of glutathione (Rowden *et al*., 2005). During hepatic glutathione depletion, NAPQI cannot be detoxified, hence covalently binds to cysteine groups on hepatic proteins and forms NAPQI-protein adducts (James *et al*., 2003). This results in hepatic cell death brought on by oxidation of enzymes, DNA fragmentation, mitochondrial injury, and the increased production of nitrogen and oxygen species (Bertolini *et al*., 2006). A report by Reid and co-workers (2005) revealed that mitochondrial permeability transition, after injury by NAPQI, leads to oxidative stress. These results were further confirmed after NAC, the known antidote to paracetamol overdose, eliminated the loss of mitochondrial membrane potential, and consequently relieved oxidative stress (Reid *et al*., 2005).

Drugs such as isoniazid, which is known to induce CYP450 metabolism, may increase the incidence of paracetamol-induced liver injury. Furthermore, chronic alcoholics face a greater risk of developing paracetamol-induced liver injury, as ethanol is known to induce CYP2E1 (Bertolini *et al*., 2006).

2.4 THE LINK BETWEEN THE METABOLIC AND IMMUNE RESPONSE IN DRUG-INDUCED LIVER INJURY

2.4.1 Idiosyncratic drug reactions

The term idiosyncratic drug reaction can be defined as an adverse drug reaction that does not occur in most patients at any readily achieved dose of a drug, and it does not involve the known pharmacological characteristics of the drug (Uetrecht, 2008). Drug-induced liver injury, as seen with isoniazid and nevirapine use, is a good example of an idiosyncratic adverse drug reaction (Walgren *et al*., 2005). Despite extensive research on this topic, idiosyncratic drug reactions are still not well understood, and are therefore unpredictable with regards to the chemistry of the drug and the biology of the patient. Furthermore, under different conditions, the same drug can cause direct metabolite-mediated toxicity, or immune-mediated toxicity (Naisbitt *et al*., 2003). In general, these reactions are divided into two categories, namely, immune-mediated reactions, and metabolic idiosyncrasy

reactions, however an overlap between the two categories may occur (Andrade *et al.*, 2009).

2.4.1.1 Immune-mediated reactions

In general, immune cells have been implicated to play a pivotal role in idiosyncratic drug-induced liver injury. More so, the direct effects of drugs, or a reactive metabolite, on hepatocytes may trigger an immune response, and this will determine the degree of liver injury (Adams *et al.*, 2010). However, the immune response may vary between individuals (Andrade *et al.*, 2009).

The most remarkable evidence to support immune-mediated drug-induced liver injury is the time to onset, time of recovery and response to rechallenge of a drug (Uetrecht, 2009). Typically, drug-induced liver injury takes 1 – 3 months to develop, which is similar to immune mechanism as it takes time for lymphocytes to grow in numbers to produce a clinical response to the drug insult. Most commonly, recovery from the liver injury occurs quickly after the drug is stopped, but in some cases the injury continues. It is likely due to an autoimmune component that was initiated by the drug, before withdrawal. Many a time when a patient is re-introduced to the drug, the time to onset is very short, resulting in a more severe form of liver injury. This response is driven by T- and B cells and, again, the outcome might be dependent on the autoimmune component as mentioned earlier (Uetrecht, 2009). In addition, the liver injury is usually accompanied by fever, a rash and eosinophilia (Boelsterly and Lim, 2007), and even anti-drug antibodies have been detected (Adams *et al.*, 2010).

Furthermore, it has been proposed that molecules such as cytokines released or produced by cells undergoing either stress, due to disease, or necrotic cell death, due to injury, can activate resting antigen presenting cells, which initiate an immune response (Naisbitt *et al*., 2003). This was observed for both isoniazid and nevirapine, where tuberculosis- and HIV patients, respectively, were co-infected with HBV and HCV, which predisposed these patients to drug-induced liver injury (Dieterich *et al*., 2004). Furthermore, nevirapine-induced liver injury was more common in patients with increased CD4 counts (Patel *et al*., 2004), and antibodies against isoniazid-modified proteins in patients with isoniazid-induced liver failure could be detected (Metushi *et al.*, 2014 a).

Regarding necrotic cell death, it was observed that hepatocellular death due to NAPQI, the toxic metabolite of paracetamol may trigger the activation of Kupffer cells which will result in the release of cytokines, and recruitment of inflammatory cells (James *et al*., 2003; Liu and Kaplowitz, 2006).

2.4.1.2 Metabolic idiosyncrasy reactions

Most drugs associated with idiosyncratic reactions, have been shown to undergo metabolic activation by enzymes to a protein-reactive intermediate (Naisbitt *et al*., 2003). These reactive intermediates/metabolites have the ability to attack and irreversibly bind to proteins, leading to the formation of protein adducts (Liu and Kaplowitz, 2007). Some of these adducts are antigenic, thereby stimulating the immune system which starts a process to eliminate hepatocytes expressing these adducts. Subsequently, hepatotoxicity develops due to liver injury (Walgren *et al*., 2005).

Recent research has indicated that hydrazine is the toxic metabolite responsible for isoniazid-induced liver injury (Tostmann *et al*., 2008). The toxicity of isoniazid is also associated with CYP2E1, which is a well known producer of free radicals (Poloyac *et al*., 2001). CYP2E1 is induced by isoniazid, and hydrazine, therefore the increased enzyme activity leads to abundant free radical production, which in turn promotes the development of liver injury (Yue *et al*., 2004; Tostmann *et al*., 2008).

It was reported that the metabolite, 12-hydroxynevirapine, is involved in nevirapine-induced liver injury. This metabolite is converted to a reactive quinone methidide, and to 4-carboxynevirapine, which has the potential to bind to cellular proteins (Wen *et al*., 2009). In a recent departmental study, it was observed that the co-administration of nevirapine and LPS led to increased nevirapine plasma concentrations, implying that less nevirapine was metabolised to immunogenic metabolites, hence little or no liver injury occurred (Bekker *et al.*, 2012). Furthermore, increased nevirapine metabolism due to auto-enzyme induction within the first two weeks of therapy could be responsible for perpetuating liver injury/toxicity (Walubo *et al*., 2006).

During hepatic glutathione depletion, after paracetamol overdose, the toxic metabolite NAPQI cannot be detoxified, hence covalently binds to cysteine groups on hepatic proteins and forms NAPQI-protein adducts (James *et al*., 2003). This results in hepatic cell death brought on by oxidation of enzymes, and increased production of nitrogen and oxygen species, *i.e.*, oxidative stress (Reid *et al*., 2005; Bertolini *et al*., 2006). Lastly, drugs and substances (nicotine) which are known to induce CYP1A2, CYP2E1, and CYP3A4, which are involved in paracetamol metabolism to NAPQI, may increase the incidence of paracetamol-induced liver injury (Bertolini *et al*., 2006).

2.5 CONCLUSION

With regards to the review, it is concluded that both immune stimulation and metabolic activation play integral roles in the initiation and development of drug-induced liver injury. Of note, the mechanisms for the same idiosyncratic adverse reaction, in this case liver injury/toxicity, caused by different drugs can be different for each drug (Uetrecht, 2008). Therefore, blood biomarkers to monitor immune stimulation and metabolic activation should be established so that liver injury can be identified early (Adams *et al*., 2010). As such, a time profile response of the immune system is necessary to determine when the respective markers would be of use in predicting the extent of liver injury. Understanding the pattern of changes in drug metabolism, and the immune system during drug therapy/toxicity, is vital to the development of preventative and curative strategies for drug toxicity.

Here, it was proposed to study the pattern and time profile changes of some cytokines and other immune markers, as well as metabolic activity of rat CYP1A2, CYP2E1 and CYP3A2, during prolonged administration of isoniazid, nevirapine and paracetamol.

CHAPTER 3

REVIEW OF ANALYTICAL METHODS

3.1 REVIEW OF ANALYTICAL METHODS FOR THE DETERMINATION OF ISONIAZID, NEVIRAPINE AND PARACETAMOL IN PLASMA

Many analytical methods for the determination of isoniazid, nevirapine, and paracetamol in plasma are described. These include liquid chromatography tandem mass spectrometry (LC-MS), gas chromatography (GC), spectrophotometry, thin layer chromatography (TLC) and high performance liquid chromatography (HPLC). Although LC-MS, GC, spectrophotometry and TLC hold certain advantages over HPLC, HPLC is the method of choice as it is available in our setup.

In this study isoniazid, nevirapine, and paracetamol are the drugs of interest. Accordingly, many reports are available on the determination of isoniazid, nevirapine and paracetamol, respectively, by HPLC. However, the development of an HPLC method for the simultaneous analysis of isoniazid, nevirapine and paracetamol in plasma, is considered more appropriate to accommodate the large number of samples to be analysed. To date no such method is available for adoption, therefore a new method will have to be developed. A method by Smith and colleagues (1999) for the determination of rifampicin, isoniazid and pyrazinamide in plasma, will be used as a starting point for method development.

3.1.1 Isoniazid

Different methods such as spectrophotometry, GC, LC-MS and HPLC can be used to measure isoniazid concentrations in plasma (Aït Moussa *et al.*, 2001).

Spectrophotometry methods hold a certain advantage as they are simple, rapid, sensitive and economical to execute (Barsoum *et al.*, 2008; Oga, 2010; Arifa Begum *et al.*, 2013). Unfortunately, the majority of methods are aimed at detection of isoniazid in pharmaceutical formulations, which is not in accordance with the aim of the study. Although a spectrophotometer is available in our laboratory, this method of analysis will not be used. Both GC and LC-MS are extremely sensitive methods of

analysis, especially for a low molecular weight compound such as isoniazid (Huang *et al.*, 2009). These methods require the use of expensive equipment not available in our setup, therefore cannot be considered for analysis.

HPLC is very commonly used for the analysis of isoniazid in plasma. Simple and rapid methods are described by Aït Moussa and colleagues (2001), and Gârbuleț and Dorneanu (2009), but are both rejected based on large plasma volumes (500 µl) used, since rats have a limited amount of blood available. However, the mobile phase and sample extraction used by Aït Moussa and colleagues (2001) will be considered during method development (Chapter 5). More intricate procedures are reported by Delahunty and colleagues (1998) and Walubo and co-workers (1991 a; 1991 b), but neither can be adopted because of large plasma volumes, complex sample processing, and rare/expensive chemicals and instrumentation used.

3.1.2 Nevirapine

Many analytical methods for the determination of nevirapine in plasma are described, and include: TLC, LC-MS and HPLC (Cressey *et al.*, 2007).

TLC is an inexpensive, simple and rapid assay, but requires large plasma volumes (Dubuisson *et al.*, 2004), which is inappropriate for method development of this study. Furthermore, an immunochromatographic strip test based on TLC principles described by Cressey and co-wokers (2007), is unable to provide information regarding the minimum effective concentration of nevirapine. As for isoniazid (Section 3.1.1), LC-MS holds many advantages and promises to quantitate nevirapine in plasma, but this instrument is not available in our laboratory.

HPLC is considered the “golden standard” for antiretroviral drug level measurement (Cressey *et al.*, 2007). Fortunately, an HPLC method for the determination of nevirapine in plasma was developed in our laboratory in a previous study, and was adopted from the report by Hollanders and co-workers (2000). In short, it involved protein precipitation, with perchloric acid, of nevirapine and chlorzoxazone (internal standard) spiked plasma. The supernatant was purified by solid phase extraction, and injected into the HPLC. Elution was achieved with an isocratic mobile phase of tetraethylammoniumphosphate buffer and acetonitrile (60:40, v/v) over a C_{18} (4.6 x

150 mm) 5 µ analytical column at a flow rate of 1 ml/min and wavelength of 210 nm. This method will be taken into account for method development (Chapter 5).

3.1.3 Paracetamol

The determination of paracetamol in plasma can be carried out by using the following techniques: TLC, spectrophotometry, GC and HPLC (Bosch *et al*., 2006).

As mentioned in Section 3.1.2, TLC methods are rapid and usually inexpensive to perform. However, most methods are aimed at quality testing of pharmaceutical formulations of paracetamol (Roy *et al.*, 1997), and cannot be considered for use. As for isoniazid (Section 3.1.1), spectrophotometry methods are used for the measurement of paracetamol in pharmaceutical formulations (Delvadiya *et al.*, 2011), and will not be utilised. Again, the use of GC methods is rejected owing to a lack of instrumentation in our laboratory.

HPLC is the analytical method of choice to measure plasma paracetamol concentrations (Bosch *et al*., 2006). Gotelli and colleagues (1977), Nagaralli and colleagues (2003) and Jensen and co-workers (2004) all described simple and rapid methods for the determination of paracetamol. None of the methods can be adopted due to large sample volumes and equipment used.

3.2 REVIEW OF ANALYTICAL METHODS FOR THE MEASUREMENT OF RAT CYTOKINES AND IMMUNOGLOBULINS

A few methods for the measurement of rat cytokines and immunoglobulins are available, and include: antibody array, cytometric bead array (CBA) and enzyme-linked immunosorbent assay (ELISA).

Antibody array allows the simultaneous measurement of multiple cytokines from a single sample. It entails the use of an array membrane, embedded with antibody array chips, specific to multiple cytokines. After incubation and washing steps, cytokines on the membrane are detected by chemiluminescence and subsequently exposed in a chemiluminescence blot documentation system, or to X-ray film (Abcam Inc., 2012).

CBAs provide a method of capturing a cytokine, or a set of cytokines, with beads of known size and fluorescence, making it possible to detect using flow cytometry. Each capture bead is conjugated with an antibody specific to a certain cytokine, and multiple beads can be mixed for a single sample. Therefore, multiple cytokine proteins can be simultaneously detected in research samples (Becton Dickinson and Company, 2010).

ELISA makes use of a micro plate which is already coated with a capture antibody, or to which the capture antibody is added, and this capture antibody is specific to the cytokine/immunoglobulin to be analysed. After multiple incubation and washing steps, the specific cytokine/immunoglobulin is detected by ultra violet (UV) light using a micro plate reader. This assay allows the analysis of a single cytokine/immunoglobulin for a large number of samples (Invitrogen, 2010; BenderMed System, 2013).

The antibody array is appealing due to its promise of high sensitivity, compared to ELISA, but is not at all cost effective as a large number of samples will have to be analysed. CBA will be the first option, as it allows the analysis of multiple cytokines in a large number of samples, and proves to be very sensitive. Although a flow cytometer is not available in our setup, collaboration with a fellow department in the Faculty of Health Sciences is possible. ELISA will be the second option, should the CBA not be successful, as a microplate reader is available in our laboratory, and this method is cost effective for the analysis of a large number of samples.

CHAPTER 4

OBSERVATIONS FROM THE REVIEW

4.1 OBSERVATIONS FROM THE REVIEW

In summary, it was observed that:

- Drug-induced liver injury has hampered the use of efficacious drugs such as isoniazid and nevirapine, and is a life threatening adverse effect after paracetamol overdose.
- Metabolic activation of a drug leads to formation of reactive metabolites.
- Reactive metabolites attack proteins and form antigenic metabolite-protein adducts, which stimulate the immune system to eliminate hepatocytes expressing these adducts.
- Paracetamol-induced liver injury is attenuated by specific immune suppressants, while chronic administration leads to tolerance to high doses, without toxicity.
- Prolonged nevirapine administration correlates with progressive IL-2 stimulation.
- Immune activation is associated with isoniazid-induced liver injury.
- There is limited information on the correlation of immune markers with one another and drug-induced liver injury.
- During liver injury the immune system is in a dynamic state in which some immune markers increase, while other decrease.
- A time profile response of the immune system is necessary to determine when the respective markers would be of use in predicting the extent of liver injury during prolonged administration of isoniazid, nevirapine and paracetamol.
- Understanding immunological changes during drug toxicity will be helpful in the development of immune-based preventative and curative strategies.

4.2 HYPOTHESIS

The CYP450 system creates reactive metabolites, which provoke an immune response, leading to isoniazid, nevirapine, and paracetamol-induced liver injury after prolonged administration.

4.3 SPECIFIC OBJECTIVES

- To develop an HPLC assay for the simultaneous analysis of isoniazid, nevirapine and paracetamol in plasma.
- To study the pattern of the immune response to prolonged drug therapy, and the trend of each immune marker during subclinical drug-induced liver injury.
- To determine the status of rat CYP1A2, CYP2E1 and CYP3A2 activity *in vivo* during isoniazid, nevirapine and paracetamol-induced liver injury.

CHAPTER 5

SIMULTANEOUS DETERMINATION OF ISONIAZID, NEVIRAPINE AND PARACETAMOL IN PLASMA BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

5.0 SUMMARY

A high performance liquid chromatography (HPLC) method for the simultaneous determination of isoniazid, nevirapine and paracetamol in plasma was developed. It involved protein precipitation of 100 µl plasma, spiked with isoniazid, nevirapine and paracetamol, with zinc sulphate and methanol, followed by centrifugation. The supernatant was purified by solid phase extraction on C_{18} cartridges, and 50 µl was injected into the HPLC. Thereafter, the sample was eluted with a gradient mobile phase system of 0.06% trifluoroacetic acid (A) and acetonitrile (B) over a C_{18} (4.60 x 250 mm) 5 µ analytical column at 1 ml/min and detected by UV at 260 nm. Sulphapyridine was used as the internal standard. Isoniazid, paracetamol, sulphapyridine and nevirapine eluted at retention times of 3.1, 9.9, 10.6 and 11.6 minutes, respectively. The average calibration curves of isoniazid (0 – 10 µg/ml), nevirapine (0 – 10 µg/ml), and paracetamol (0 – 20 µg/ml) were linear with regression equations of $y = 0.029x + 0.025$ ($r^2 = 0.9954$), $y = 0.043x + 0.127$ ($r^2 = 0.9968$), and $y = 0.097x + 0.070$ ($r^2 = 0.9997$), respectively. The method was used successfully in animal experiments to monitor isoniazid, nevirapine and paracetamol in the plasma of treated rats.

5.1 INTRODUCTION

In this chapter, the development and validation of a high performance liquid chromatography method for the simultaneous determination of isoniazid, nevirapine and paracetamol in plasma is described. Here, the selection of a mobile phase and internal standard, preparation of standard solutions, as well as the development of a sample preparation and extraction procedure, is discussed under method development. For the method validation section, calibration, accuracy and stability are described. Finally, successful application of the validated method is demonstrated.

5.2 METHODS

A. Materials

5.2.1 Apparatus

For weighing gram and milligram quantities of reagents and drug standards, a precision balance (SPB 52, Scaltec Instruments, Goettingen, Germany) and an analytical balance (AS 220/C/2, Radwag, Radom, Poland) were used, respectively. A vortex mixer (Vortex Genie 2, Scientific Industries Inc., Bohemia, NY, U.S.A) and a micro centrifuge (Minispin, Eppendorf, Hamburg, Germany) were used for mixing and spinning of the samples. Solid phase extraction was done with C_{18} cartridges (Sep-Pak®, Waters Corporation, Milford, MA, U.S.A) in a vacuum manifold (Visiprep-DL™, Bellefonte, PA, U.S.A).

5.2.2 Reagents and chemicals

All standards and chemicals used were of analytical grade. Carbamazepine, chlorpropamide, chlorzoxazone, debrisoquine, diclofenac, diphenylcarbazide, glacial acetic acid ($C_2H_4O_2$), isoniazid, nevirapine, omeprazole, paracetamol, phenacetin, phenytoin, propionamidophenyl acetate, sodium hydroxide (NaOH), theophylline, tolbutamide, and valproic acid were obtained from Sigma-Aldrich Inc. (St. Louis, MO, U.S.A). Orthophosphoric acid (H_3PO_4), perchloric acid ($HClO_4$), potassium dihydrogen phosphate (KH_2PO_4), tetraethylammoniumhydroxide ($C_8H_{21}NO$), 70% trichloroacetic acid ($C_2HCl_3O_2$), and trifluoroacetic acid ($C_2HF_3O_2$) were purchased from Merck Laboratories (Darmstadt, Germany). HPLC grade acetonitrile (C_2H_3N), diethyl ether ($(C_2H_5)_2O$), and methanol (CH_4O) were from Honeywell Burdick and Jackson (Muskegon, MI, U.S.A). BDH Lab Supplies (Poole, England, U.K) provided zinc sulphate ($ZnSO_4$), while ammonia solution (NH_4OH) was from NT Laboratory Supplies Pty Ltd. (Johannesburg, South Africa). The following analytical standards were from the Department of Pharmacology, Toxicology Laboratory reference substances: bezafibrate, furosemide, hydrochlorothiazide, mafenide, mefenamic acid, phenolphthalein, ranitidine, salicylic acid, sulphadoxine, sulphafurazole, sulphamoxole, sulphapyridine, sulphasomidine, and tolmetin. Fresh plasma was obtained from healthy volunteers after informed consent.

5.2.3 Chromatographic system

The HPLC system was an Agilent, Hewlett Packard 1100 Series, equipped with a 1260 Infinity quaternary pump (Waldbronn, Germany) with a 1260 Infinity degasser attached to a G1313A autosampler (Waldbronn, Germany), and a G1314A UV wavelength detector (Tokyo, Japan). Data were collected using ChemStation software.

5.3 PRELIMINARY EXPERIMENTS

5.3.1 Selection of a mobile phase

Aim: To select a mobile phase and gradient conditions by which to elute and lead to optimum separation of the four compounds using a given analytical column.

A mobile phase of tetraethylammoniumphosphate (TEAP) buffer (solvent A) and acetonitrile (solvent B) was tried using different gradients of solvent A and B, but with no success, as isoniazid peaks remained unresolved.

In a similar way, a mobile phase of 0.05 M ammonium acetate buffer (solvent A) and pure acetonitrile (solvent B; Aït Moussa *et al.*, 2001) did not yield good results, as nevirapine eluted very late and the mobile phase had to be altered so much during the run that it led to inconsistent results. As such, the above-mentioned method could not be adopted successfully, and it was decided to move on to another method.

Thereafter, a mobile phase of 0.06% trifluoroacetic acid (solvent A) and pure acetonitrile (solvent B) was tried (Smith *et al.*, 1999) with different gradients. Eventually, isoniazid, nevirapine, paracetamol and sulphapyridine were separated, and all peaks were sharp and well resolved. Therefore this mobile phase was selected for further evaluation in the subsequent experiments.

5.3.2 Preparation of standard solutions

Aim: To select an appropriate solvent in which all four compounds can dissolve.

It is well known that isoniazid is a hydrophilic drug, nevirapine is a weak base that is highly lipophilic, while paracetamol is a lipophilic weak acid.

First, a 1 mg/ml stock solution of each drug was prepared in methanol, and this was further diluted to 100 µg/ml with methanol. This led to very broad and unresolved peaks. It was decided to prepare each drug standard's stock solution (1 mg/ml) in methanol, after which it was diluted with mobile phase (97% solvent A and 3% solvent B) to working solutions as follows: 100 µg/ml isoniazid, 100 µg/ml nevirapine, and 200 µg/ml paracetamol.

5.3.3 Selection of an internal standard

Aim: To select an appropriate drug or chemical to standardise the extraction and HPLC analytical conditions.

The selection of a suitable internal standard for this method was challenging, as the drug had to be stable, not be regularly prescribed and should not interact or interfere with the elution of isoniazid, nevirapine and paracetamol. Therefore the following drugs were tested: bezafibrate, carbamazepine, chlorpropamide, chlorzoxazone, debrisoquine, diclofenac, diphenylcarbazide, furosemide, hydrochlorothiazide, mafenide, mefenamic acid, omeprazole, phenacetin, phenolphthalein, phenytoin, propionamidophenyl acetate, ranitidine, salicylic acid, sulphadoxine, sulphafurazole, sulphamoxole, sulphapyridine, sulphasomidine, theophylline, tolbutamide, tolmetin, and valproic acid. Finally, sulphapyridine was selected as the appropriate internal standard, because it dissolved well in methanol, was easily diluted with mobile phase and showed no interference with any of the three drugs (Figure 5.1 c).

5.3.4 Sample preparation and extraction

Aim: To select the best extraction method by which to obtain acceptable peaks of all the four compounds, isoniazid, nevirapine, paracetamol and internal standard.

5.3.4.1 Protein precipitation with perchloric acid and solid phase extraction

Here, perchloric acid was used for protein precipitation of plasma, and the supernatant purified by solid phase extraction (SPE). Thus, to plasma spiked with isoniazid, nevirapine and paracetamol, 60 µl of 2 M (70%) perchloric acid was

added, the sample was vortexed for 30 seconds and centrifuged for 7 minutes at 7026 *g* (13,400 r.p.m). C_{18} SPE cartridges (1 ml) were conditioned with 2 ml deionised water, followed by 2 ml methanol. The supernatant was placed on the column to elute, and washed with 500 µl of deionised water. Finally, 200 µl of an acetonitrile:water (80:20, v/v) solution was used to elute the compounds, and 40 µl was injected into the HPLC. Samples were analysed with the TEAP buffer:acetonitrile mobile phase, described in Section 5.3.1. Chromatographically, the peaks of paracetamol, nevirapine and sulphapyridine could be distinguished. However, for isoniazid there was interference from plasma peaks, and the compound could not be detected. It was decided to try a liquid-liquid extraction of the sample, as described below.

5.3.4.2 Liquid-liquid extraction

Two basic liquid-liquid extractions using sodium hydroxide and acetonitrile, and sodium hydroxide and diethyl ether were tried. In both extractions there were interfering peaks, and the isoniazid peak was not visible. Thereafter, an acidic liquid-liquid extraction using perchloric acid and diethyl ether was tried, but with no success. As a result, the liquid-liquid extraction was abandoned.

5.3.4.3 Extraction by centrifugation with trichloroacetic acid

To a spiked plasma sample, 50 µl of 10% trichloroacetic acid (Aït Moussa *et al.*, 2001) was added for protein precipitation, the sample vortexed for 30 seconds and centrifuged for 10 minutes at 7026 *g* (13,400 r.p.m). The supernatant was transferred to a fresh test tube, to which 50 µl of ammonium acetate was added in order to neutralise any excess trichloroacetic acid, and 50 µl was injected into the HPLC. A mobile phase of 0.05 M ammonium acetate buffer and acetonitrile, as described in Section 5.3.1, was used. Poor results were obtained as the peaks were broad and plasma peaks interfered with the compounds of interest.

5.3.4.4 Protein precipitation with zinc sulphate and methanol, followed by solid phase extraction

This was based on a method reported by Smith and colleagues (1999). A mobile phase of 0.06% trifluoroacetic acid:acetonitrile was used as discussed in Section 5.3.1. In the original method, the C_{18} SPE cartridges were conditioned with 2 ml

methanol, followed by 2 ml deionised water and 2 ml potassium phosphate buffer (pH 4.5), after which the plasma sample (unextracted) was placed on the column to elute. The column was washed with 500 µl of potassium phosphate buffer, and to a fresh test tube, the compounds were eluted with 100 µl acetonitrile followed by 100 µl methanol, and 50 µl of the filtered eluent was injected into the HPLC.

Unfortunately, although all peaks separated well, the chromatogram lacked sensitivity, but most importantly, the use of unextracted plasma posed a danger to the lifetime of the SPE cartridges and the analytical column.

Therefore it was decided to first precipitate the proteins with 10% trichloroacetic acid before solid phase extraction. This produced a cleaner chromatogram, but the peak of isoniazid was very small, implying that isoniazid did extract into the supernatant under acidic conditions. Thereafter methanol was tried as protein precipitant. Again, all peaks eluted, but that of isoniazid remained very small.

The possibility of isoniazid being lost during the washing step of the solid phase extraction was considered, as isoniazid is a hydrophilic molecule. Thus, the washing step was reduced to 300 µl and 200 µl, respectively. With the 300 µl wash still too much isoniazid was lost, but the peaks of paracetamol and nevirapine looked good. On the other hand, with the 200 µl wash, the isoniazid peak appeared larger, but the peaks of paracetamol and nevirapine lost sensitivity. It was concluded that the washing step could not be solely blamed for the loss of isoniazid, and that maybe methanol should not be used as the only protein precipitant. Regarding the hydrophilic properties of isoniazid, the addition of an aqueous protein precipitant was considered.

Here, 15% zinc sulphate was used as the only protein precipitant, and the washing step was again tested at 300 µl and 200 µl, respectively. The 200 µl wash produced a clean chromatogram and a sensitive isoniazid peak, but nevirapine struggled to extract under the aqueous conditions.

Finally, it was decided to use zinc sulphate followed by methanol for protein precipitation during sample preparation to accommodate the extraction of both

isoniazid and nevirapine. It was also decided to reduce the washing step to 100 µl, as this would further minimize isoniazid loss during solid phase extraction. Therefore, these conditions were set as final for purification of plasma samples after protein precipitation, and proved to be reproducible.

5.4 FINAL CONDITIONS

5.4.1 Sample preparation

Standard plasma calibration samples were prepared by spiking 1 ml of plasma with appropriate volumes of isoniazid, nevirapine and paracetamol standard solutions, respectively, to obtain final concentrations as follows:

- isoniazid: 1, 2, 4, 6 and 10 µg/ml
- nevirapine: 1, 3, 5, 8 and 10 µg/ml, and
- paracetamol: 1, 5, 10, 15 and 20 µg/ml

To 100 µl of the spiked plasma sample, 20 µl of internal standard was added, and the sample was vortexed for 15 seconds. Then, 50 µl of 15% zinc sulphate was added, followed by 50 µl methanol to precipitate the proteins. The sample was then vortexed for 30 seconds, and centrifuged at 7026 *g* (13,400 r.p.m) for 15 minutes, after which the supernatant was further purified by solid phase extraction as described below.

5.4.2 Solid phase extraction

Solvents and samples were eluted over the C_{18} SPE (1 ml) cartridges assembled in a vacuum manifold using a vacuum created by running tap water. The cartridges were conditioned with 2 ml HPLC grade methanol followed by 2 ml deionised water, and 2 ml 0.05 M potassium phosphate buffer (pH 4.5). The sample (supernatant from Section 5.4.1) was placed on the column and allowed to elute for 30 seconds. Thereafter, the column was washed with 100 µl of potassium phosphate buffer. Finally, to a fresh test tube, the compounds were eluted with 100 µl HPLC grade acetonitrile, followed by 100 µl methanol and 50 µl was injected into the HPLC for analysis.

5.4.3 Chromatographic conditions

Chromatographic separation of isoniazid, nevirapine, paracetamol and sulphapyridine was achieved by running the mobile phase at a flow rate of 1 ml/min. over a Phenomenex® Luna C_{18} (4.60 x 250 mm) 5 micron analytical column, coupled to a Phenomenex® SecurityGuard™ C_{18} (4 x 3 mm) guard column (Torrance, CA, U.S.A). Compounds were detected by UV at a wavelength of 260 nm.

The mobile phase consisted of 0.06% trifluoroacetic acid in distilled water (solvent A) and 100% HPLC grade acetonitrile (solvent B) run by a gradient programmer. For gradient separation, the proportion of solvent A and B was initially 97:3 for 3 minutes. This was changed to 60:40 over 5 minutes, and finally maintained for 5 minutes. For re-equilibration purposes a post-run of 2 minutes was performed at the initial ratio of 97:3. In total, the final run time of the method was 15 minutes.

5.5 METHOD VALIDATION

The validation of an analytical method aims to demonstrate that the method measures the test substance accurately and consistently. It also helps the analyst to understand the behaviour of the method, and to establish limits of the method (Singh, 2013). The results from method validation can be implemented to evaluate the quality, reliability and consistency of results. Various validation parameters include: calibration/linearity, accuracy, precision, ruggedness, robustness, limit of detection, limit of quantitation, selectivity/specificity, and stability. For purposes of this study, only calibration/linearity, accuracy and stability were determined.

5.5.1 Calibration/linearity

A linear relationship should be evaluated across the range of the analytical procedure. Calibration, or linearity, of an HPLC method refers to its ability to obtain results that are directly proportional to the concentration, or amount of analyte in the sample. Linearity is determined by a series of five to six injections of five or more calibration standards. The response, determined from peak area, of the calibration standards should be directly proportional to their concentrations.

For evaluation purposes, a calibration curve is constructed by plotting the calibration concentration range on the x-axis, versus response on the y-axis. Acceptability of

linearity data is dependent on the correlation coefficient (r^2) and y-intercept of the regression equation (y = mx + c) for the plot. A r^2 value of greater than 0.998 is considered acceptable, and the y-intercept should not be significantly different from 0 (Shabir, 2004).

Here, calibration was performed by analysing plasma samples spiked with isoniazid, nevirapine and paracetamol at the following concentration ranges: 1, 2, 4, 6 and 10 µg/ml isoniazid, 1, 3, 5, 8 and 10 µg/ml nevirapine, and 1, 5, 10, 15 and 20 µg/ml paracetamol, on different days for 5 days. Calibration curves were created by plotting the peak area ratio of isoniazid, nevirapine, and paracetamol, respectively, to sulphapyridine, versus the spiked concentrations of isoniazid, nevirapine, and paracetamol. The respective curves were analysed by linear regression using the GraphPad® Instat statistical program.

5.5.2 Accuracy

Accuracy of an analytical method can be defined as the closeness of agreement between the conventional true value (accepted reference value) and the value found. It is a qualitative characteristic, and has an inverse relation to errors, where higher accuracy means lower errors (Singh, 2013).

Accuracy is determined by analysing a sample with known concentration, *i.e.*, blank sample (plasma) spiked with a known concentration of a substance, and comparing the measured value with the true (known) value. Since accuracy is also a measurement of the effectiveness of sample preparation, care should be taken to mimic the sample preparation for each sample as closely as possible. It is recommended that three different concentrations be selected from the calibration concentration range, and that these samples should be prepared in triplicate. Usually, nine determinations, or runs of the specified samples are performed (Singh, 2013), however a number of five runs are still statistically significant. Accuracy should be reported as percentage recovery.

For this study, accuracy was tested at 1, 4 and 10 µg/ml for isoniazid, 1, 5 and 10 µg/ml for nevirapine, and 1, 10 and 20 µg/ml for paracetamol. The test was repeated five times for each sample, after which accuracy values were derived from a

calibration curve. The results obtained were used to calculate the coefficient of variation using the following formula: (standard deviation/mean) x 100.

5.5.3 Stability

Chemical compounds under investigation can decompose during storage, prior to HPLC analysis. Therefore, the stability of the compounds of interest should be determined. Stability is a measure of the bias in assay results generated during a preselected time interval, and is important for estimating the permissible time span between sample collection and sample analysis. Experiments should be conducted under real sample storage conditions, such as temperature and time intervals of storage. Quantitation of components should be determined by comparing the stability samples to freshly prepared standards. Stability is considered acceptable when the change in the standard or sample response is within 2% relative to freshly prepared standards (Shabir, 2004).

Here, stability of the three drugs in plasma was determined at low and high concentrations. The low concentration sample contained 4 µg/ml isoniazid, 5 µg/ml nevirapine and 10 µg/ml paracetamol, while the high concentration sample contained 10 µg/ml isoniazid, 10 µg/ml nevirapine and 20 µg/ml paracetamol. Both the low and high concentration samples were stored at room temperature, 4ºC and -20ºC and analysed after 8, 12 and 24 hours (short-term stability), and 7, 30 and 60 days (long-term stability).

5.5.4 Application of the validated method

The method was tested by analysing plasma samples of rats, after oral administration of isoniazid, nevirapine, and paracetamol, respectively. All details on the animal study and procedures are described in Chapter 6.

5.6 RESULTS

5.6.1 Chromatographic performance

Figures 5.1 a – e are the representative chromatograms for the standard solutions, blank plasma and spiked plasma. From the standard solutions it was observed that the peaks were well resolved, with the drugs eluting at the following retention times (in minutes): isoniazid, 3.1; paracetamol, 9.8; sulphapyridine (internal standard), 10.4; and nevirapine, 11.6. The blank plasma showed no interference from plasma. This observation was also evident in the spiked plasma samples. Retention times for plasma spiked with isoniazid, paracetamol, internal standard and nevirapine were 3.1, 9.8, 10.4 and 11.6 minutes, respectively. The total run time was 15 minutes.

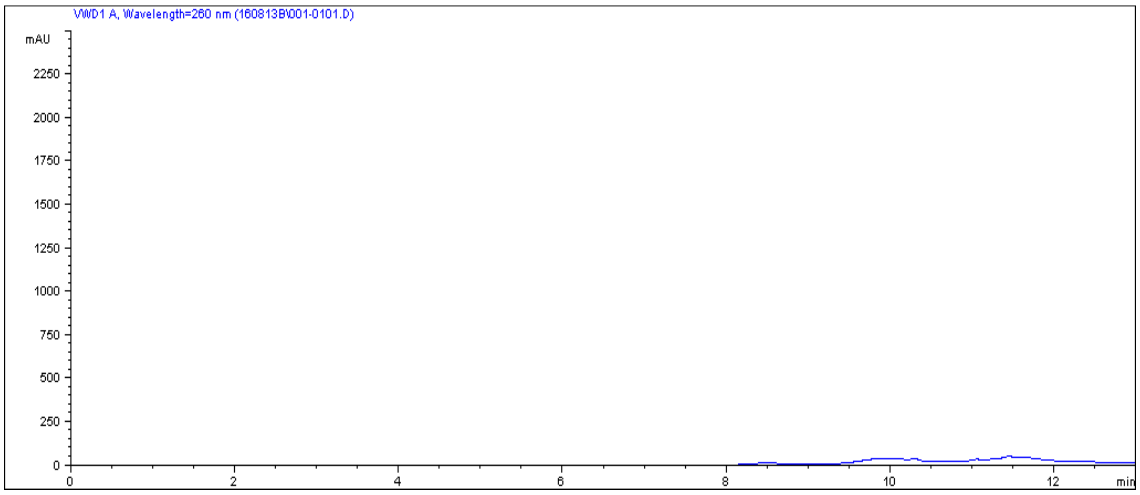

Figure 5.1 a): Chromatogram of mobile phase alone

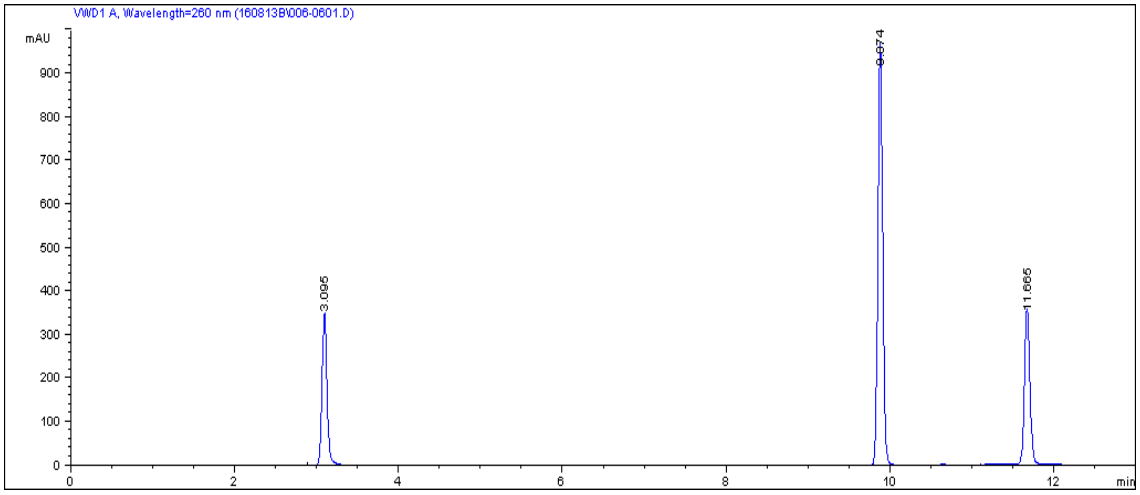

Figure 5.1 b): Chromatogram of mobile phase spiked with isoniazid, paracetamol and nevirapine

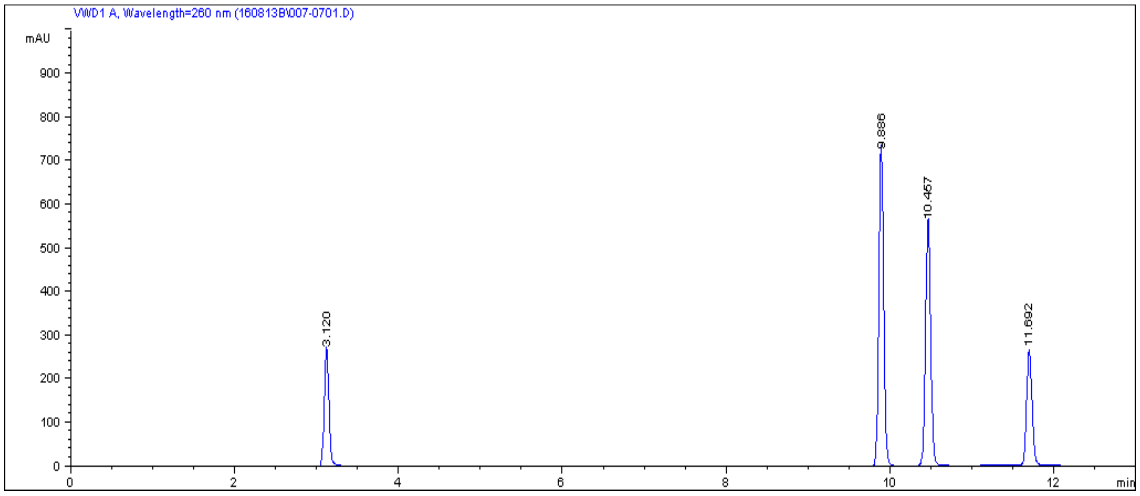

Figure 5.1 c): Chromatogram of mobile phase spiked with isoniazid, paracetamol, nevirapine and internal standard

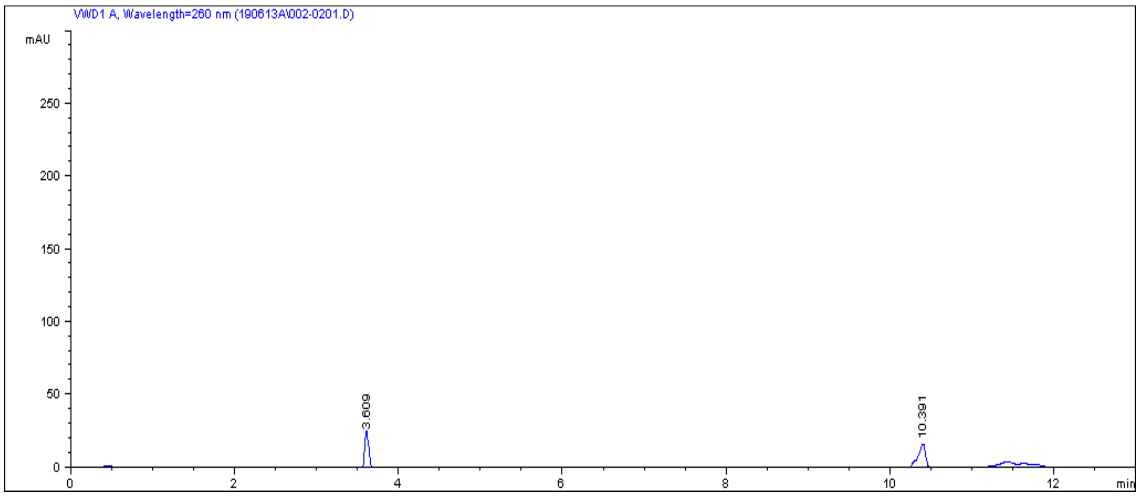

Figure 5.1 d): Chromatogram of a blank plasma sample

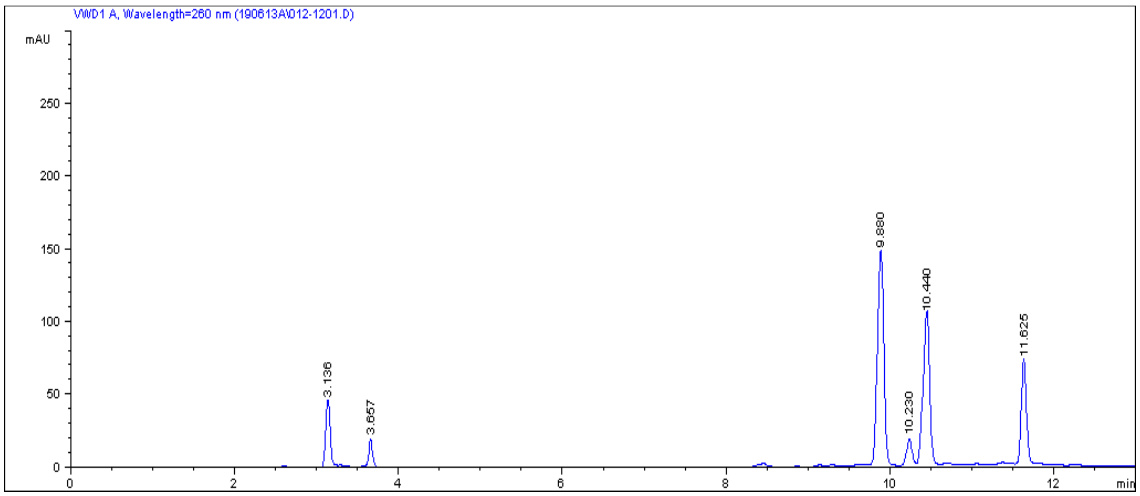

Figure 5.1 e): Chromatogram of a plasma sample spiked with internal standard, 10 µg/ml isoniazid, 20 µg/ml paracetamol and 10 µg/ml nevirapine

A. Standardisation of isoniazid assay

5.6.2 Calibration

The summary data for the isoniazid calibration over five days is shown in Table 5.1, while the average calibration curve is shown in Figure 5.2 (see Appendix A for individual calibrations). The results showed that the calibration curve was linear with a regression equation of y = 0.029x + 0.025 and correlation coefficient (r^2) of 0.9954, while the coefficient of variation (CV %) was less than 20%.

Table 5.1: HPLC calibrations for isoniazid using ratio isoniazid/ratio internal standard

Conc. (µg/ml)	Cal. Day 1	Cal. Day 2	Cal. Day 3	Cal. Day 4	Cal. Day 5	Mean	SD	CV (%)
1	0.051	0.044	0.066	0.041	0.048	**0.050**	0.01	19.4
2	0.070	0.062	0.100	0.078	0.082	**0.078**	0.01	18.3
4	0.187	0.127	0.156	0.157	0.136	**0.153**	0.02	15.2
6	0.226	0.148	0.203	0.250	0.204	**0.206**	0.04	18.3
10	0.330	0.248	0.334	0.355	0.302	**0.314**	0.04	13.2

Conc. = concentration; Cal. = calibration; SD = standard deviation; CV = coefficient of variation

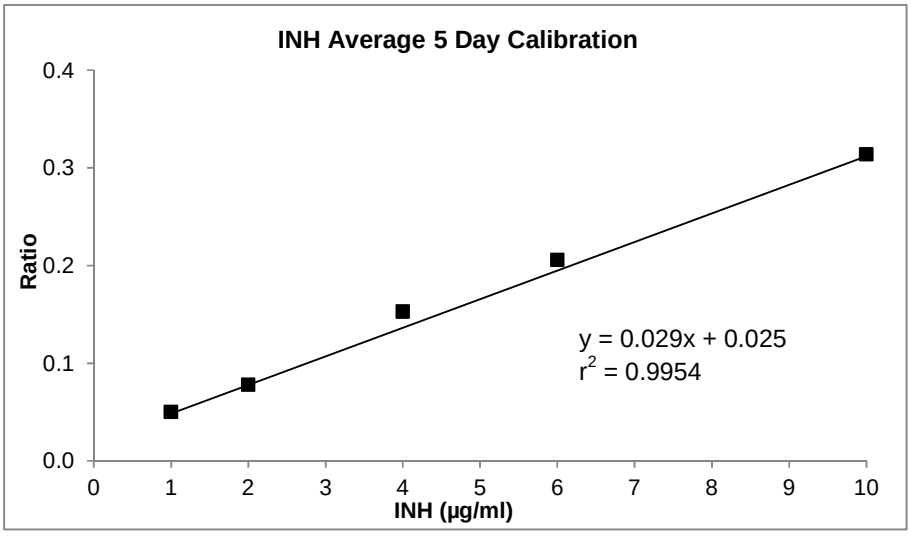

Figure 5.2: Average 5 day calibration curve of isoniazid

5.6.3 Accuracy

According to the data in Table 5.2, accuracy was 102%, 98% and 101% at 1, 4 and 10 µg/ml, respectively (see appendix B for detailed accuracy results). The CV % was less than 10% for all samples.

Table 5.2: Summary of accuracy data of isoniazid in plasma at 1, 4 and 10 µg/ml

Conc. prepared (µg/ml)	Conc. measured (µg/ml)	Mean accuracy (%)	SD	CV (%)
1	1.017	**102**	0.04	4.1
4	3.905	**98**	0.30	7.7
10	10.120	**101**	0.31	3.0

Conc. = concentration; SD = standard deviation; CV = coefficient of variation

5.6.4 Stability

5.6.4.1 Short-term stability

A variation in isoniazid stability was observed over 24 hours at both low (4 µg/ml) and high (10 µg/ml) concentrations (Table 5.3). It would be advisable to freeze samples immediately after blood collection, and to analyse these samples within 1 – 2 days (refer to Appendix C-1 – C-6 for detailed stability results).

5.6.4.2 Long-term stability

In view of the long-term stability of isoniazid, it remained unstable over 60 days at both low (4 µg/ml) and high (10 µg/ml) concentrations (Table 5.3). At both concentrations, isoniazid showed a marked decay over 60 days, hereby further emphasising the importance of freezing samples immediately after blood collection. It is not recommended to store the samples for longer than 1 – 2 days, as samples have to be analysed as soon as possible (see Appendix C-7 – C-8 for detailed stability results).

Table 5.3: Summary of short- and long-term stability data of 4 and 10 µg/ml isoniazid in plasma at ambient temperature, 4ºC and -20ºC measured after 0, 8, 12 and 24 hours and 7, 30 and 60 days

Temp.	0 Hours		8 Hours		12 Hours		24 Hours		7 Days		30 Days		60 Days	
	Conc. measured	Stab. (%)	Conc. measured	Stab. (%)	Conc. measured	Stab. (%)	Conc. measured	Stab. (%)	Conc. measured	Stab. (%)	Conc. measured	Stab. (%)	Conc. measured	Stab. (%)
4 µg/ml														
Ambient	4.609	**115**	2.446	**61**	2.122	**53**	2.347	**59**	**-**	**-**	**-**	**-**	**-**	**-**
4℃	-	-	2.825	**71**	1.854	**46**	2.223	**56**	**-**	**-**	**-**	**-**	**-**	**-**
-20℃	-	-	1.924	**48**	1.307	**33**	2.811	**70**	2.059	**51**	0.285	**7**	0.869	**22**
10 µg/ml														
Ambient	8.863	**89**	7.463	**75**	7.799	**78**	9.415	**94**	**-**	**-**	**-**	**-**	**-**	**-**
4℃	-	-	6.803	**68**	8.244	**82**	8.082	**81**	**-**	**-**	**-**	**-**	**-**	**-**
-20℃	-	-	7.584	**76**	8.432	**84**	9.576	**96**	2.895	**29**	2.507	**25**	4.866	**49**

Temp. = temperature; Conc. = concentration; Stab. = stability

B. Standardisation of nevirapine assay

5.6.5 Calibration

The summary data for the nevirapine calibration over five days is shown in Table 5.4, while the average calibration curve is shown in Figure 5.3 (see Appendix A for individual calibrations). The results indicated that the calibration curve was linear with a regression equation of y = 0.043x + 0.127 and correlation coefficient (r^2) of 0.9968, while the CV % was less than 20%.

Table 5.4: HPLC calibrations for nevirapine using area nevirapine/area internal standard

Conc. (µg/ml)	Cal. Day 1	Cal. Day 2	Cal. Day 3	Cal. Day 4	Cal. Day 5	Mean	SD	CV (%)
1	0.170	0.142	0.164	0.177	0.144	**0.159**	0.02	9.8
3	0.254	0.262	0.257	0.282	0.292	**0.269**	0.02	6.2
5	0.324	0.326	0.366	0.340	0.361	**0.343**	0.02	5.7
8	0.420	0.465	0.523	0.393	0.539	**0.468**	0.06	13.5
10	0.484	0.524	0.626	0.465	0.668	**0.553**	0.09	16.1

Conc. = concentration; Cal. = calibration; SD = standard deviation; CV = coefficient of variation

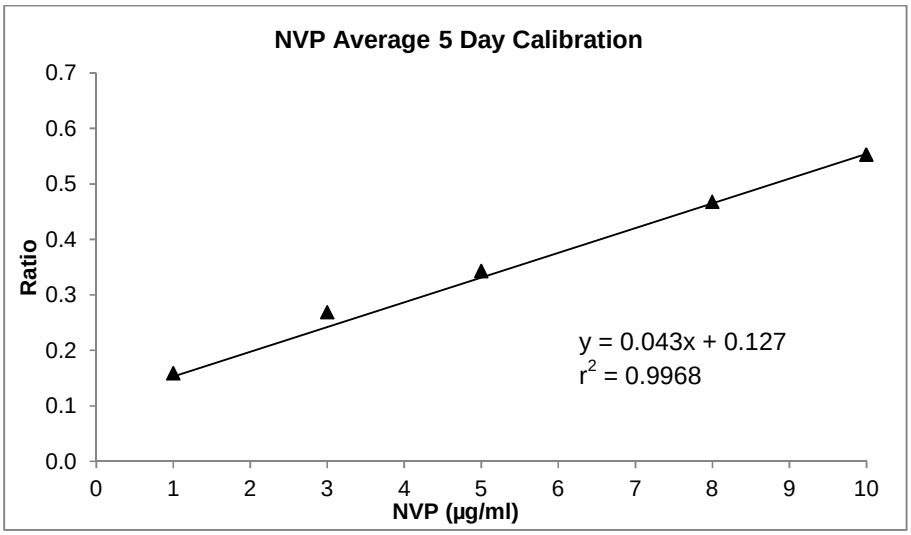

Figure 5.3: Average 5 day calibration curve of nevirapine

5.6.6 Accuracy

According to the data in Table 5.5, accuracy was 94%, 96% and 100% at 1, 5 and 10 µg/ml, respectively (see appendix B for detailed accuracy results). The CV % was less than 15% for the 5 and 10 µg/ml samples, while for the 1 µg/ml sample it was higher, owing to the small volume of nevirapine in the sample.

Table 5.5: Summary of accuracy data of nevirapine in plasma at 1, 5 and 10 µg/ml

Conc. prepared (µg/ml)	Conc. measured (µg/ml)	Mean accuracy (%)	SD	CV (%)
1	0.942	**94**	0.14	15.2
5	4.807	**96**	0.26	5.5
10	10.042	**100**	0.32	3.2

Conc. = concentration; SD = standard deviation; CV = coefficient of variation

5.6.7 Stability

5.6.7.1 Short-term stability

Nevirapine proved to be more stable at a high concentration (10 µg/ml) over 24 hours when compared to a low concentration (5 µg/ml; Table 5.6). Although nevirapine was stable in the short-term, it is recommended to freeze samples after blood collection (See Appendix C-9 – C-14 for detailed stability results).

5.6.7.2 Long-term stability

Nevirapine was more stable at a high concentration (10 µg/ml) over 60 days in comparison to the low concentration (5 µg/ml; Table 5.6). It is advisable to freeze samples after blood collection and to store at -20ºC until analysis (Refer to Appendix C-15 – C-16 for detailed stability results).

Table 5.6: Summary of short- and long-term stability data of 5 and 10 µg/ml nevirapine in plasma at ambient temperature, 4ºC and -20ºC measured after 0, 8, 12 and 24 hours and 7, 30 and 60 days

Temp.	0 Hours		8 Hours		12 Hours		24 Hours		7 Days		30 Days		60 Days	
	Conc. measured	Stab. (%)	Conc. measured	Stab. (%)	Conc. measured	Stab. (%)	Conc. measured	Stab. (%)	Conc. measured	Stab. (%)	Conc. measured	Stab. (%)	Conc. measured	Stab. (%)
5 µg/ml														
Ambient	4.791	**96**	2.555	**51**	3.577	**72**	2.958	**59**	**-**	**-**	**-**	**-**	**-**	**-**
4℃	-	-	5.444	**109**	4.609	**92**	3.577	**72**	**-**	**-**	**-**	**-**	**-**	**-**
-20℃	-	-	5.251	**105**	3.764	**75**	4.465	**89**	4.418	**88**	2.247	**45**	3.814	**76**
10 µg/ml														
Ambient	9.629	**96**	8.905	**89**	10.386	**104**	10.024	**100**	**-**	**-**	**-**	**-**	**-**	**-**
4℃	-	-	8.685	**87**	10.199	**102**	9.640	**96**	**-**	**-**	**-**	**-**	**-**	**-**
-20℃	-	-	8.751	**88**	11.132	**111**	10.046	**100**	8.100	**81**	9.360	**94**	7.641	**76**

Temp. = temperature; Conc. = concentration; Stab. = stability

C. Standardisation of paracetamol assay

5.6.8 Calibration

The summary data for the paracetamol calibration over five days is shown in Table 5.7, while the average calibration curve is shown in Figure 5.4 (see Appendix A for individual calibrations). The results showed that the calibration curve was linear with a regression equation of y = 0.097x + 0.070 and correlation coefficient (r^2) of 0.9997, while the CV % was less than 15%.

Table 5.7: HPLC calibrations for paracetamol using area paracetamol/area internal standard

Conc. (µg/ml)	Cal. Day 1	Cal. Day 2	Cal. Day 3	Cal. Day 4	Cal. Day 5	Mean	SD	CV (%)
1	0.167	0.165	0.163	0.151	0.124	**0.154**	0.02	11.6
5	0.539	0.532	0.542	0.660	0.529	**0.560**	0.06	10.0
10	1.139	0.909	1.039	1.207	1.000	**1.059**	0.12	11.0
15	1.338	1.411	1.573	1.688	1.575	**1.517**	0.14	9.3
20	2.034	1.846	2.012	2.283	1.842	**2.003**	0.18	9.0

Conc. = concentration; Cal. = calibration; SD = standard deviation; CV = coefficient of variation

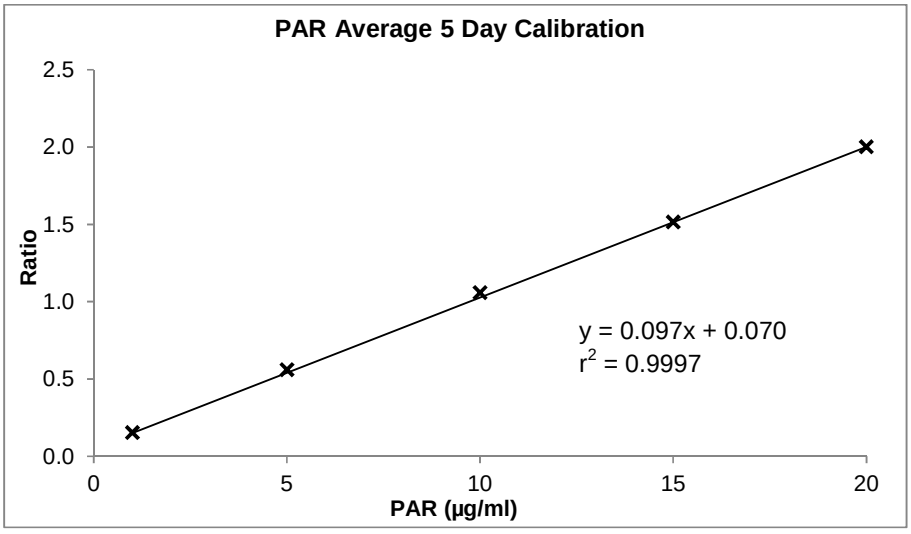

Figure 5.4: Average 5 day calibration curve of paracetamol

5.6.9 Accuracy

According to the data in Table 5.8, accuracy was 99%, 97% and 99% at 1, 10 and 20 µg/ml, respectively (see appendix B for detailed accuracy results). The CV % was less than 10% for the 10 and 20 µg/ml samples, while for the 1 µg/ml sample it was slightly higher, owing to the small volume of paracetamol in the sample.

Table 5.8: Summary of accuracy data of paracetamol in plasma at 1, 10 and 20 µg/ml

Conc. prepared (µg/ml)	Conc. measured (µg/ml)	Mean accuracy (%)	SD	CV (%)
1	0.989	**99**	0.11	11.0
10	9.686	**97**	0.73	7.5
20	19.742	**99**	1.14	5.8

Conc. = concentration; SD = standard deviation; CV = coefficient of variation

5.6.10 Stability

5.6.10.1 Short-term stability

Paracetamol was very stable at both low (10 µg/ml) and high (20 µg/ml) concentrations, and at all temperatures (Table 5.9). Although paracetamol is stable in the short-term, it is still recommended to freeze samples after blood collection (Refer to Appendix C-17 – C-22 for detailed stability results).

5.6.10.2 Long-term stability

Paracetamol exhibited good stability over 60 days at both low (10 µg/ml) and high (20 µg/ml) concentrations (Table 5.9). It is advisable to freeze samples after blood collection and to store at -20ºC until analysis (See Appendix C-23 – C-24 for detailed stability results).

Table 5.9: Summary of short- and long-term stability data of 10 and 20 µg/ml paracetamol in plasma at ambient temperature, 4ºC and -20ºC measured after 0, 8, 12 and 24 hours and 7, 30 and 60 days

Temp.	0 Hours		8 Hours		12 Hours		24 Hours		7 Days		30 Days		60 Days	
	Conc. measured	Stab. (%)	Conc. measured	Stab. (%)	Conc. measured	Stab. (%)	Conc. measured	Stab. (%)	Conc. measured	Stab. (%)	Conc. measured	Stab. (%)	Conc. measured	Stab. (%)
10 µg/ml														
Ambient	10.581	**106**	10.007	**100**	10.232	**102**	10.190	**102**	**-**	**-**	**-**	**-**	**-**	**-**
4℃	-	-	10.136	**101**	9.950	**99**	11.814	**118**	**-**	**-**	**-**	**-**	**-**	**-**
-20℃	-	-	10.292	**103**	11.166	**112**	10.898	**109**	10.315	**103**	12.261	**123**	6.573	**66**
20 µg/ml														
Ambient	19.925	**100**	17.226	**86**	18.820	**94**	17.941	**90**	**-**	**-**	**-**	**-**	**-**	**-**
4℃	-	-	15.688	**78**	19.324	**97**	17.757	**89**	**-**	**-**	**-**	**-**	**-**	**-**
-20℃	-	-	16.130	**81**	19.195	**96**	18.380	**92**	17.942	**90**	13.821	**69**	8.545	**43**

Temp. = temperature; Conc. = concentration; Stab. = stability

5.6.11 Application of the method

Figure 5.5 a illustrates a chromatogram of blank rat plasma, while Figures 5.5 b – d show the chromatograms of isoniazid, nevirapine, and paracetamol in rat plasma, respectively. Isoniazid was administered orally at a dosage of 20 mg/kg/day, nevirapine at 200 mg/kg/day, and paracetamol at 500 mg/kg/day, respectively, over a 42 day period. In Figure 5.5 b isoniazid concentration was calculated as 6.035 µg/ml after 7 days. Nevirapine concentration was calculated as 12.849 µg/ml after 7 days, while paracetamol concentration after 28 days was calculated as 0.963 µg/ml (Figures 5.5 c and d).

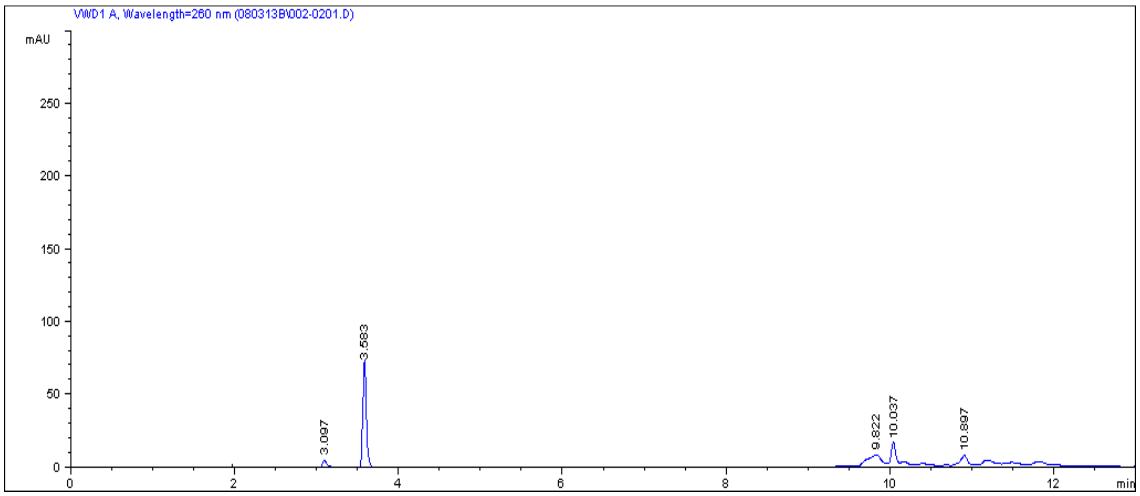

Figure 5.5 a): Chromatogram of blank rat plasma

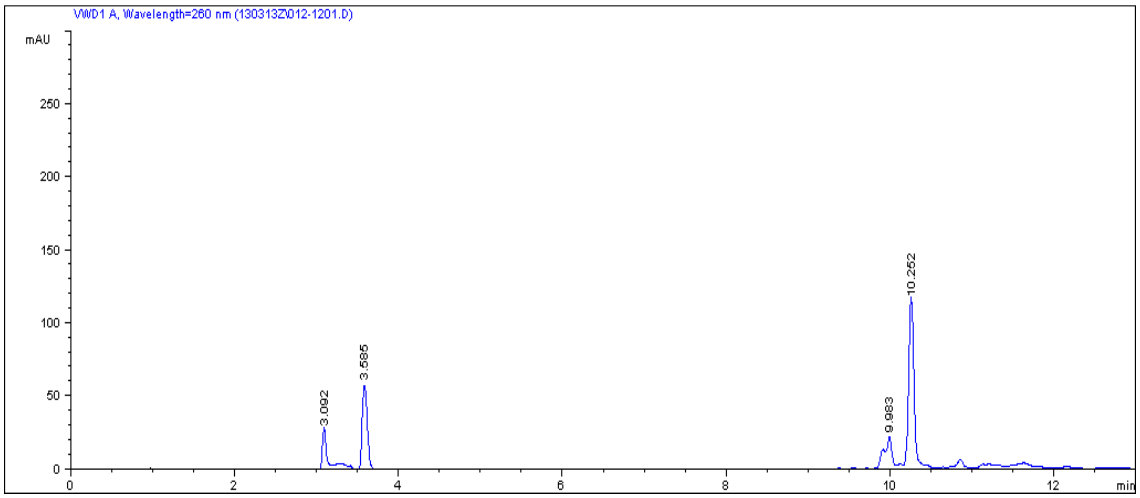

Figure 5.5 b): Chromatogram of isoniazid (6.035 µg/ml) in rat plasma after 7 days of 20 mg/kg/day oral administration

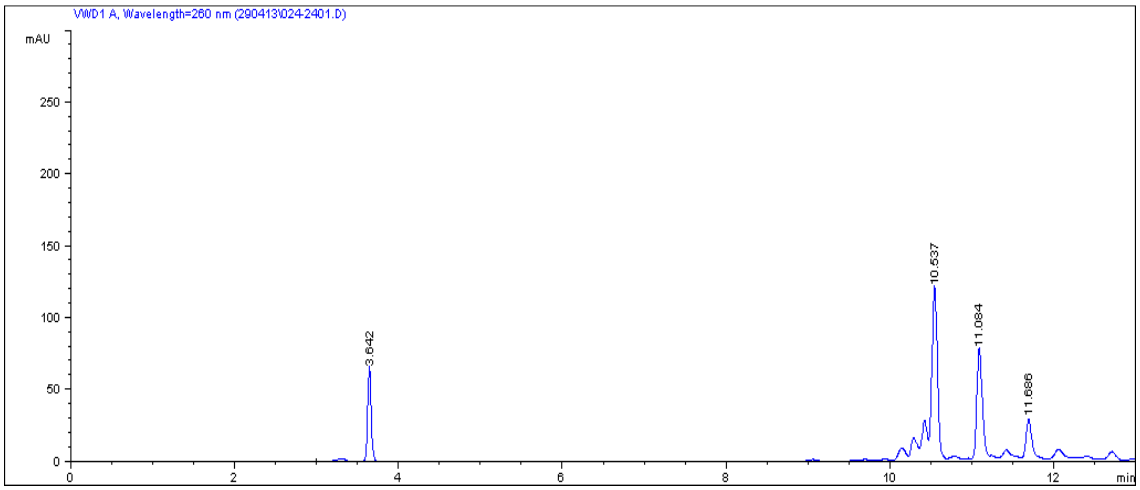

Figure 5.5 c): Chromatogram of nevirapine (12.849 µg/ml) in rat plasma after 7 days of 200 mg/kg/day oral administration

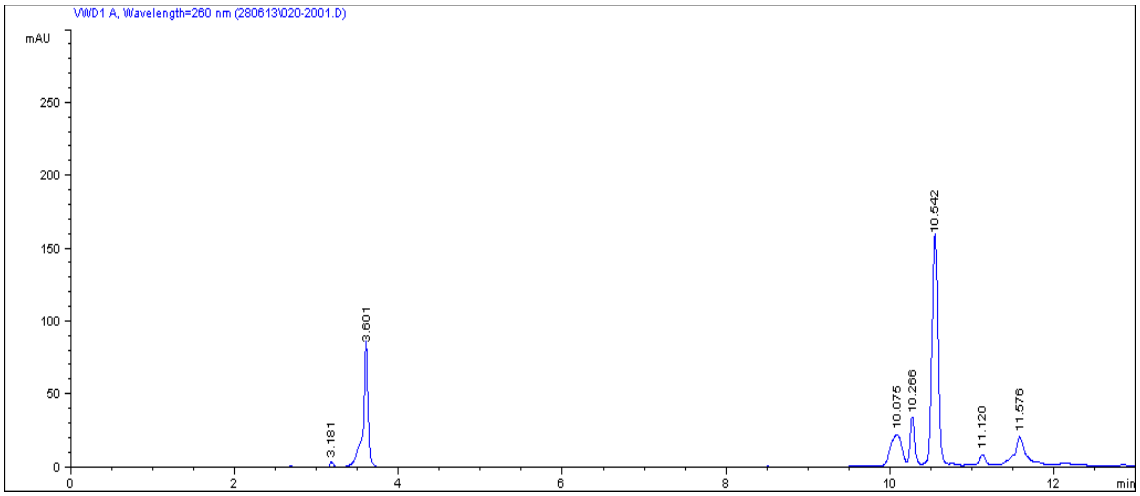

Figure 5.5 d): Chromatogram of paracetamol (0.963 µg/ml) in rat plasma after 28 days of 500 mg/kg/day oral administration

5.7 DISCUSSION

A robust and accurate HPLC method for simultaneous determination of isoniazid, nevirapine and paracetamol in plasma was successfully developed. Sharp, symmetrical peaks of isoniazid, nevirapine, paracetamol and the internal standard were observed in the chromatograms produced. The average 5 days calibration curve was linear for isoniazid ($y = 0.029x + 0.025$; $r = 0.9977$), nevirapine ($y = 0.043x + 0.127$; $r = 0.9984$) and paracetamol ($y = 0.097x + 0.070$; $r = 0.9998$) with a CV% < 20%. Accuracy at low, medium and high concentrations was 102%, 98% and 101% (isoniazid), 94%, 96% and 100% (nevirapine), and 99%, 97% and 99% (paracetamol), respectively. Paracetamol was most stable at ambient temperature, 4ºC and -20ºC. The method was used to monitor isoniazid, nevirapine and paracetamol concentrations in the plasma of treated rats.

Care should be taken when performing the solid phase extraction, as it is a small sample volume containing the compounds of interest. Unfortunately, isoniazid proved to be unstable, delivering poor results. Thus, it is advisable to analyse isoniazid plasma samples as soon as possible after blood collection. In addition, repeated freeze-thaw cycles should be avoided. In spite of this shortcoming the method produced satisfactory results as attention was paid to the time of storage of the samples.

This method will be useful for plasma drug monitoring in patients suffering from acquired immune deficiency syndrome (AIDS), and/or tuberculosis, as they may be on nevirapine and isoniazid treatment, with their pain being managed with an analgesic such as paracetamol. The concurrent use of the three drugs also increases the potential for development of hepatotoxicity and, as such, plasma drug monitoring would be appropriate.

CHAPTER 6

CYTOCHROME P450 AND THE IMMUNE RESPONSE TO PROLONGED ADMINISTRATION OF ISONIAZID

6.0 SUMMARY

Introduction: Isoniazid use for the treatment of tuberculosis poses a risk for the development of severe liver injury or toxicity. Both the immune system and CYP450 enzymes have been implicated in the development of the injury, hence were investigated during prolonged isoniazid administration.

Methods: Ethical approval was obtained and male Sprague-Dawley (SPD) rats (200 – 250 g) were used. The animal experiment was divided into three phases. In Phase I, two groups of 25 rats each were daily administered with saline solution (S) or INH alone (20 mg/kg), while for Phase II, two groups of 25 rats each received daily S or INH in combination with an immune stimulant, levamisole (LMS; 2.5 mg/kg). Lastly, during Phase III, two groups of 25 rats each received daily S or INH along with a CYP450 inducer, carbamazepine (CBZ; 60 mg/kg). In each group, five rats were sacrificed after 2, 7, 14, 28 and 42 days. Blood was analysed for full blood count, CD4 and CD8 counts, liver function, renal function, IL-2, IL-10, IgG, IgM and isoniazid concentrations. A piece of liver was sent for histopathology testing, and the activity of rat CYP1A2, CYP2E1 and CYP3A2 analysed.

Results: Isoniazid alone caused liver injury up to 28 days and this was associated with high isoniazid concentrations, CYP2E1 induction and early increase in CD4 count and IL-2 concentrations. By day 42 the injury had improved and was associated with increased IL-10 and IgG levels. Co-administration with levamisole caused more severe liver injury and was associated with high isoniazid concentrations, and increased IL-10 and IgM concentrations. Co-treatment with carbamazepine caused only minimal liver injury and exhibited low isoniazid concentrations, less CYP2E1 induction and higher IL-10 concentrations throughout the treatment period.

Conclusion: It was demonstrated that the immune system is involved in the initial liver injury caused by isoniazid treatment.

6.1 INTRODUCTION

Isoniazid is liable to cause severe liver injury/toxicity in 1 – 2% of patients (Shen *et al*., 2006). Here, involvement of the immune system was implicated, but remains to be proven. This chapter describes the role of the CYP450 and immune response in isoniazid-induced liver injury. The results are reported under the following parameters: physiological observations (function tests), *i.e.*, full blood count, renal function tests, liver function tests and liver histopathology; isoniazid concentrations; specific immune tests, *i.e.*, direct observations, cytokines, CD4 and CD8 counts and immunoglobulins; and activity of rat CYP1A2, CYP2E1 and CYP3A2.

6.2 METHODS

A. Materials

6.2.1 Apparatus

Rats were weighed with a precision balance (1213 MP, Sartorius, Göttingen, Germany), and feeding needles (16 G-3", curved 3 mm ball; Poppers and sons, Inc., NY, U.S.A) were used for oral gavage. Anaesthesia was administered with a gas anaesthetic machine (Ugo Basile, Comerio, VA, Italy), while blood was collected in the following tubes (Vacuette®, Greiner Bio-One): 4 ml K2EDTA tubes (Kremsmünster, Austria), 4 ml lithium heparin separator tubes and 5 ml Z serum separator clot activator tubes (Chonburi, Thailand). Rat surgery was performed with a dissection kit (Lasec S.A., Bloemfontein, South Africa). For analysis of isoniazid concentrations in rat plasma, the apparatus are the same as discussed in Chapter 5. Cytokines and immunoglobulins were analysed by ELISA, using a microplate reader (Multiskan Ascent®, Thermo Electron Corporation, Vantaa, Finland) and washer (93PW; Tecan, Grödig, Austria) coupled to a vacuum pump (KNF Neuberger, Freiburg, Germany). Rat liver microsomes were prepared with a sonicator (Janke and Kunkel, Scönaich, Germany), low centrifuge (Sorvall® RC2-B, Ivan Sorvall Inc., Newtown, CT, U.S.A), and ultracentrifuge (Optima™ L-100 XP, Beckman Coulter, Fullerton, CA, U.S.A), while protein concentrations were measured with an UV spectrophotometer (Libra S12, Biochrom Ltd., Cambridge, England). Analysis of rat CYP1A2 activity was done with a fluorescence spectrophotometer (Cary Eclipse, Varian, Victoria, Australia) whilst that of CYP2E1 and CYP3A2 was performed by the same HPLC system as described in Chapter 5, over a C_{18} (4.60 x 150 mm) 5 micron,

and a C_{18} (4.60 x 150 mm) 3 micron analytical column (Phenomenex® Luna, Torrance, CA, U.S.A) for CYP2E1 and CYP3A2, respectively.

6.2.2 Chemicals

All standards and chemicals used were of analytical grade. Carbamazepine, chlorzoxazone, *D*-glucose-6-phosphate monosodium salt, ethoxyresorufin, ethylenediaminetetraacetic acid (EDTA), formaldehyde, glucose-6-phosphate dehydrogenase (from *S. cerevisae*), hydroxyethyl piperazineethanesulphonic acid potassium salt (HEPES), isoniazid, resorufin, and trizma base, were supplied by Sigma-Aldrich Inc. (St. Louis, MO, U.S.A). β-nicotinamide adenine dinucleotide phosphate, orthophosphoric acid and trifluoroacetic acid were obtained from Merck Laboratories (Darmstadt, Germany). Sulphapyridine was from the Department of Pharmacology, Toxicology Laboratory reference substances, and Toronto research Chemicals Inc. (North York, ON, Canada) supplied the following analytical standards: 6-hydroxychlorzoxazone, 1-hydroxymidazolam and midazolam. ELISA kits for rat IL-2 and IL-10 were purchased from RayBio® (RayBiotech Inc., Norcross, GA, U.S.A) and Invitrogen™ (Invitrogen Corporation, Camarillo, CA, U.S.A), while ELISA kits for rat IgG and IgM were from eBioscience™ (Bender MedSystems, Vienna, Austria).

Ripercol®-*l* (levamisole) powder (Janssen Pharmaceutica (Pty) Ltd, Woodmead, Gauteng, South Africa) was purchased from a local agricultural business, while saline solution (0.9%; Adcock Ingram Critical Care (Pty) Ltd, Johannesburg, South Africa) was kindly sponsored by the Department of Pharmacology, Toxicology Laboratory. ISOFOR (isoflurane) inhalation anaesthetic was obtained from Safe Line Pharmaceuticals (Pty) Ltd (Roodepoort, Gauteng, South Africa).

6.2.3 Preparation of drugs for oral administration

20 mg/kg isoniazid was prepared in saline solution, while 2.5 mg/kg levamisole was in distilled water, and 60 mg/kg carbamazepine in olive oil.

6.2.4 Buffers and reagents

Neutral buffered formalin (10%) was used to fixate liver sections, and consisted of formaldehyde (10%) in phosphate buffer. Phosphate buffered saline (pH 7.4) was

used as a wash buffer for the immunoglobulin ELISA. Sodium phosphate buffer (pH 7.4) was used in the CYP2E1 and CYP3A2 assays, while HEPES buffer (pH 7.4) was used in the CYP1A2 assay. The NADP regenerating system was used to initiate metabolism in the CYP450 assays, and consisted of *D*-glucose-6-phosphate monosodium salt, β-nicotinamide adenine dinucleotide phosphate and glucose-6-phosphate dehydrogenase in distilled water.

B. Procedures

6.2.5 Experimental design

A total of 155 rats were used. Five rats were not treated with any drug, and used for baseline data. Furthermore, the study was divided into three phases.

6.2.5.1 Phase I – Treatment with isoniazid alone

Rats were weighed and divided into two groups of 25 animals each, namely the S group (control) and the INH group (test). Rats received saline solution or isoniazid once daily for 2, 7, 14, 28, and 42 days, respectively. At each time frame, five rats were sacrificed (Section 6.2.7) on the day following the last day of dosing. Saline solution and isoniazid were administered orally on a daily basis, as follows (Figure 6.1):

- S group: saline solution (1 ml, orally)
- INH group: 20 mg/kg isoniazid (1 ml, orally)

The dose of isoniazid was from a previous departmental study (Walubo *et al.*, 2004).

6.2.5.2 Phase II – Co-treatment with an immune stimulant

Rats were weighed and divided into two groups of 25 animals each, namely the S+LMS group (control) and the INH+LMS group (test). Rats received saline solution plus levamisole or isoniazid plus levamisole for 2, 7, 14, 28, and 42 days, respectively. Levamisole administration was begun on the day before the start of saline or isoniazid dosing, thereafter saline and isoniazid were administered daily in the morning, and levamisole daily in the afternoon to avoid a potential drug interaction. At each time frame, five rats were sacrificed on the day following the last day of dosing. Saline solution, isoniazid and levamisole were administered orally, as follows (Figure 6.1):

- S+LMS group: saline solution (1 ml, orally) and 2.5 mg/kg levamisole (500 µl, orally)
- INH+LMS group: 20 mg/kg isoniazid (1 ml, orally) and 2.5 mg/kg levamisole (500 µl, orally)

The dose of levamisole was from a report by Gautam *et al*. (2009).

6.2.5.3 Phase III – Co-treatment with a CYP450 inducer

Rats were weighed and divided into two groups of 25 animals each, namely the S+CBZ group (control) and the INH+CBZ group (test). Rats received saline solution plus carbamazepine or isoniazid plus carbamazepine for 2, 7, 14, 28, and 42 days, respectively. Carbamazepine administration commenced on the day before the start of saline or isoniazid dosing, thereafter saline and isoniazid were administered daily in the morning, and carbamazepine daily in the afternoon to avoid a potential drug interaction. At each time frame, five rats were sacrificed on the day following the last day of dosing. Saline solution, isoniazid and carbamazepine were administered orally as follows (Figure 6.1):

- S+CBZ group: saline solution (1 ml, orally) and 60 mg/kg carbamazepine (500 µl, orally)
- INH+CBZ group: 20 mg/kg isoniazid (1 ml, orally) and 60 mg/kg carbamazepine (500 µl, orally)

The dose of carbamazepine was from a report by Tateishi and co-workers (1999).

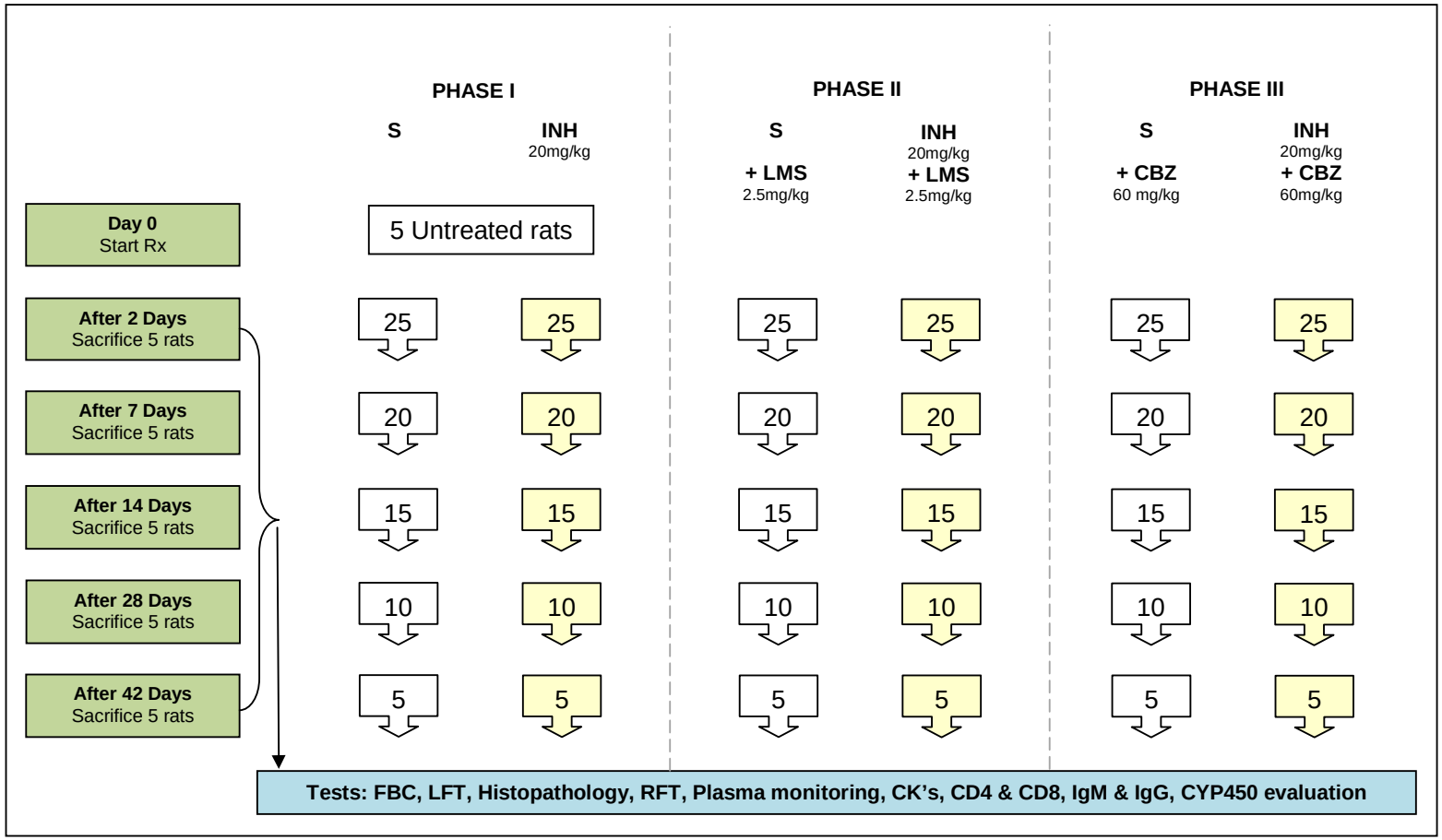

Figure 6.1: A schematic illustration of the experimental design of Phase I, II and III of prolonged isoniazid administration

6.2.6 Animal care

Ethical approval (Animal Experiment NR 09/2012) was obtained from the Animal Ethics Committee of the University of the Free State. Male SPD rats with a weight range of 200 – 250 g were used. Animals were housed at the Animal House of the University of the Free State, where they were fed and looked after by qualified staff, and their cages, which allowed a maximum capacity of four rats, were cleaned once a week. Standard rat chow and water was available to the animals *ad libitum*. All drug administration took place at the Animal House, and animals were inspected for skin lesions and other visible adverse events every day.

6.2.7 Animal weighing, blood collection and liver removal

For all groups, animals were weighed before the day of the start of the dosing period, in order to prepare the required drug dosage per kg of body weight, and weighed again on the day of sacrificing. During the treatment period, the respective drugs were administered by oral gavage. Rats were sacrificed on the day following the last day of dosing. Anaesthesia was performed with isoflurane. Firstly, anaesthesia was induced in a gas chamber at a concentration of 4% isoflurane, after which the rat was transferred and secured to a surgical board. Secondly, anaesthesia was maintained with a cone diaphragm at a concentration of 2% isoflurane. Under anaesthesia, central blood was drawn by direct cardiac puncture (Figure 6.2 a). Blood was collected in yellow top serum separator tubes for serum, green top lithium heparin tubes for plasma, and purple top EDTA tubes for full blood count. The plasma and serum tubes were centrifuged and stored at -20ºC and -85ºC for plasma and serum, respectively, until analysis. Blood for full blood count and CD4 and CD8 counts were sent off to independent laboratories for analysis (Section 6.2.8).

After blood collection the abdomen was opened and the liver exposed. A liver section was cut and stored in 10% formalin (Figure 6.2 b). The remainder of the liver was excised, removed and washed in a 1.5% potassium chloride solution, frozen with liquid nitrogen and stored at -85℃. Animals were sacrificed by exsanguination whilst still under isoflurane anaesthesia.

Figure 6.2 a): Photograph of a rat under isoflurane anaesthesia while blood is being drawn by cardiac puncture

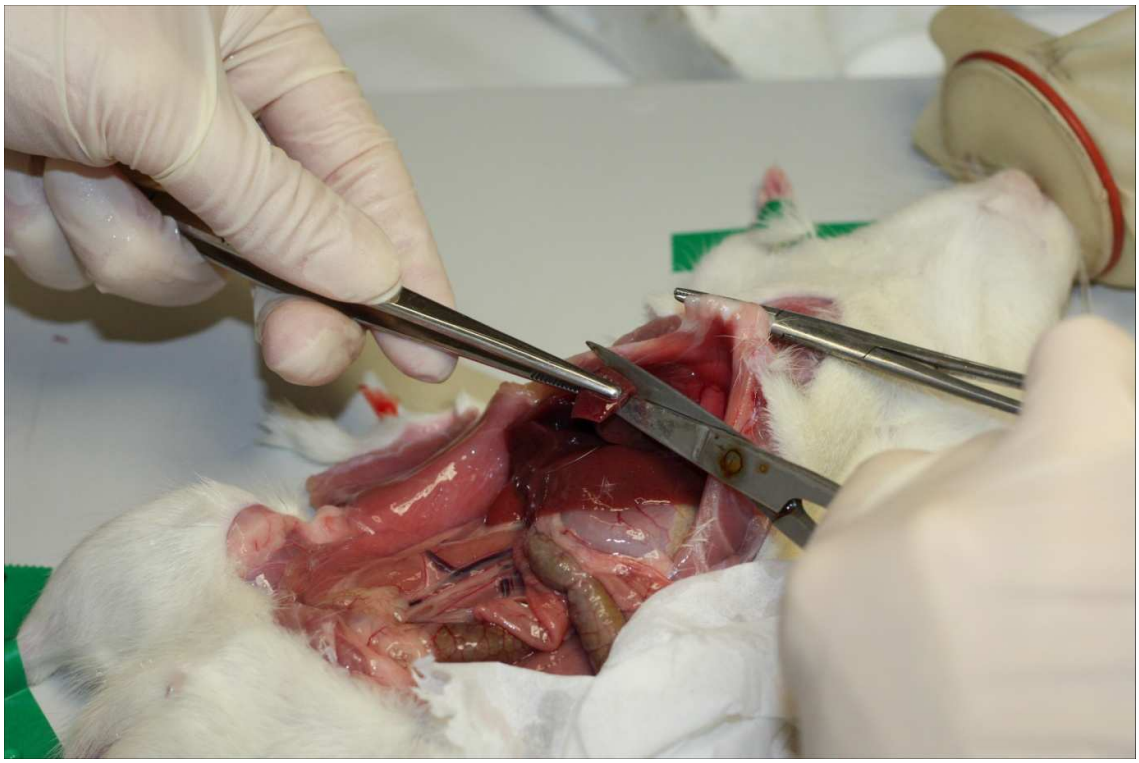

Figure 6.2 b): Photograph of a rat under isoflurane anaesthesia with an open abdomen while a liver section is cut

6.2.8 Analysis of function tests

Full blood count (red blood cells, white blood cells, platelets and differential count) was determined by the National Health Laboratory Services (NHLS, Universitas Academic Hospital, Bloemfontein, South Africa). CD4 and CD8 counts were measured by the Department of Haematology and Cell Biology, University of the Free State (Bloemfontein, South Africa).

Liver and renal function tests were conducted by the Toxicology Laboratory of the Department of Pharmacology, University of the Free State (Bloemfontein, South Africa). The following serum transaminase enzyme levels were measured: alanine aminotransferase (ALT), aspartate aminotransferase (AST) and alkaline phosphatase (ALP). For renal function, urea and creatinine levels were measured.

Histopathology of the rat livers was performed and reported by an independent veterinary pathologist (Idexx Laboratories, Johannesburg, South Africa).

6.2.9 Analysis of cytokines by enzyme-linked immunosorbent assay

ELISA was used for the quantitative detection of rat IL-2 and IL-10 in serum. The assay was performed using commercial ELISA kits according to the manufacturer's instruction, as follows:

The ELISA plate was already coated with a capture antibody, specific to the cytokine to be analysed. To the wells were added incubation buffer, respective standards and samples, and biotin-conjugate secondary antibody. This was left to incubate for 2 hours at room temperature. Thereafter, streptavidin-horseradish peroxidase was added and the plate incubated for 30 minutes at room temperature. Lastly, a chromogen substrate was added and the plate left to incubate for 30 minutes at room temperature and in the dark. The reaction was stopped by the addition of acid, and absorption measured at 450 nm with a microplate reader. A standard curve was prepared from rat cytokine standard dilutions and the rat cytokine sample concentration derived from the standard curve in pg/ml. For IL-2 the standard curve concentration range was 23.4, 46.9, 93.7, 187, 375, 750 and 1500 pg/ml, while for IL-10 it was 15.6, 31.2, 62.5, 125, 250, 500 and 1000 pg/ml.

6.2.10 Analysis of immunoglobulins by enzyme-linked immunosorbent assay

ELISA was used for the quantitative detection of rat IgM and IgG in serum. The assay was performed using commercial ELISA kits according to the manufacturer's instruction, as follows:

An ELISA plate was coated with coating buffer, containing the specific capture antibody, and incubated overnight at 4℃. The coated plate was blocked at room temperature for 2 hours, after which respective standards and samples were added, and the plate left to incubate at room temperature for 2 hours. Thereafter, streptavidin-horseradish peroxidase detection antibody was added to all wells and incubation carried out at room temperature for 1 hour. Lastly, substrate solution was added, incubated at room temperature for 15 minutes, and the reaction stopped by the addition of acid. Absorption was measured at 450 nm with a microplate reader. A standard curve was prepared from rat immunoglobulin standard dilutions and the rat immunoglobulin sample concentration was derived from the standard curve. For IgM the standard curve concentration range was 0.3125, 0.625, 1.25, 2.5, 5, 10 and 20 ng/ml, while for IgG it was 1.6, 3.125, 6.25, 12.5, 25, 50 and 100 ng/ml.

6.2.11 Analysis of isoniazid concentrations in rat plasma

Plasma concentrations of isoniazid in rats were monitored using the HPLC assay as developed and described in Chapter 5. A standard curve was generated from five known calibration standards, from which isoniazid concentrations in rat plasma were derived.

6.2.12 Preparation of rat liver microsomes

Rat livers were removed and stored as explained in Section 6.2.7. From the frozen liver samples, microsomes were extracted according to a standardised procedure used in our laboratory as, follows:

Livers of randomly selected rats were placed on ice to thaw. Only half of the liver (1 – 2 g) was used, and the remainder stored at -85ºC. The piece of liver was cut into smaller sections, placed in cold homogenising buffer 1, and manually homogenised to a mesh. Here, the mesh was blended with a sonicator until a homogenate was formed. The homogenate was centrifuged for 25 minutes at 26964 *g* (15,000 r.p.m)

and -4ºC. For further extraction, the supernatant was removed and ultracentrifuged for 60 minutes at 106,750 *g* (25,000 r.p.m) and -4℃ to form a concentrated pel let of microsomes. Thereafter, the supernatant was discarded, a second homogenising buffer added, and the pellet manually homogenised. Again, the sample was ultracentrifuged at the above mentioned settings, the supernatant poured off, and the pellet rinsed with storage buffer. The pellet and storage buffer were homogenised and the microsomal mixture transferred to respective tubes, quick frozen with liquid nitrogen, and finally stored at -85ºC.

6.2.13 Protein assay

To evaluate the activity of rat microsomal CYP1A2, CYP2E1 and CYP3A2, the reaction conditions have to be standardised to a point at which optimum enzyme activity is attained. For each microsomal sample the concentration of proteins was determined by the Biuret protein assay. Here, the microsomal protein concentration was determined to standardise each microsomal solution for use in reactions.

For analysis of the microsomal samples, six calibration samples were prepared by adding different volumes of bovine serum albumin (BSA) to distilled water to achieve the following concentrations: 0, 0.5, 1, 2.5, 5 and 10 mg/ml. Each microsomal sample was diluted 10x with distilled water before protein analysis. To a fresh test tube, 100 µl of sample was added, followed by 900 µl of Biuret reagent and the test tube vortexed immediately. The test tube was covered with parafilm and the sample left to incubate at room temperature for 20 minutes. Thereafter, the sample was transferred to a cuvette and absorption read at 550 nm. For each batch of microsomes, a calibration was performed, and the linear equation used to derive the protein concentration of the microsomes.

6.2.14 Determination of rat CYP1A2, CYP2E1 and CYP3A2 activity *in vivo*

6.2.14.1 CYP1A2 assay

(a) Sample preparation

To 210 µl of 0.1 M HEPES potassium salt buffer (pH 7.4) was added final concentrations: 0.2 mg/ml microsomes, 60 µM EDTA, 5 mM magnesium sulphate and 20 nmol ethoxyresorufin. Six calibration samples were prepared by adding different volumes of resorufin to achieve the following concentration range: 0, 50,

100, 150, 200 and 250 pmol/ml. Samples were pre-incubated for 5 minutes at 37 ºC, while the reaction was started by the addition of the NADP regenerating system. The total reaction volume of the sample was 500 µl. Ultimate incubation was continued for 10 minutes at 37 ºC and stopped by the addition of 2.5 ml cold acetonitrile. All samples were prepared in duplicate.

(b) Spectrophotometric conditions

The sample was transferred to a quarts cuvette and resorufin absorption read by fluorescence at wavelengths of excitation of 560 nm and emission of 585 nm, respectively.

6.2.14.2 CYP2E1 assay

(a) Sample preparation

To 250 µl of 0.1 M sodium phosphate buffer (pH 7.4) was added final concentrations: 0.2 mg/ml microsomes and 505 nmol chlorzoxazone. Seven calibration samples were prepared by adding different volumes of 6-hydroxychlorzoxazone to achieve the following concentration range: 0, 1.078, 2.155, 4.310, 6.466, 8.621 and 10.776 nmol/ml. Samples were pre-incubated for 5 minutes at 37 ºC, while the reaction was started by the addition of the NADP regenerating system. The total reaction volume of the sample was 500 µl. Ultimate incubation was continued for 10 minutes at 37 ºC and stopped with 40 µl of 0.1 M hydrochloric acid and 15 µl paracetamol (internal standard). All samples were prepared in duplicate.

(b) Sample extraction

A C_{18} solid phase extraction cartridge (1 ml) was conditioned with 1 ml HPLC grade methanol and 1 ml deionised water. The microsomal mixture was placed on the column and allowed to elute. Thereafter, the column was washed with 500 µl of deionised water. Finally, to a fresh test tube, the compounds were eluted with 200 µl sodium phosphate buffer (pH 4.5):acetonitrile (55:45). Of the collected eluent, 50 µl was injected into the HPLC for analysis.

(c) Chromatographic conditions

An HPLC system, as described in Section 6.2.1, was used for analysis. Chromatographic separation of chlorzoxazone, 6-hydroxychlorzoxazone and

paracetamol (internal standard) was achieved by running the mobile phase at a flow rate of 1 ml/min. The mobile phase consisted of solvent A, sodium phosphate buffer, pH 4.5, and solvent B, HPLC grade acetonitrile. For gradient separation, the proportion of solvent A and B was initially 70:30 for 3 minutes. This was changed to 60:40 over 1 minute, after which it was changed to 50:50 over 1 minute, and finally maintained for 5 minutes. For re-equilibration purposes a post-run of 2 minutes was performed at the initial ratio of 70:30. Compounds were detected by UV at a wavelength of 280 nm.

6.2.14.3 CYP3A2 assay

(a) Sample preparation

To 260 µl of 0.1 M sodium phosphate buffer (pH 7.4) was added final concentrations: 0.8 mg/ml microsomes, 4 mM magnesium chloride and 61.3 nmol midazolam. Six calibration samples were prepared by adding different volumes of 1-hydroxymidazolam to achieve the following concentration range: 0, 1.25, 2.5, 5.00, 7.50, and 10.00 nmol/ml. Samples were pre-incubated for 5 minutes at 37 ºC, while the reaction was started by the addition of the NADP regenerating system. The total reaction volume of the sample was 500 µl. Ultimate incubation was continued for 20 minutes at 37 ºC and stopped with 250 µl of cold HPLC grade acetonitrile and 50 µl carbamazepine (internal standard). All samples were prepared in duplicate.

(b) Sample extraction

The sample was alkalinised with sodium hydroxide and extracted with diethyl ether by liquid-liquid extraction. After extraction, the supernatant was removed and evaporated to dryness under a stream of nitrogen, reconstituted with 150 µl of mobile phase, and 100 µl was injected into the HPLC for analysis.

(c) Chromatographic conditions

An HPLC system, as described in Section 6.2.1, was used for analysis. Chromatographic separation of midazolam, 1-hydroxymidazolam and carbamazepine (internal standard) was achieved by running the mobile phase at a flow rate of 1 ml/min. The mobile phase consisted of solvent A, sodium acetate buffer, pH 4.0, and solvent B, HPLC grade acetonitrile. An isocratic mixture was

prepared by mixing solvent A and B to a ratio of 55:45. Compounds were detected by UV at a wavelength of 220 nm.

6.2.15 Statistical analysis of results

Data were analysed by non-parametric methods using the GraphPad Instat statistical program. Accordingly, parameters were reported as mean and standard deviation (SD), and the Mann-Whitney Test was used for data comparison with the level of significance set at $p < 0.05$.

6.3 RESULTS

The results are divided into three phases: Phase I – treatment with isoniazid alone, Phase II – co-treatment with an immune stimulant, and Phase III – co-treatment with a CYP450 inducer, and all parameters are reported as such.

A. Phase I: Treatment with isoniazid alone

6.3.1 Physiological observations (function tests)

6.3.1.1 Full blood count

Table 6.1 shows results of the full blood count of the S and INH groups. In both groups, the red cell count, haemoglobin and mean corpuscular haemoglobin concentrations (MCHC) increased ($p = 0.0500$) over the 42 day treatment period, while mean corpuscular volume (MCV) and mean corpuscular haemoglobin (MCH) values decreased ($p = 0.0500$), implying that red blood cell development with age was characterised by reduced cell size (MCV), which was associated with increased red cell count and haemoglobin concentrations most probably to maintain body oxygen requirements. On the other hand, a comparable pattern of change in the mentioned parameters was observed with saline treatment, meaning that these parameters most probably had no clinical significance. Furthermore, neutrophils were elevated in the INH group by day 42 ($p = 0.0500$), while in the S group the white cell count, lymphocytes and monocytes had become lower ($p = 0.0500$).

Table 6.1: Average (mean ± SD) full blood count and platelets results of the S and INH groups

Group (n = 3)	RCC ($x10^{12}$/l)	Hb (g/dl)	Hct (l/l)	MCV (fl)	MCH (pg)	MCHC (g/dl)	Plt ($x10^9$/l)	WCC ($x10^9$/l)	Neu ($x10^9$/l)	Ly ($x10^9$/l)	Mo ($x10^9$/l)	Eos ($x10^9$/l)	Bas ($x10^9$/l)
Untreated													
0 Days	6.28±0.2	12.9±0.3	0.398±0.01	63.5±2.5	20.5±0.4	32.3±0.8	860±221.1	6.95±2.7	0.77±0.2	4.67±1.8	0.19±0.1	0.02±0.0	0.00±0.0
S													
2 Days	6.67±0.2	13.7±0.1	0.422±0.01	63.3±2.3	20.6±0.6	32.4±0.6	849±81.6	6.50±0.9	0.60±0.2	5.18±0.7	0.21±0.0	0.50±0.2	0.01±0.0
7 Days	7.53±0.9	15.3±1.7	0.451±0.04	60.1±2.5	20.3±0.2	33.9±1.2	1033±79.8	5.44±2.4	1.03±0.8	4.07±2.0	0.30±0.3	0.04±0.0	0.01±0.0
14 Days	6.77±0.6	13.9±1.1	0.417±0.03	61.8±2.8	20.5±0.6	33.2±0.6	721±196.4	5.22±1.2	0.63±0.5	4.21±0.7	0.18±0.1	0.18±0.1	0.05±0.0
28 Days	7.07±0.7	13.9±1.3	0.390±0.04	55.1±1.0	19.7±0.1	35.8±0.6	961±172.5	7.38±1.0	0.91±0.2	6.15±0.8	0.24±0.1	0.07±0.0	0.01±0.0
42 Days	6.93±0.8	13.4±1.8	0.374±0.05	53.9±1.0	19.3±0.4	35.8±0.2	839±166.0	3.93±0.3	0.54±0.1	3.23±0.3	0.11±0.0	0.04±0.0	0.01±0.0
INH													
2 Days	6.57±0.3	13.4±0.4	0.416±0.01	63.4±2.0	20.5±0.3	32.3±0.7	880±54.5	5.86±1.1	0.59±0.0	5.02±1.1	0.21±0.1	0.03±0.0	0.00±0.0
7 Days	6.05±0.6	12.7±1.1	0.381±0.04	63.0±0.6	21.0±0.2	33.3±0.2	478±19.1	5.98±0.8	0.37±0.3	5.10±0.8	0.19±0.1	0.02±0.0	0.01±0.0
14 Days	6.65±0.6	13.1±1.2	0.394±0.04	59.2±2.7	19.8±0.6	33.4±0.7	494±50.2	5.78±1.1	0.61±0.1	4.91±0.9	0.23±0.1	0.03±0.0	0.00±0.0
28 Days	7.27±0.4	14.3±0.8	0.406±0.02	55.9±0.6	19.7±0.1	35.3±0.4	845±116.0	5.44±0.4	0.66±0.1	4.56±0.5	0.17±0.1	0.04±0.0	0.01±0.0
42 Days	7.25±0.7	13.9±1.2	0.390±0.04	53.8±1.9	19.2±0.4	35.6±0.8	785±152.7	5.11±0.9	0.84±0.1	4.05±0.6	0.15±0.1	0.07±0.0	0.01±0.0

RCC = red cell count; Hb = haemoglobin; Hct = haematocrit; MCV = mean corpuscular volume; MCH = mean corpuscular haemoglobin; MCHC = mean corpuscular haemoglobin concentration; Plt = platelets; WCC = white cell count; Neu = neutrophils; Ly = lymphocytes; Mo = monocytes; Eos = eosinophils; Bas = basophils; S = saline; INH = isoniazid

6.3.1.2 Renal function tests

Table 6.2 shows the changes of blood urea nitrogen (BUN) and creatinine (Cr) of the S and INH groups. In both groups BUN and Cr levels were normal, in spite of the spike on day 28 ($p = 0.0500$), as this was still within the normal range, *i.e.*, similar to the Untreated group.

Table 6.2: Average (mean ± SD) renal function test results of the S and INH groups

Group	**RFT**	
	BUN	**Cr**
(n = 3)	**(mmol/l)**	**(µmol/l)**
Untreated		
0 Days	7.2 ± 1	37 ± 8
S		
2 Days	7.3 ± 1	39 ± 2
7 Days	8.1 ± 0	46 ± 7
14 Days	7.5 ± 1	39 ± 3
28 Days	10.6 ± 2	73 ± 17
42 Days	5.8 ± 1	38 ± 9
INH		
2 Days	6.7 ± 0	34 ± 6
7 Days	6.6 ± 0	36 ± 2
14 Days	6.2 ± 1	43 ± 8
28 Days	7.3 ± 1	69 ± 4
42 Days	5.8 ± 0	34 ± 21

RFT = renal function test; BUN = blood urea nitrogen; Cr = creatinine; S = saline; INH = isoniazid

6.3.1.3 Liver function tests

Table 6.3 shows the changes of ALT, AST and ALP of the S and INH groups. Liver function was normal as there were no differences in ALT, AST and ALP between the two groups.

Table 6.3: Average (mean ± SD) liver function test results of the S and INH groups

Group	LFT		
	ALT	AST	ALP
(n = 3)	(U/l)	(U/l)	(U/l)
Untreated			
0 Days	50 ± 5	88 ± 14	352 ± 76
S			
2 Days	46 ± 2	90 ± 7	400 ± 7
7 Days	49 ± 10	103 ± 25	304 ± 13
14 Days	58 ± 4	127 ± 37	508 ± 37
28 Days	47 ± 2	115 ± 44	216 ± 19
42 Days	46 ± 6	76 ± 28	109 ± 76
INH			
2 Days	46 ± 4	104 ± 7	335 ± 32
7 Days	53 ± 13	233 ± 223	369 ± 10
14 Days	43 ± 4	98 ± 29	364 ± 38
28 Days	46 ± 4	143 ± 36	220 ± 29
42 Days	51 ± 2	86 ± 14	127 ± 78

LFT = liver function test; ALT = alanine aminotransferase; AST = aspartate aminotransferase; ALP = alkaline phosphatase; S = saline; INH = isoniazid

6.3.1.4 Liver histopathology

(a) Liver histopathology reports

Liver sections for histopathology (Figures 6.3 a – k) were randomly selected, and the main histopathology lesions are summarised in the tally table (Table 6.4). The following report is a summary of the features of the lesion:

i. Figure 6.3 a: Liver section from an untreated rat at day 0

A representative photograph of a rat liver from an untreated rat. The report: “No pathology appears to be present in the control (untreated) animals.”

ii. Figures 6.3 b and c: Liver sections A and B from the INH group after 2 days of isoniazid alone treatment

Representative photographs of rat livers, after 2 days of daily isoniazid alone treatment. The report: “Moderate granular vacuolar degeneration and cell swelling were observed in both liver sections, A and B, along with a loss of coordinated and well-organized hepatocytic cords. The cytoplasm appeared to be cloudy and granular. Only minimal cytonecrosis was present in both liver sections. Although centrilobular zonal necrosis was minimal in both liver sections, it appeared slightly

more prominent in liver section A, as characterised by loss of nuclei and sinusoidal dilatation."

iii. Figures 6.3 d and e: Liver sections A and B from the INH group after 7 days of isoniazid alone treatment

Representative photographs of rat livers, after 7 days of daily isoniazid alone treatment. The report: "Severe degeneration of vacuoles and osmotic swelling of the mitochondria were observed in liver sections A and B. Cytonecrosis was mild to moderate in both liver sections, and was characterised by loss of cell boundaries and nuclei. Centrilobular necrosis was absent, while one mitotic figure was present in section A."

iv. Figures 6.3 f and g: Liver sections A and B from the INH group after 14 days of isoniazid alone treatment

Representative photographs of rat livers, after 14 days of daily isoniazid alone treatment. The report: "Degenerative changes, such as moderate granular vacuolar degeneration and cell swelling were seen in both liver sections, A and B. Cytonecrosis was present in both liver sections, and was characterised by mild loss of cell boundaries, disruption of the cytoplasm and irregular appearance of the hepatic parenchyma. Furthermore, minimal zonal necrosis was observed in liver section A."

v. Figures 6.3 h and i: Liver sections A and B from the INH group after 28 days of isoniazid alone treatment

Representative photographs of rat livers, after 28 days of daily isoniazid alone treatment. The report: "Mild to moderate granular vacuolar degeneration and cell swelling were observed in sections A and B, along with mild cytonecrosis. In liver section A hepatocytes appear swollen with granular cytoplasm. No centrilobular zonal necrosis or hepatocyte mitosis was recorded."

vi. Figures 6.3 j and k: Liver sections A and B from the INH group after 42 days of isoniazid alone treatment

Representative photographs of rat livers, after 42 days of daily isoniazid alone treatment. The report: "The histopathological lesions had improved as granular

vacuolar degeneration and cell swelling were only minimal, with minimal to mild cytonecrosis in sections A and B. Centrilobular zonal necrosis was completely absent, although one mitotic figure was evident in section B."

In view of the histopathology photographs (Figures 6.3 a – k), reports and tally table (Table 6.4), it was concluded that treatment with isoniazid alone caused liver injury up to 28 days, and by day 42 this had improved.

(b) Liver histopathology photographs

Figures 6.3 a – k are representative of randomly selected liver sections of untreated rats, as well as the INH group after isoniazid alone treatment.

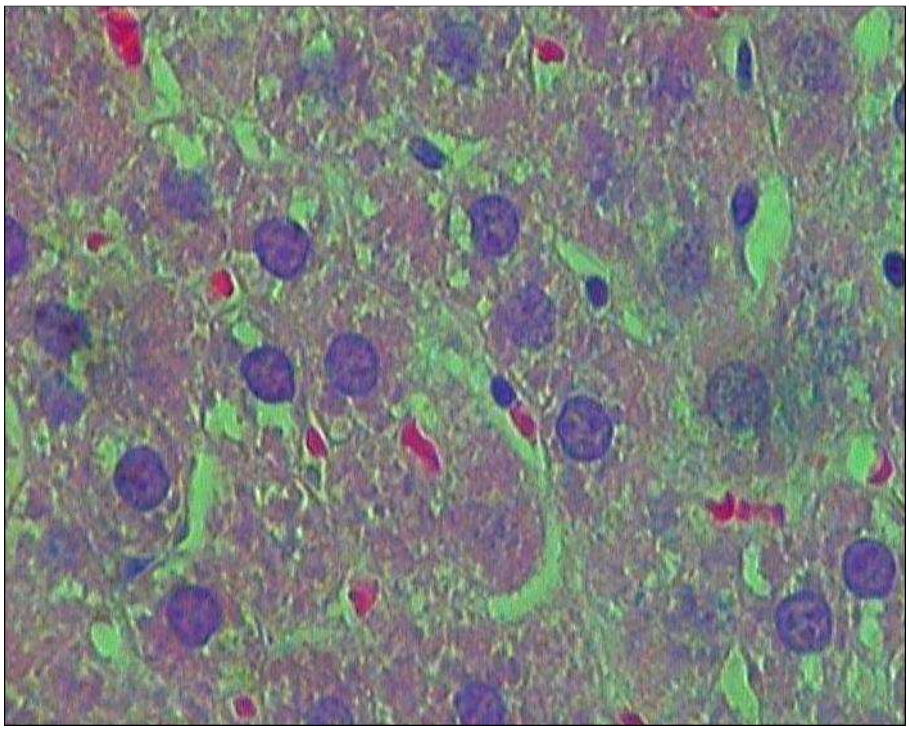

Figure 6.3 a): Liver section from an untreated rat at day 0, showing a normal liver with no inflammation

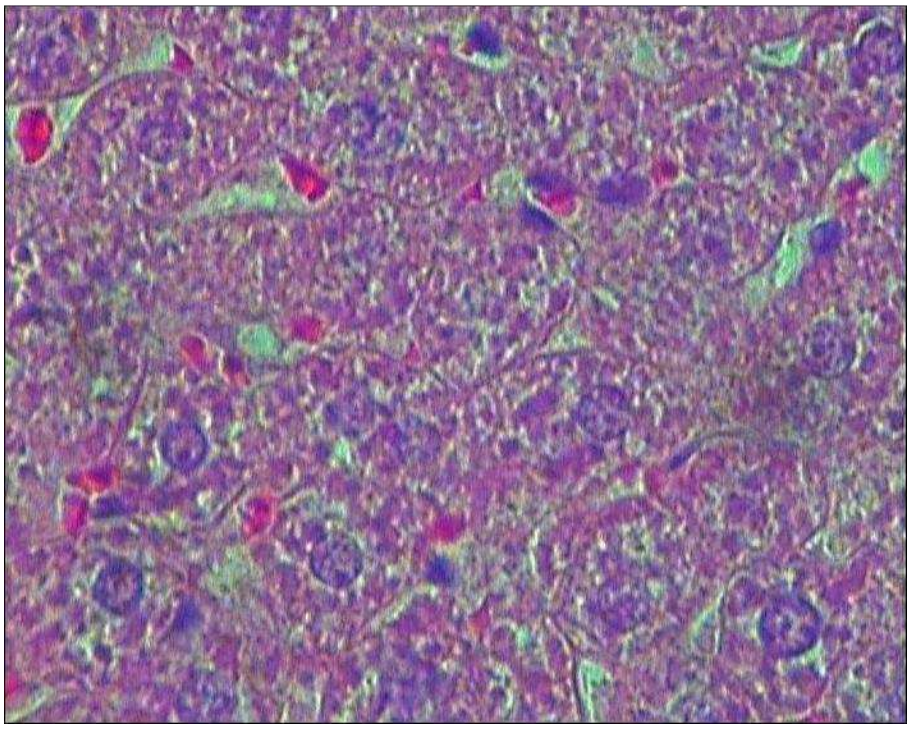

Figure 6.3 b): Liver section A from the INH group after 2 days, showing centrilobular zonal necrosis with loss of nuclei and sinusoidal dilatation

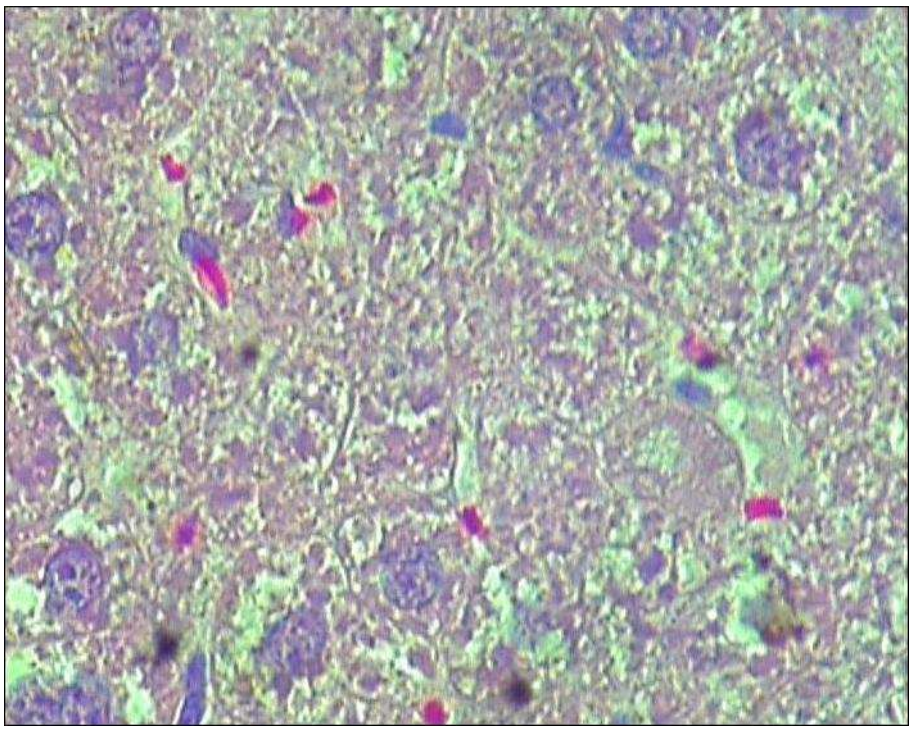

Figure 6.3 c): Liver section B from the INH group after 2 days, showing moderate granular vacuolar degeneration and cell swelling with loss of coordinated hepatocytic cords

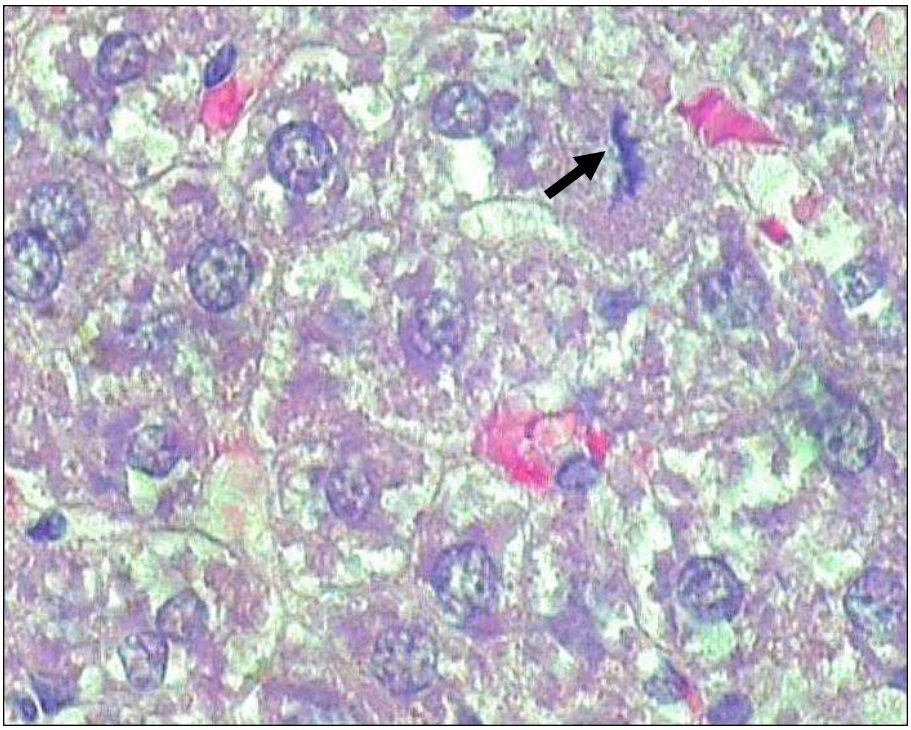

Figure 6.3 d): Liver section A from the INH group after 7 days of treatment, showing severe granular vacuolar degeneration of hepatocytes, karyopyknosis, and one mitotic figure

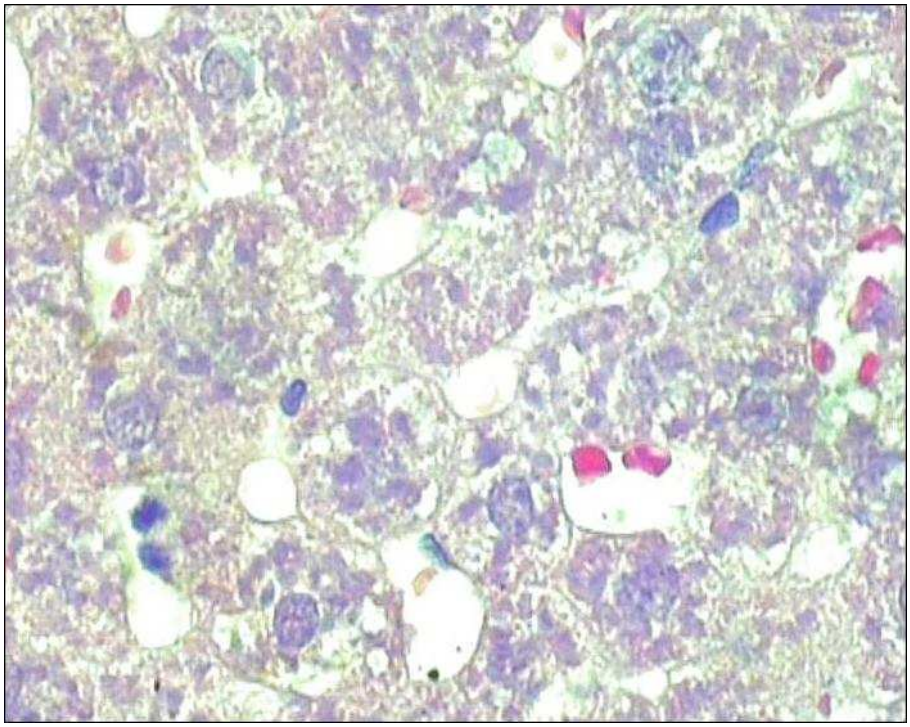

Figure 6.3 e): Liver section B from the INH group after 7 days of treatment, showing severe hepatocyte degeneration, as well as nuclear loss and cytonecrosis in the parenchyma

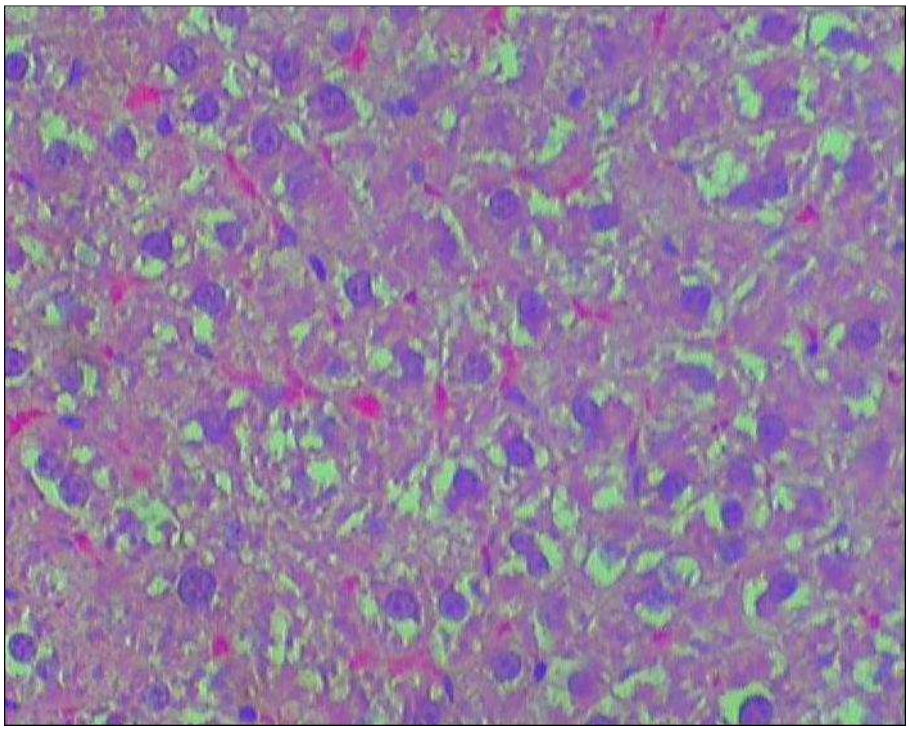

Figure 6.3 f): Liver section A from the INH group after 14 days of treatment, showing moderate granular vacuolar degeneration, cell swelling, and minimal zonal necrosis

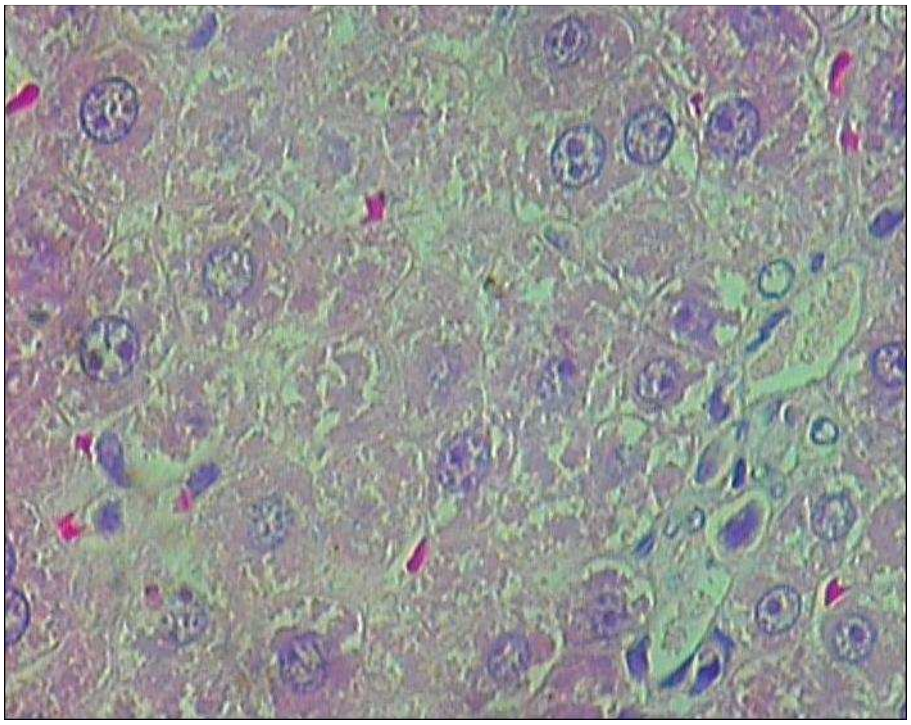

Figure 6.3 g): Liver section B from the INH group after 14 days of treatment, showing cytonecrosis with loss of cell boundaries, disrupted cytoplasm and irregular appearance of hepatic parenchyma

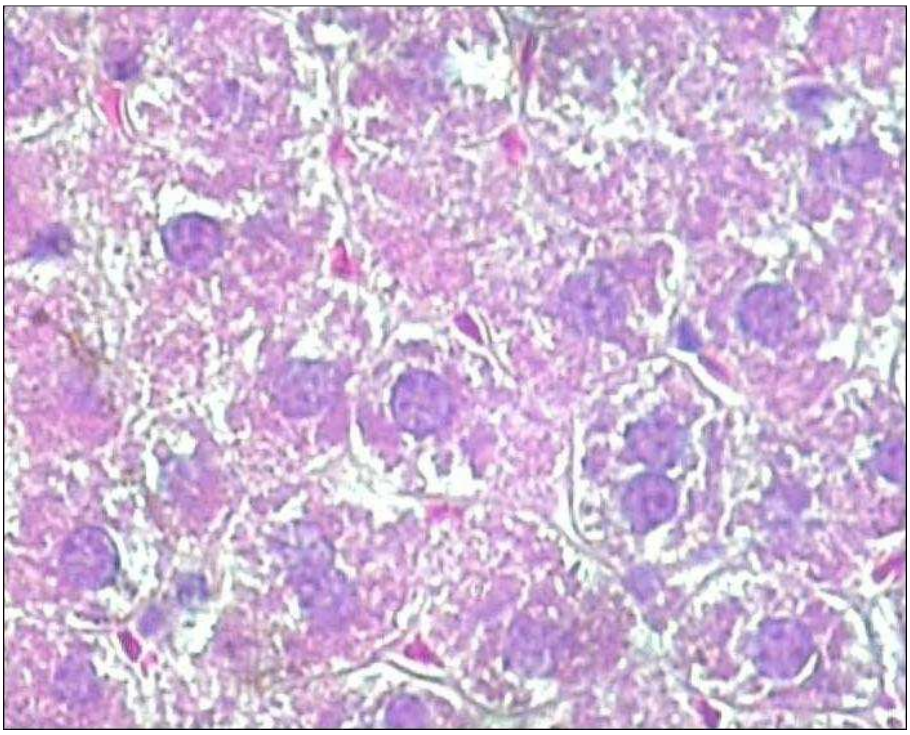

Figure 6.3 h): Liver section A from the INH group after 28 days of treatment, showing moderate vacuolar granular degeneration and cell swelling, with mild cytonecrosis

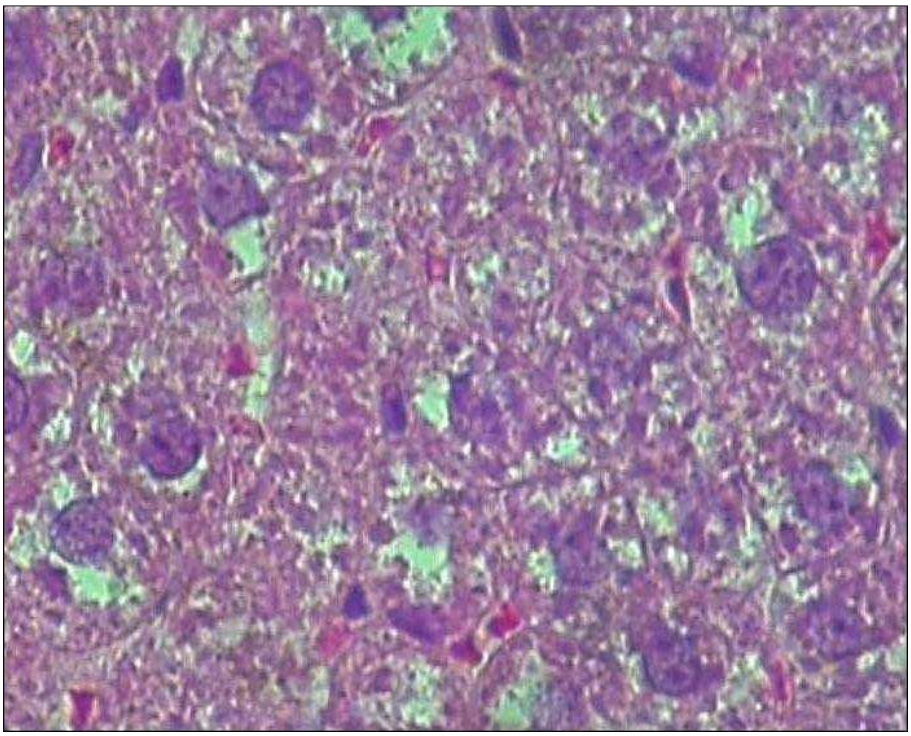

Figure 6.3 i): Liver section B from the INH group after 28 days of treatment, showing mild vacuolar degeneration in hepatocytes

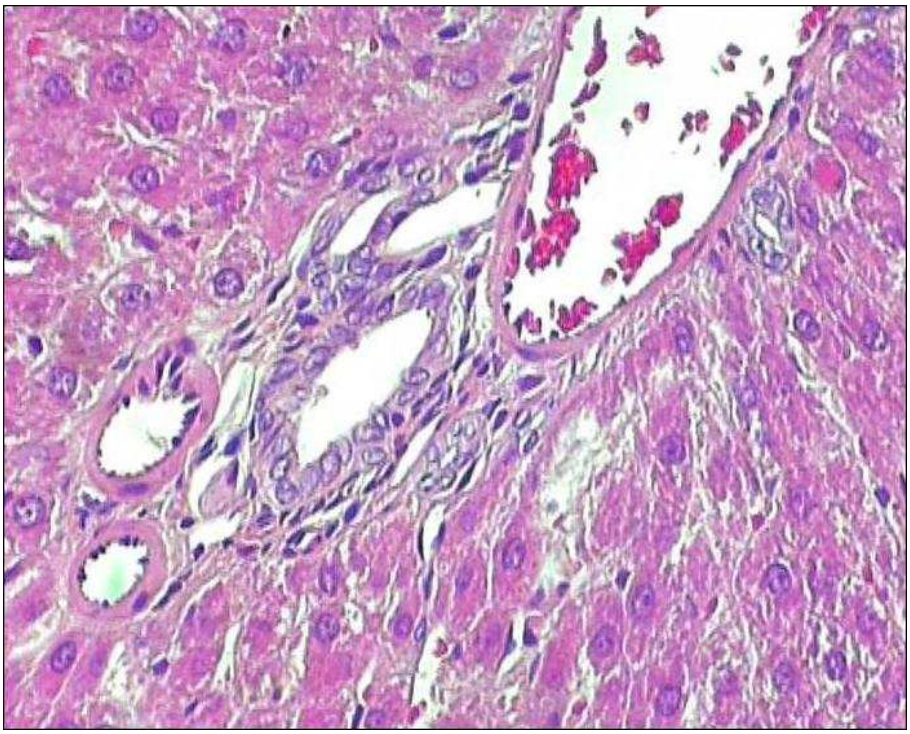

Figure 6.3 j): Liver section A from the INH group after 42 days of treatment, showing a normal portal area

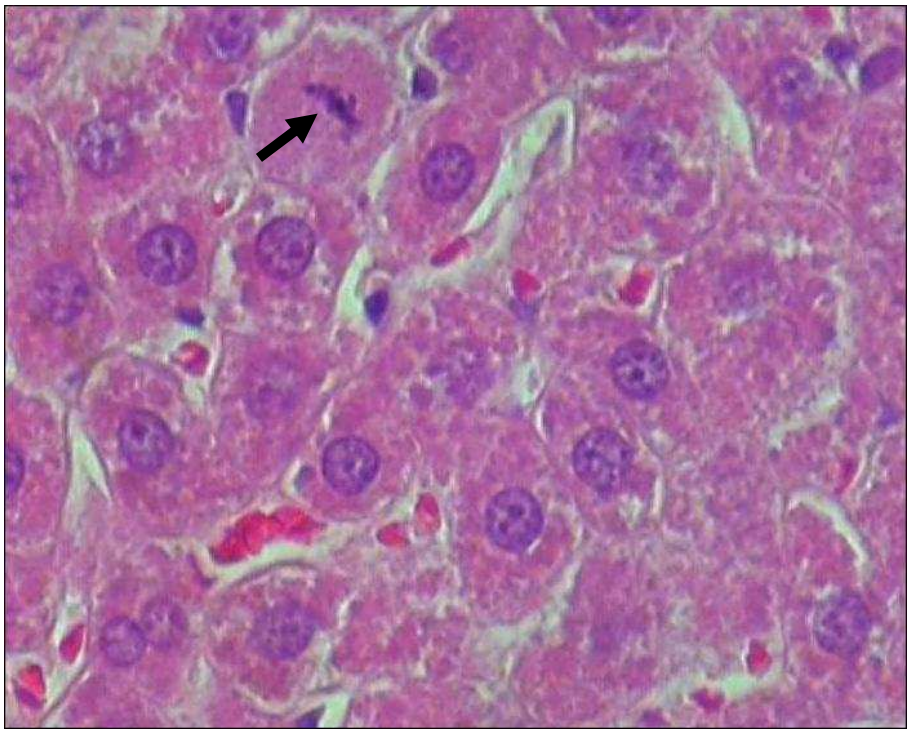

Figure 6.3 k): Liver section B from the INH group after 42 days of treatment, showing one mitotic figure

Table 6.4: Tally of main pathology lesions (lesions score) in livers of untreated rats and the INH group

Group	UnRx	INH									
		2 Days		7 Days		14 Days		28 Days		42 Days	
(n = 2)	Fig.6.3a	Fig.6.3b	Fig.6.3c	Fig.6.3d	Fig.6.3e	Fig.6.3f	Fig.6.3g	Fig.6.3h	Fig.6.3i	Fig.6.3j	Fig.6.3k
Histopathological lesions											
Vacuolar degeneration	0	3+	3+	4+	4+	3+	3+	3+	2+	1+	1+
Cell swelling	0	3+	3+	4+	4+	3+	3+	3+	2+	1+	1+
Cytonecrosis	0	1+	1+	2+	3+	2+	1+	2+	2+	2+	1+
Centrilobular necrosis	0	1+	1+	0	0	1+	0	0	0	0	0
Hepatocyte mitosis	0	0	0	1+	0	0	0	0	0	0	1+
Average lesions score											
Vacuolar degeneration	0	3+		4+		3+		2.5+		1+	
Cell swelling	0	3+		4+		3+		2.5+		1+	
Cytonecrosis	0	1+		2.5+		1.5+		2+		1.5+	
Centrilobular necrosis	0	1+		0		0.5+		0		0	
Hepatocyte mitosis	0	0		0.5+		0		0		0.5+	
Total lesion score	**0**	**8+**		**11+**		**8+**		**7+**		**4+**	

UnRx = untreated; INH = isoniazid

6.3.2 Isoniazid concentrations

Table 6.5 shows isoniazid concentrations of the INH group, while Figure 6.4 is a graphical illustration of the same. In the INH group, concentrations increased up to day 14 ($p = 0.0317$), and thereafter dropped ($p = 0.0159$). The increase in isoniazid appeared to correlate with the subclinical liver injury as seen in the histopathology sections (Table 6.4).

Table 6.5: Average (mean ± SD) isoniazid concentrations of the INH group

Group	**INH**
	INH concentration
(n = 5)	**(µg/ml)**
2 Days	1.891 ± 0.57
7 Days	4.287 ± 1.50
14 Days	8.628 ± 6.82
28 Days	2.642 ± 0.81
42 Days	1.607 ± 1.19

INH = isoniazid

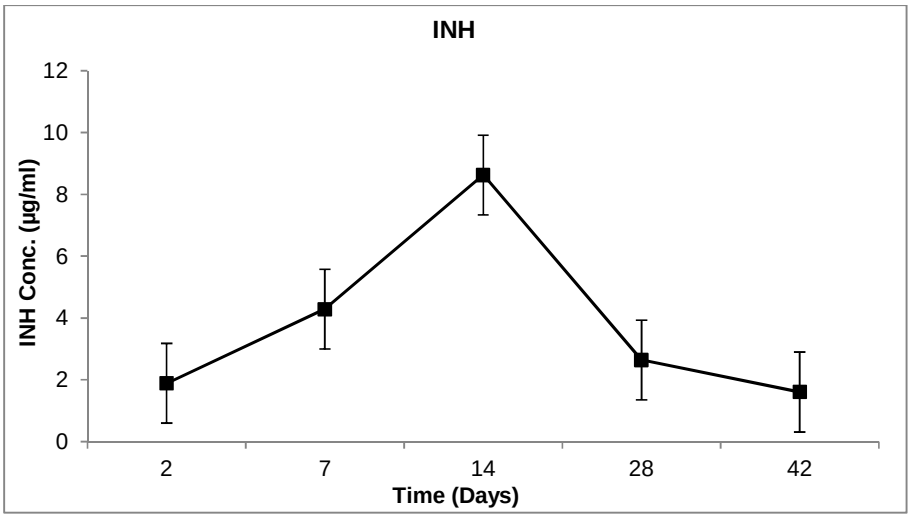

Figure 6.4: Isoniazid concentrations of the INH group over 42 days

6.3.3 Specific immunology tests

6.3.3.1 Direct observations

Table 6.6 shows changes in body weight of the S and INH groups over the treatment period. Both groups showed weight gain at all times, but the weight gain was greater in the S group from days 14 to 42 (Refer to Appendix H-1 and H-2 for baseline weights).

Table 6.6: Average (mean ± SD) change in rat weights of the S and INH groups

Group (n = 5)	S change in weight (g)	INH change in weight (g)
2 Days	9.2±4	7.0±6
7 Days	35.7±8	31.0±19
14 Days	84.6±5	55.0±10
28 Days	107.8±10	111.9±12
42 Days	171.4±27	141.3±14

S = saline; INH = isoniazid

6.3.3.2 Cytokines

Table 6.7 shows IL-2 and IL-10 concentrations of the S and INH groups, while Figures 6.5 a – b are graphical illustrations of the same. There was an increase in IL-2 from 2 to 7 days (p = 0.0500). Thereafter, IL-2 concentrations were the same and declined until day 42. IL-10 was similar in both groups, but that of the INH group tended to be lower most of the time.

Table 6.7: Average (mean ± SD) cytokine concentrations of the S and INH groups

Group	Cytokine	
(n = 3)	IL-2 (pg/ml)	IL-10 (pg/ml)
Untreated		
0 Days	65.46 ± 2.0	31.08 ± 1.2
S		
2 Days	74.87 ± 6.5	29.96 ± 2.8
7 Days	77.26 ± 5.8	34.57 ± 0.7
14 Days	77.58 ± 6.6	35.69 ± 5.4
28 Days	78.81 ± 4.6	32.46 ± 4.2
42 Days	74.39 ± 5.7	32.03 ± 2.5
INH		
2 Days	70.56 ± 1.5	30.26 ± 6.0
7 Days	83.80 ± 1.7	31.96 ± 0.7
14 Days	78.38 ± 5.8	32.46 ± 4.8
28 Days	77.42 ± 6.0	28.51 ± 5.2
42 Days	72.32 ± 5.9	34.32 ± 6.8

IL = interleukin; S = saline; INH = isoniazid

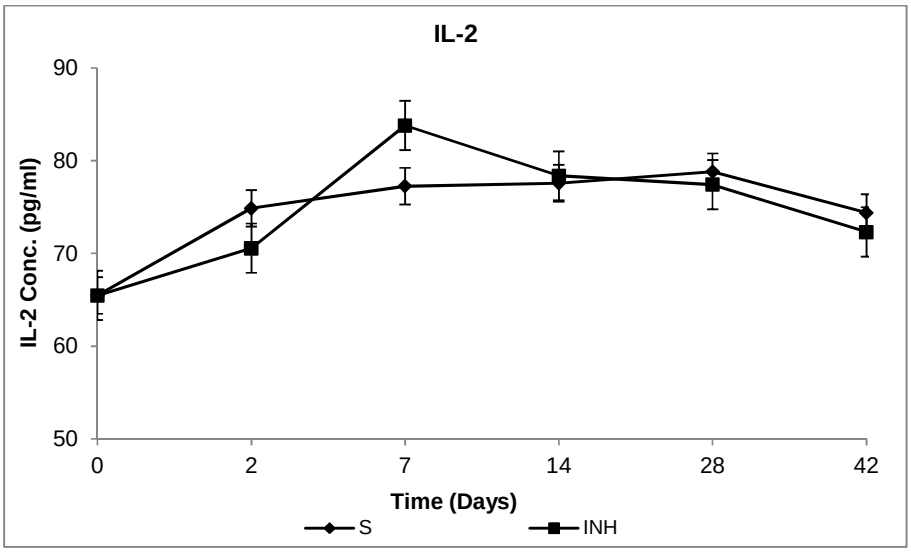

Figure 6.5 a): IL-2 concentrations of the S and INH groups over 42 days

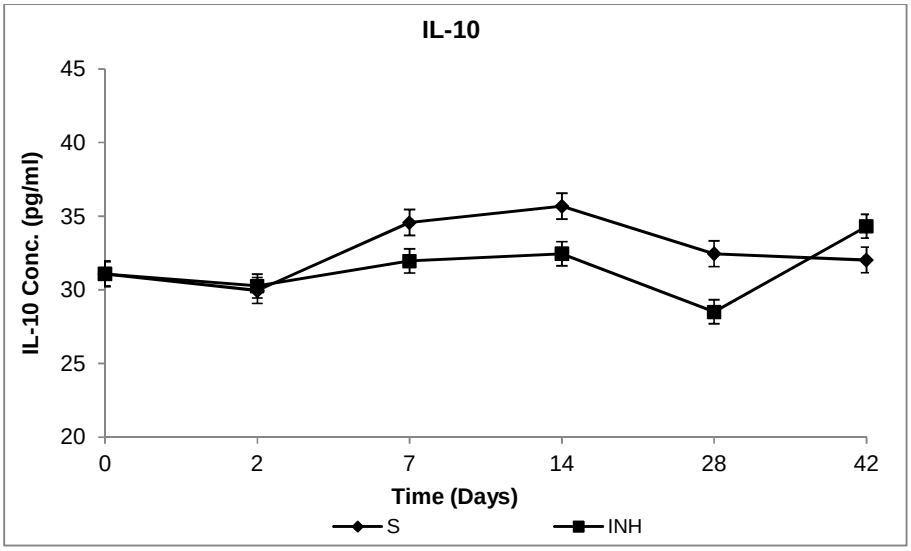

Figure 6.5 b): IL-10 concentrations of the S and INH groups over 42 days

6.3.3.3 CD4 and CD8 counts

Table 6.8 shows CD4 and CD8 counts of the S and INH groups, while Figures 6.6 a – b are graphical illustrations of the same. Over the 42 days, CD4 and CD8 counts of both groups showed some fluctuations, but these were not statistically significant. As lymphocyte and CD4 counts of the S group showed the same pattern of change, this was not observed in the INH group. Here, the CD4 count increased steadily between days 2 and 14, and then declined (p = 0.0500).

Table 6.8: Average (mean ± SD) CD4 and CD8 counts of the S and INH groups

Group	**Ly**	**T-Ly**	
		CD4	**CD8**
(n = 3)	**(x10^9/l)**	**(x10^9/l)**	**(x10^9/l)**
Untreated			
0 Days	4.67±1.8	2.23±1.3	1.42±0.7
S			
2 Days	5.18±0.7	2.27±0.3	1.35±0.2
7 Days	4.07±2.0	1.72±0.8	1.07±0.5
14 Days	4.21±0.7	1.69±0.2	1.17±0.2
28 Days	6.15±0.8	2.45±0.2	1.58±0.3
42 Days	3.23±0.3	1.47±0.1	0.79±0.2
INH			
2 Days	5.02±1.1	1.95±0.4	1.27±0.2
7 Days	5.10±0.8	2.20±0.5	1.28±0.3
14 Days	4.91±0.9	2.28±0.4	1.08±0.5
28 Days	4.56±0.5	1.76±0.4	1.24±0.1
42 Days	4.05±0.6	1.50±0.2	1.06±0.2

Ly = lymphocytes; CD = cluster of differentiation; S = saline; INH = isoniazid

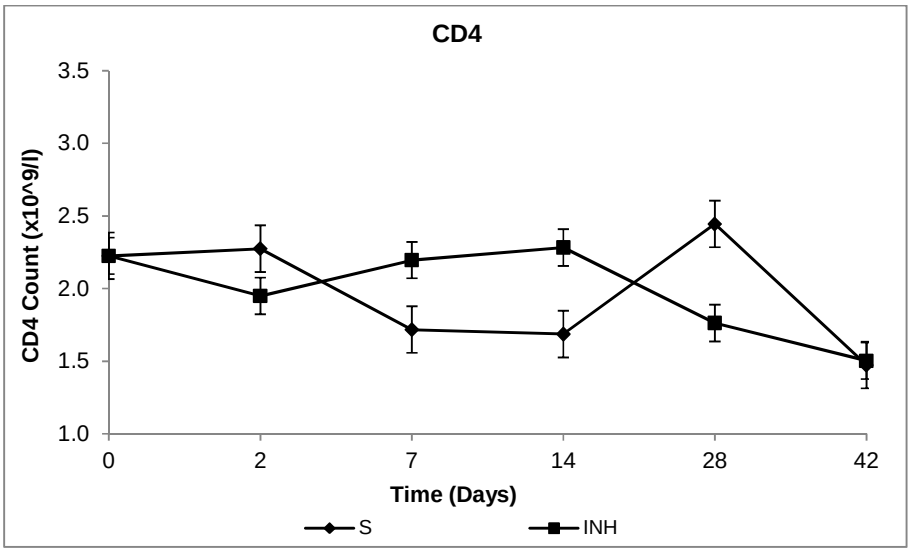

Figure 6.6 a): CD4 counts of the S and INH groups over 42 days

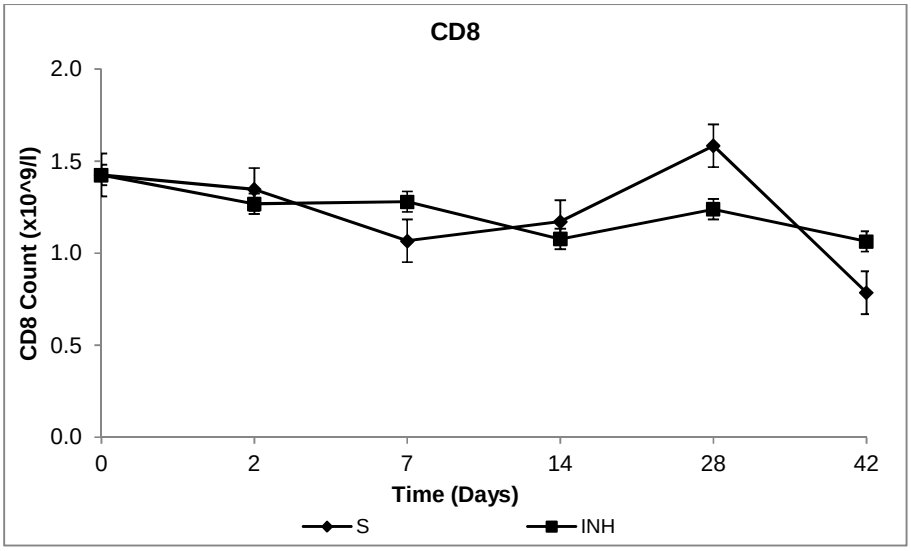

Figure 6.6 b): CD8 counts of the S and INH groups over 42 days

6.3.3.4 Immunoglobulins

Table 6.9 shows concentrations of IgM and IgG of the S and INH groups, while Figures 6.7 a – b are graphical illustrations of the same. IgM concentrations were lower in the INH group than in the S group, but were not statistically different. For both groups, IgG concentrations increased between days 14 and 42 ($p = 0.0500$).

Table 6.9: Average (mean ± SD) immunoglobulin concentrations of the S and INH groups

Group	**Immunoglobulin**	
	IgM	**IgG**
(n = 3)	**(mg/ml)**	**(mg/ml)**
Untreated		
0 Days	0.109 ± 0.02	14.434 ± 1.10
S		
2 Days	0.104 ± 0.04	14.137 ± 0.91
7 Days	0.110 ± 0.04	14.302 ± 0.70
14 Days	0.110 ± 0.03	12.617 ± 0.29
28 Days	0.075 ± 0.03	16.350 ± 1.00
42 Days	0.046 ± 0.01	17.109 ± 0.26
INH		
2 Days	0.057 ± 0.03	12.849 ± 0.34
7 Days	0.040 ± 0.02	14.765 ± 0.40
14 Days	0.046 ± 0.01	12.321 ± 1.24
28 Days	0.029 ± 0.01	13.707 ± 2.61
42 Days	0.027 ± 0.01	18.299 ± 0.94

IgM = immunoglobulin M; IgG = immunoglobulin G; S = saline; INH = isoniazid

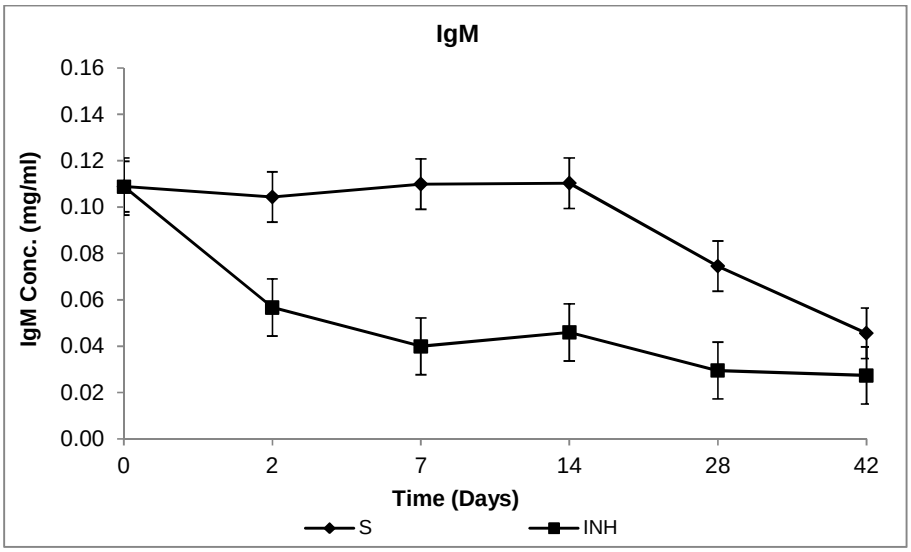

Figure 6.7 a): IgM concentrations of the S and INH groups over 42 days

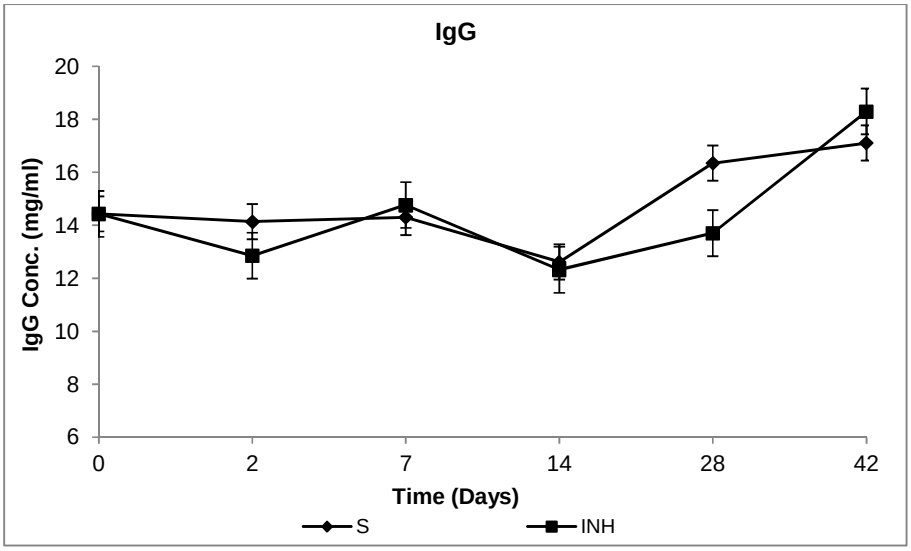

Figure 6.7 b): IgG concentrations of the S and INH groups over 42 days

6.3.4 Activity of rat CYP1A2, CYP2E1 and CYP3A2 *in vivo*

6.3.4.1 Protein concentrations

Table 6.10 and Figure 6.8 show the results of bovine serum albumin (BSA) calibration samples, used for the protein assay, while Table 6.11 is that of microsomal protein concentrations of untreated rats and the INH group. The absorption plot (Figure 6.8) is linear with a correlation coefficient (r^2) of 0.9937 and regression equation of y = 0.22x + 0.007. Final protein concentrations of microsomal liver samples from selected rats from the untreated and INH groups were calculated as indicated in Table 6.11. It appears that isoniazid resulted in a decrease in protein concentration after 14 days of treatment.

Table 6.10: Protein assay calibration data

BSA concentration (mg/ml)	Absorption (nm)
0.5	0.01
1	0.03
2.5	0.07
5	0.13
10	0.22

BSA = bovine serum albumin

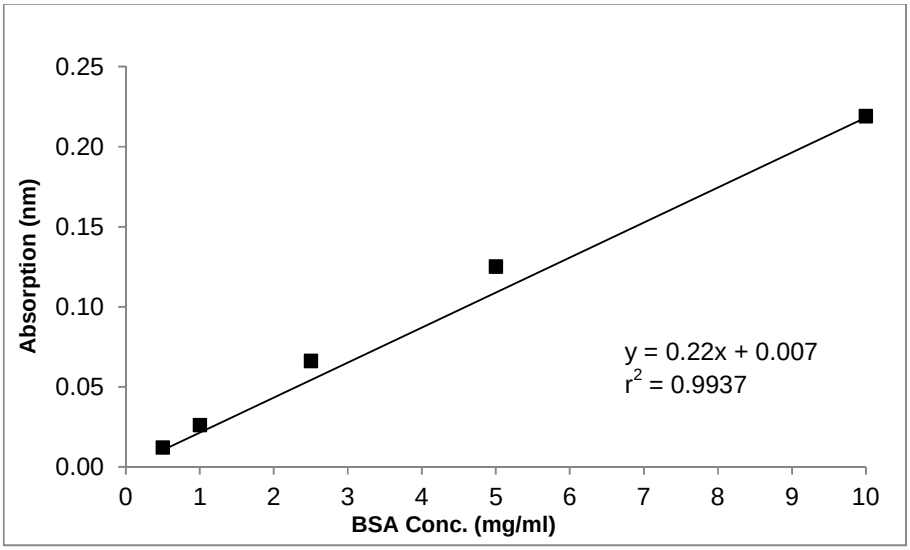

Figure 6.8: Calibration curve of BSA standards

Table 6.11: Average (mean ± SD) microsomal protein concentrations of the untreated and INH groups

Selected liver (n = 3)	Protein concentration (mg/ml)	Absorption (nm)
Untreated		
Rat 1	49.40 ± 6.8	0.132 ± 0.02
Rat 2	55.02 ± 1.1	0.146 ± 0.00
Rat 4	51.00 ± 5.1	0.136 ± 0.01
INH-2D		
Rat 1	31.05 ± 1.8	0.059 ± 0.00
Rat 2	38.52 ± 0.3	0.077 ± 0.00
Rat 3	51.76 ± 3.3	0.108 ± 0.01
INH-7D		
Rat 1	31.50 ± 0.3	0.078 ± 0.00
Rat 3	42.21 ± 2.2	0.105 ± 0.01
Rat 5	40.07 ± 2.5	0.100 ± 0.01
INH-14D		
Rat 1	20.00 ± 0.3	0.051 ± 0.00
Rat 4	23.91 ± 0.7	0.059 ± 0.00
Rat 5	34.73 ± 2.9	0.083 ± 0.01

INH = isoniazid; D = days

6.3.4.2 CYP1A2, CYP2E1 and CYP3A2 activity in vivo

Table 6.12 shows CYP1A2, CYP2E1 and CYP3A2 activity after 2, 7 and 14 days of isoniazid alone treatment, while Figures 6.9 a – c are graphical illustrations of the same. Treatment with isoniazid alone increased CYP1A2 activity, and this was different from the normal on days 7 and 14 ($p = 0.0286$). CYP2E1 activity was also increased, and was significantly higher than the normal at each time interval ($p = 0.0119$). CYP3A2 activity was not affected by INH alone treatment.

Table 6.12: Average (mean ± SD) CYP1A2, CYP2E1 and CYP3A2 activity

Group (n = 3)	CYP1A2 (pmol/min*mg)	CYP2E1 (nmol/min*mg)	CYP3A2 (pmol/min*mg)
Untreated			
0 Days	4.40 ± 0.8	0.77 ± 0.1	84.63 ± 6.9
INH			
2 Days	5.00 ± 0.3	1.08 ± 0.2	75.94 ± 6.6
7 Days	8.25 ± 0.4	1.30 ± 0.3	87.91 ± 20.0
14 Days	10.12 ± 1.1	2.40 ± 1.4	74.09 ± 5.8

INH = isoniazid

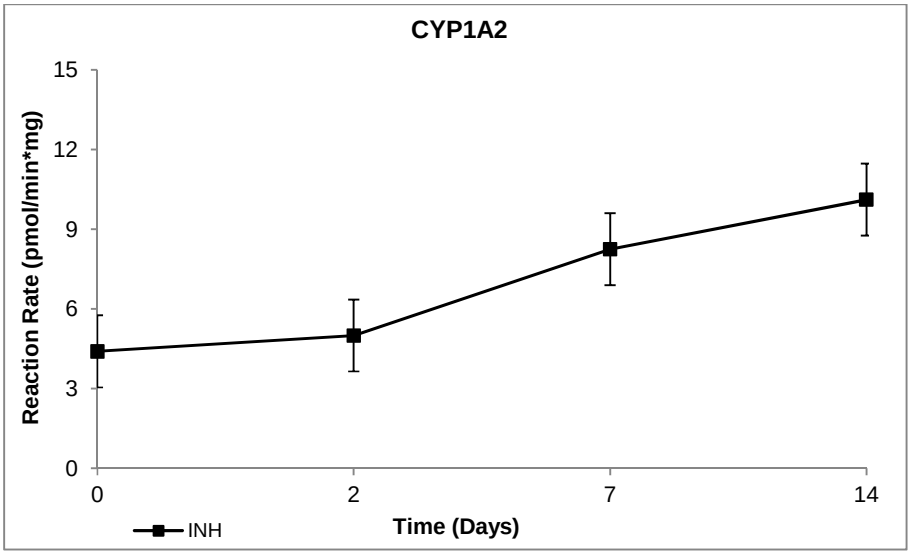

Figure 6.9 a): CYP1A2 activity after isoniazid alone treatment

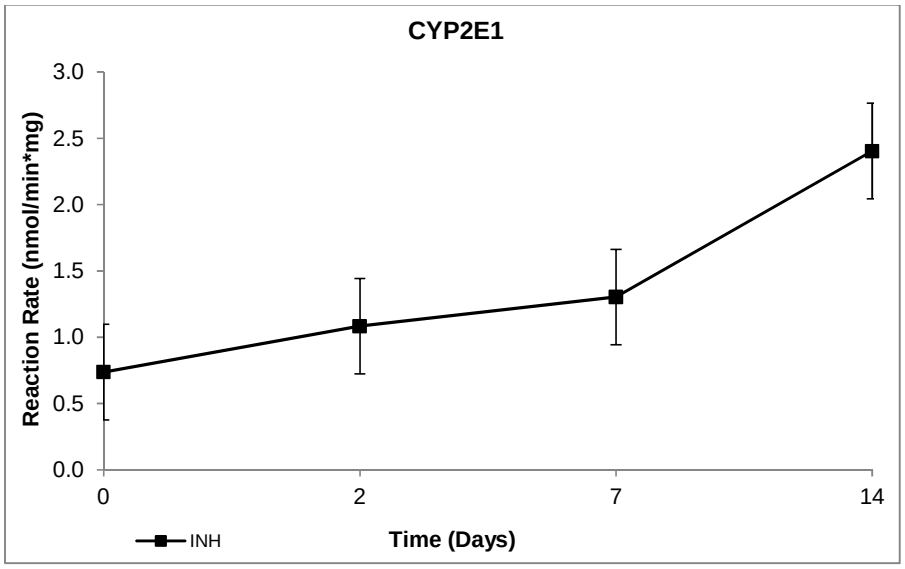

Figure 6.9 b): CYP2E1 activity after isoniazid alone treatment

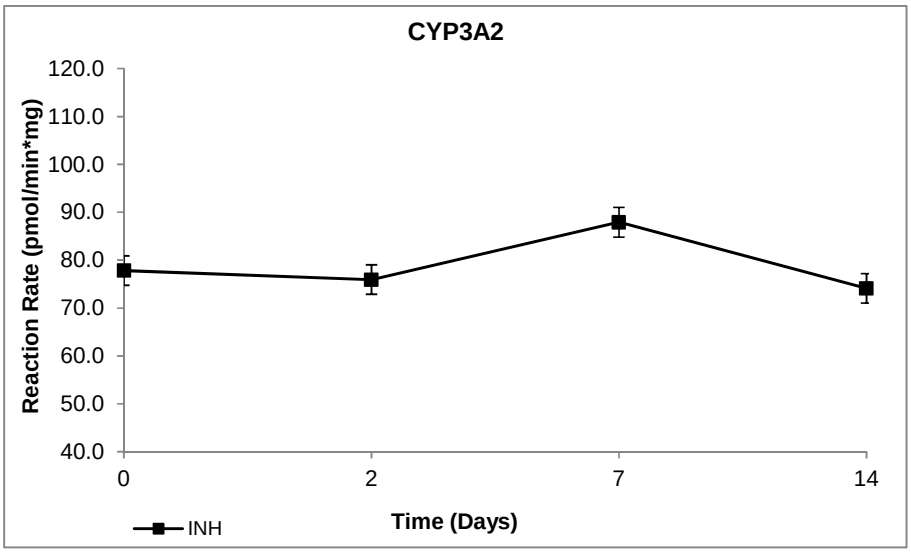

Figure 6.9 c): CYP3A2 activity after isoniazid alone treatment

6.3.5 Main observations

- ALT observations did not correlate with the histopathology changes, which implies that the liver injury was subclinical (as intended).
- From the histopathology, isoniazid caused liver injury up to 28 days, and had improved by day 42.
- The increased isoniazid concentrations seemed to correlate with the subclinical liver injury as seen in the histopathology sections.
- A Th1 response (increased IL-2) was observed early (day 7) with isoniazid alone treatment and this shifted to a later (day 42) Th2 response (increased IL-10).
- The increased CD4 count correlated with the early IL-2 levels.
- The increased IgG at a later stage of isoniazid alone treatment correlates with the increased IL-10.
- Isoniazid alone increased CYP1A2 and CYP2E1 activity, but had no effect on CYP3A2 activity.

B. Phase II: Co-treatment with an immune stimulant

6.3.6 Physiological observations (function tests)

6.3.6.1 Full blood count

Table 6.13 shows results of the full blood count of the S, S+LMS, INH and INH+LMS groups. The changes of red blood count parameters as observed for isoniazid and levamisole co-treatment share a common pattern with that of isoniazid alone (Section 6.3.1.1). By the end of treatment, haemoglobin, haematocrit, MCV and MCHC values were statistically different between the two groups ($p = 0.0500$). Furthermore, the white cell count, neutrophils and lymphocytes in the INH+LMS group had declined by day 42 ($p = 0.0500$), and this was statistically different from the INH group ($p = 0.0500$).

Table 6.13: Average (mean ± SD) full blood count and platelets results of the S, S+LMS, INH and INH+LMS groups

Group (n = 3)	RCC ($x10^{12}$/l)	Hb (g/dl)	Hct (l/l)	MCV (fl)	MCH (pg)	MCHC (g/dl)	Plt ($x10^9$/l)	WCC ($x10^9$/l)	Neu ($x10^9$/l)	Ly ($x10^9$/l)	Mo ($x10^9$/l)	Eos ($x10^9$/l)	Bas ($x10^9$/l)
Untreated													
0 Days	6.28±0.2	12.9±0.3	0.398±0.01	63.5±2.5	20.5±0.4	32.3±0.8	860±221.1	6.95±2.7	0.77±0.2	4.67±1.8	0.19±0.1	0.02±0.0	0.00±0.0
S													
2 Days	6.67±0.2	13.7±0.1	0.422±0.01	63.3±2.3	20.6±0.6	32.4±0.6	849±81.6	6.50±0.9	0.60±0.2	5.18±0.7	0.21±0.0	0.50±0.2	0.01±0.0
7 Days	7.53±0.9	15.3±1.7	0.451±0.04	60.1±2.5	20.3±0.2	33.9±1.2	1033±79.8	5.44±2.4	1.03±0.8	4.07±2.0	0.30±0.3	0.04±0.0	0.01±0.0
14 Days	6.77±0.6	13.9±1.1	0.417±0.03	61.8±2.8	20.5±0.6	33.2±0.6	721±196.4	5.22±1.2	0.63±0.5	4.21±0.7	0.18±0.1	0.18±0.1	0.05±0.0
28 Days	7.07±0.7	13.9±1.3	0.390±0.04	55.1±1.0	19.7±0.1	35.8±0.6	961±172.5	7.38±1.0	0.91±0.2	6.15±0.8	0.24±0.1	0.07±0.0	0.01±0.0
42 Days	6.93±0.8	13.4±1.8	0.374±0.05	53.9±1.0	19.3±0.4	35.8±0.2	839±166.0	3.93±0.3	0.54±0.1	3.23±0.3	0.11±0.0	0.04±0.0	0.01±0.0
S+LMS													
2 Days	6.53±0.4	13.2±0.6	0.408±0.03	62.5±0.5	20.3±0.5	32.4±0.7	755±239.0	7.33±0.2	0.66±0.0	5.99±0.4	0.35±0.0	0.34±0.1	0.01±0.0
7 Days	6.91±0.0	13.4±0.0	0.408±0.00	59.0±0.0	19.4±0.0	32.8±0.0	850±00.0	6.75±0.0	0.93±0.0	5.10±0.0	0.38±0.0	0.33±0.0	0.01±0.0
14 Days	6.99±0.4	13.8±0.8	0.427±0.02	61.1±1.6	19.8±0.1	32.4±0.8	718±204.1	4.65±1.7	0.53±0.3	3.82±1.8	0.12±0.1	0.18±0.3	0.00±0.0
28 Days	7.41±0.2	14.9±0.4	0.436±0.01	58.8±0.2	20.1±0.4	34.1±0.8	578±62.1	5.13±1.8	0.71±0.1	4.27±1.6	0.10±0.1	0.04±0.0	0.01±0.0
42 Days	7.91±0.2	15.6±0.4	0.455±0.02	57.5±0.7	19.7±0.2	34.2±0.5	701±116.7	6.76±0.8	0.85±0.1	5.69±0.9	0.16±0.1	0.04±0.0	0.00±0.0
INH													
2 Days	6.57±0.3	13.4±0.4	0.416±0.01	63.4±2.0	20.5±0.3	32.3±0.7	880±54.5	5.86±1.1	0.59±0.0	5.02±1.1	0.21±0.1	0.03±0.0	0.00±0.0
7 Days	6.05±0.6	12.7±1.1	0.381±0.04	63.0±0.6	21.0±0.2	33.3±0.2	478±19.1	5.98±0.8	0.37±0.3	5.10±0.8	0.19±0.1	0.02±0.0	0.01±0.0
14 Days	6.65±0.6	13.1±1.2	0.394±0.04	59.2±2.7	19.8±0.6	33.4±0.7	494±50.2	5.78±1.1	0.61±0.1	4.91±0.9	0.23±0.1	0.03±0.0	0.00±0.0
28 Days	7.27±0.4	14.3±0.8	0.406±0.02	55.9±0.6	19.7±0.1	35.3±0.4	845±116.0	5.44±0.4	0.66±0.1	4.56±0.5	0.17±0.1	0.04±0.0	0.01±0.0
42 Days	7.25±0.7	13.9±1.2	0.390±0.04	53.8±1.9	19.2±0.4	35.6±0.8	785±152.7	5.11±0.9	0.84±0.1	4.05±0.6	0.15±0.1	0.07±0.0	0.01±0.0
INH+LMS													
2 Days	6.73±0.4	13.6±0.7	0.404±0.02	60.2±1.1	20.2±0.3	33.6±0.3	1181±45.3	7.55±0.9	0.90±0.0	6.35±0.9	0.23±0.1	0.07±0.0	0.00±0.0
7 Days	6.49±0.2	13.1±0.4	0.397±0.01	61.1±1.1	20.1±0.3	33.0±0.2	806±63.8	6.96±1.5	0.73±0.2	5.64±1.2	0.34±0.2	0.24±0.2	0.01±0.0
14 Days	6.60±0.3	13.3±0.8	0.408±0.03	61.9±1.4	20.2±0.2	32.6±0.5	816±55.1	6.19±1.2	0.64±0.1	5.26±1.0	0.24±0.3	0.04±0.0	0.01±0.0
28 Days	7.13±0.1	16.2±0.6	0.421±0.00	59.1±1.2	22.7±1.0	38.4±1.4	562±20.6	6.03±0.2	0.64±0.6	4.81±0.3	0.17±0.1	0.04±0.0	0.00±0.0
42 Days	7.83±0.2	15.1±0.3	0.445±0.01	56.9±0.5	19.3±0.2	33.9±0.1	643±63.1	5.91±0.6	0.69±0.1	5.00±0.6	0.18±0.0	0.04±0.0	0.01±0.0

RCC = red cell count; Hb = haemoglobin; Hct = haematocrit; MCV = mean corpuscular volume; MCH = mean corpuscular haemoglobin; MCHC = mean corpuscular haemoglobin concentration; Plt = platelets; WCC = white cell count; Neu = neutrophils; Ly = lymphocytes; Mo = monocytes; Eos = eosinophils; Bas = basophils; S = saline; LMS = levamisole; INH = isoniazid

6.3.6.2 Renal function tests

Table 6.14 shows the changes of BUN and Cr of the S, S+LMS, INH and INH+LMS groups. In all groups BUN and Cr levels were normal. Here, Cr levels also spiked on day 28, but were still within the normal range (p = 0.0500).

Table 6.14: Average (mean ± SD) renal function test results of the S, S+LMS, INH and INH+LMS groups

Group	**RFT**		**Group**	**RFT**	
	BUN	**Cr**		**BUN**	**Cr**
(n = 3)	**(mmol/l)**	**(µmol/l)**	**(n = 3)**	**(mmol/l)**	**(µmol/l)**
Untreated					
0 Days	7.2 ± 1	37 ± 8			
S			**INH**		
2 Days	7.3 ± 1	39 ± 2	2 Days	6.7 ± 0	34 ± 6
7 Days	8.1 ± 0	46 ± 7	7 Days	6.6 ± 0	36 ± 2
14 Days	7.5 ± 1	39 ± 3	14 Days	6.2 ± 1	43 ± 8
28 Days	10.6 ± 2	73 ± 17	28 Days	7.3 ± 1	69 ± 4
42 Days	5.8 ± 1	38 ± 9	42 Days	5.8 ± 0	34 ± 21
S+LMS			**INH+LMS**		
2 Days	8.3 ±1	38 ± 5	2 Days	6.9 ± 1	37 ± 9
7 Days	7.8 ± 1	40 ± 18	7 Days	7.1 ± 1	33 ± 3
14 Days	6.5 ± 1	35 ± 5	14 Days	6.4 ± 0	35 ± 2
28 Days	6.1 ± 0	60 ± 3	28 Days	6.3 ± 1	63 ± 2
42 Days	6.5 ± 1	21 ± 1	42 Days	5.9 ± 0	24 ± 1

RFT = renal function test; BUN = blood urea nitrogen; Cr = creatinine; S = saline; LMS = levamisole; INH = isoniazid

6.3.6.3 Liver function tests

Table 6.15 shows the changes of ALT, AST and ALP of the S, S+LMS, INH and INH+LMS groups. Over the 42 days, the results were similar in all groups.

Table 6.15: Average (mean ± SD) liver function test results of the S, S+LMS, INH and INH+LMS groups

Group	LFT			Group	LFT		
	ALT	AST	ALP		ALT	AST	ALP
(n = 3)	(U/l)	(U/l)	(U/l)	(n = 3)	(U/l)	(U/l)	(U/l)
Untreated							
0 Days	50±5	88±14	352±76				
S				**INH**			
2 Days	46±2	90±7	400±7	2 Days	46±4	104±7	335±32
7 Days	49±10	103±25	304±13	7 Days	53±13	233±223	369±10
14 Days	58±4	127±37	508±37	14 Days	43±4	98±29	364±38
28 Days	47±2	115±44	216±19	28 Days	46±4	143±36	220±29
42 Days	46±6	76±28	109±76	42 Days	51±2	86±14	127±78
S+LMS				**INH+LMS**			
2 Days	40±3	113±53	541±9	2 Days	48±5	120±42	369±22
7 Days	52±15	90±27	483±130	7 Days	46±2	156±48	330±10
14 Days	48±12	73±16	478±105	14 Days	46±7	123±29	392±25
28 Days	50±7	75±19	127±63	28 Days	33±18	101±34	184±29
42 Days	46±2	75±6	24±11	42 Days	38±4	64±6	32±19

LFT = liver function test; ALT = alanine aminotransferase; AST = aspartate aminotransferase; ALP = alkaline phosphatase; S = saline; LMS = levamisole; INH = isoniazid

6.3.6.4 Liver histopathology

(a) Liver histopathology reports

Liver sections for histopathology (Figures 6.10 a – t) were randomly selected, and the main histopathology lesions are summarised in the tally tables (Tables 6.16 a and b). The following report is a summary of the features of the lesion:

i. Figures 6.10 a and b: Liver sections A and B from the S+LMS group after 2 days of saline and levamisole co-treatment

Representative photographs of rat livers after 2 days of daily saline and levamisole co-treatment. The report: “Sections A and B shows normal appearing hepatic parenchymal cells, as well as multiple normal nuclei.”

ii. Figures 6.10 c and d: Liver sections A and B from the S+LMS group after 7 days of saline and levamisole co-treatment

Representative photographs of rat livers after 7 days of daily saline and levamisole co-treatment. The report: “A mitotic figure is present in section A, which is indicative of normal morphology. Section B shows a mitotic figure in metaphase.”

iii. Figures 6.10 e and f: Liver sections A and B from the S+LMS group after 14 days of saline and levamisole co-treatment

Representative photographs of rat livers after 14 days of daily saline and levamisole co-treatment. The report: “Section A illustrates normal hepatic parenchyma in the portal area of the liver. In section B minimal vacuolar degeneration is observed within the hepatocytes. Loss of cell boundaries could be detected.”

iv. Figures 6.10 g and h: Liver sections A and B from the S+LMS group after 28 days of saline and levamisole co-treatment

Representative photographs of rat livers after 28 days of daily saline and levamisole co-treatment. The report: “Minimal hepatic cytoplasmic swelling and vacuolar degeneration are present in sections A and B. A mitotic figure is demonstrated in section B.”

v. Figures 6.10 i and j: Liver sections A and B from the S+LMS group after 42 days of saline and levamisole co-treatment

Representative photographs of rat livers after 42 days of daily saline and levamisole co-treatment. The report: “Sections A and B illustrate normal hepatocytic cords with minimal vacuolar changes within the cytoplasm.”

vi. Figures 6.10 k and l: Liver sections A and B from the INH+LMS group after 2 days of isoniazid and levamisole co-treatment

Representative photographs of rat livers after 2 days of daily isoniazid and levamisole co-treatment. The report: “The granular appearance of the hepatic cytoplasm is minimal, while minimal cytonecrosis and loss of nuclei could be detected in the parenchyma of sections A and B.”

vii. Figures 6.10 m and n: Liver sections A and B from the INH+LMS group after 7 days of isoniazid and levamisole co-treatment

Representative photographs of rat livers after 7 days of daily isoniazid and levamisole co-treatment. The report: “The degeneration is graded mild to moderate in sections A and B, respectively. Mild single cell necrosis (cytonecrosis) could be demonstrated in the parenchyma of both liver sections.”

viii. Figures 6.10 o and p: Liver sections A and B from the INH+LMS group after 14 days of isoniazid and levamisole co-treatment

Representative photographs of rat livers after 14 days of daily isoniazid and levamisole co-treatment. The report: “Degenerative changes, such as moderate granular vacuolar degeneration and cell swelling were seen in liver sections A and B. Mild cytonecrosis was present in both sections, and was characterised by loss of cell boundaries, disruption of the cytoplasm and irregular appearance of the hepatic parenchyma. Only minimal zonal necrosis was observed in section B. Furthermore, no hepatocyte mitosis was observed.”

ix. Figures 6.10 q and r: Liver sections A and B from the INH+LMS group after 28 days of isoniazid and levamisole co-treatment

Representative photographs of rat livers after 28 days of daily isoniazid and levamisole co-treatment. The report: “Moderate to severe granular vacuolar degeneration and cell swelling were observed in sections A and B. This was characterised by the cloudy and granular appearance of the hepatocytes, along with a loss of well-organised hepatocytic cords. Cytonecrosis was graded as mild to moderate in both sections. Centrilobular zonal necrosis was minimal, while hepatocyte mitosis was absent.”

x. Figures 6.10 s and t: Liver sections A and B from the INH+LMS group after 42 days of isoniazid and levamisole co-treatment

Representative photographs of rat livers after 42 days of daily isoniazid and levamisole co-treatment. The report: “Granular vacuolar degeneration, cell swelling and cytonecrosis are graded as mild in liver sections A and B.”

In view of the histopathology photographs (Figures 6.10 a – t), reports and tally tables (Tables 6.16 a and b), it was concluded that co-treatment with isoniazid and levamisole caused liver injury up to 28 days, and this was more severe than with isoniazid alone.

(b) Liver histopathology photographs

Figures 6.10 a – t are representative of randomly selected liver sections of the S+LMS and INH+LMS groups.

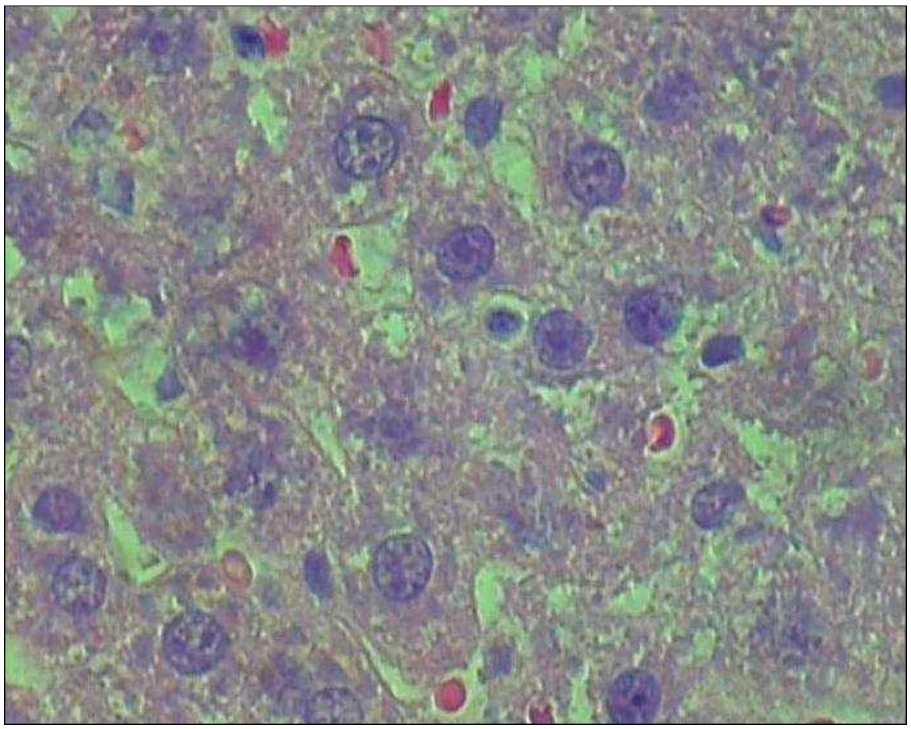

Figure 6.10 a): Liver section A from the S+LMS group after 2 days of treatment, showing the presence of nuclei and hepatic cell cords

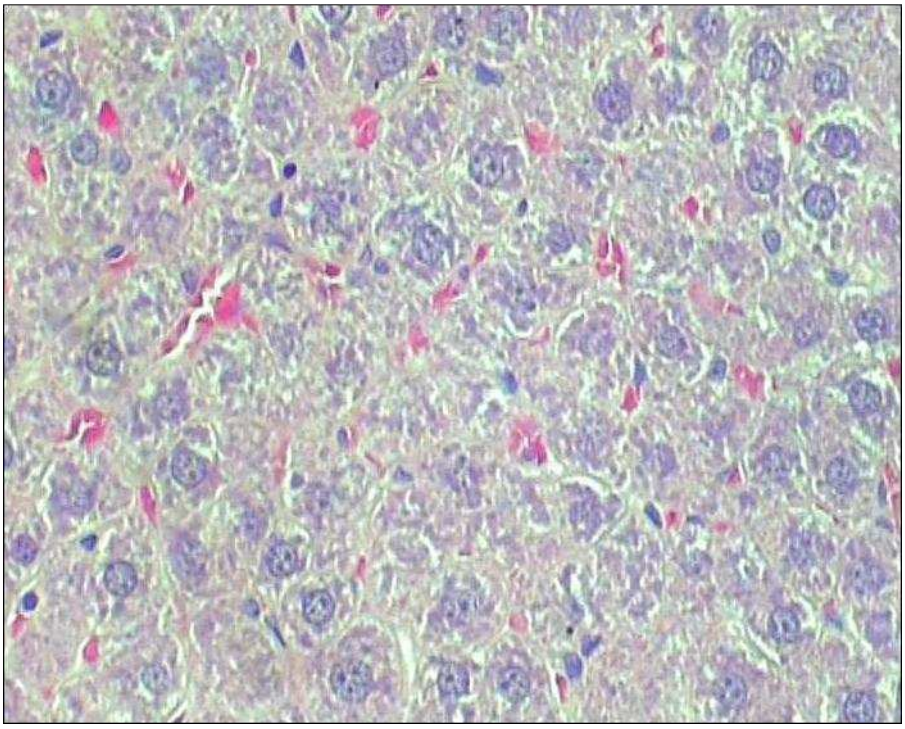

Figure 6.10 b): Liver section B from the S+LMS group after 2 days of treatment, showing normal appearing hepatic parenchymal cells and multiple normal nuclei

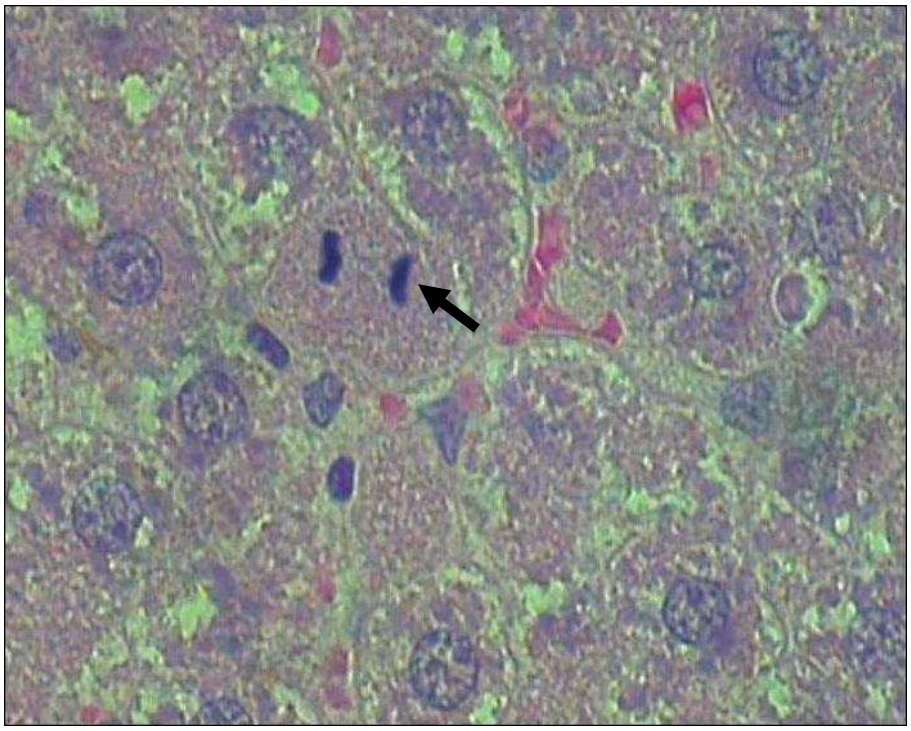

Figure 6.10 c): Liver section A from the S+LMS group after 7 days of treatment, showing a mitotic figure in the hepatocytes

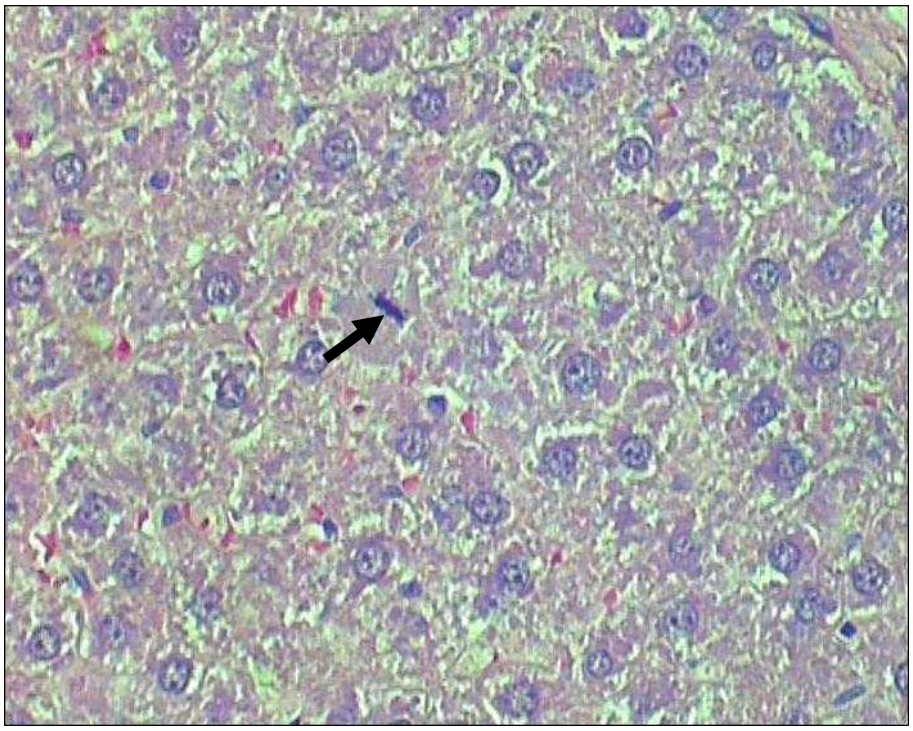

Figure 6.10 d): Liver section B from the S+LMS group after 7 days of treatment, showing a mitotic figure in metaphase

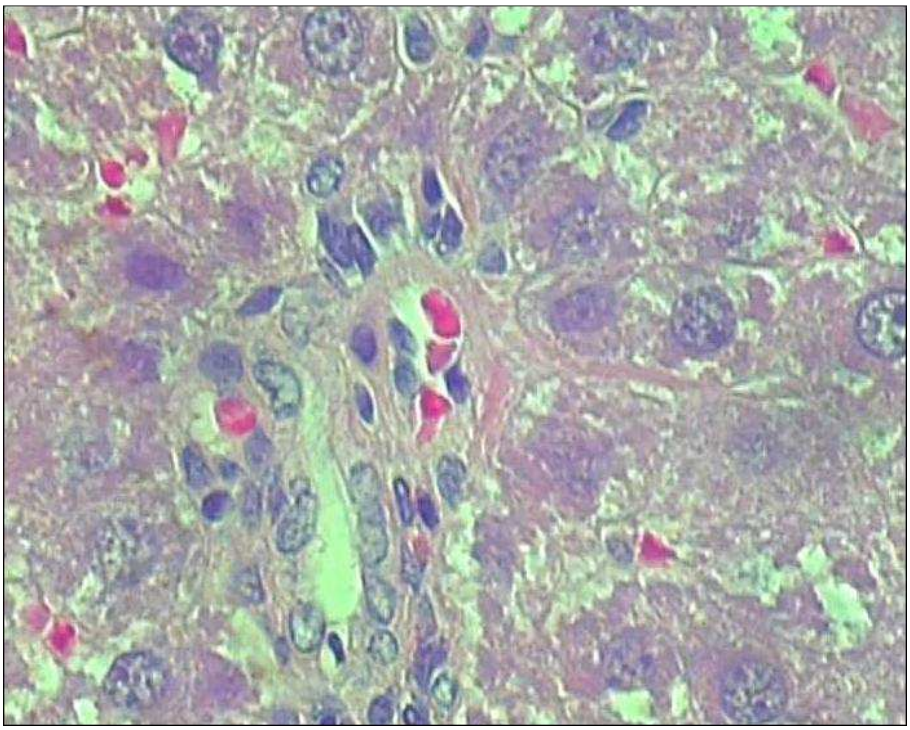

Figure 6.10 e): Liver section A from the S+LMS group after 14 days of treatment, showing normal hepatic parenchyma in the portal area of the liver

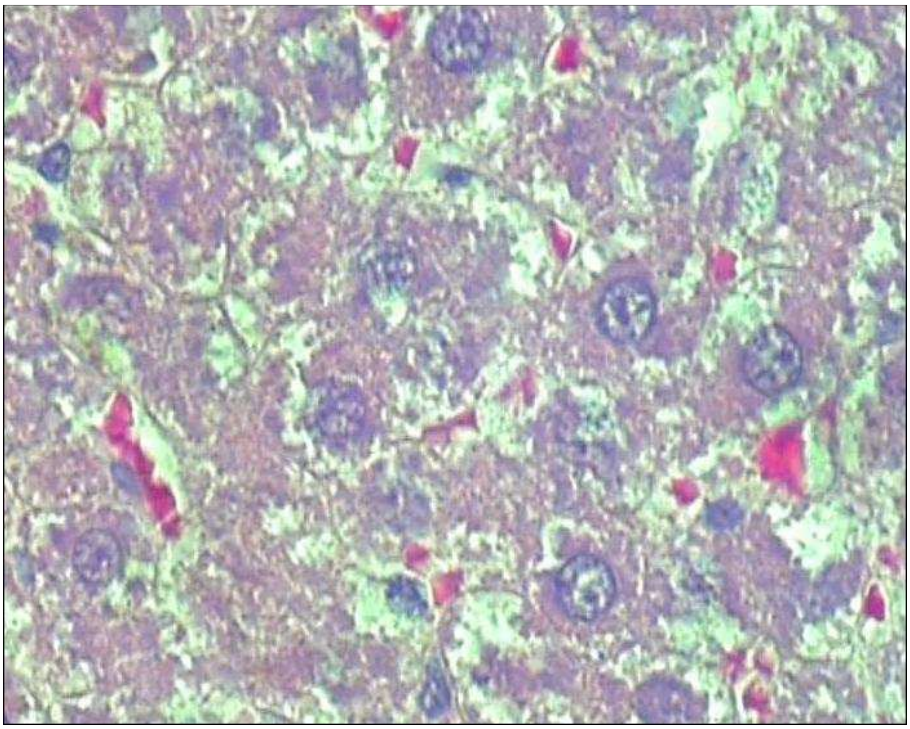

Figure 6.10 f): Liver section B from the S+LMS group after 14 days of treatment, showing minimal vacuolar degeneration with vacuolated cytoplasm, and loss of cell boundaries

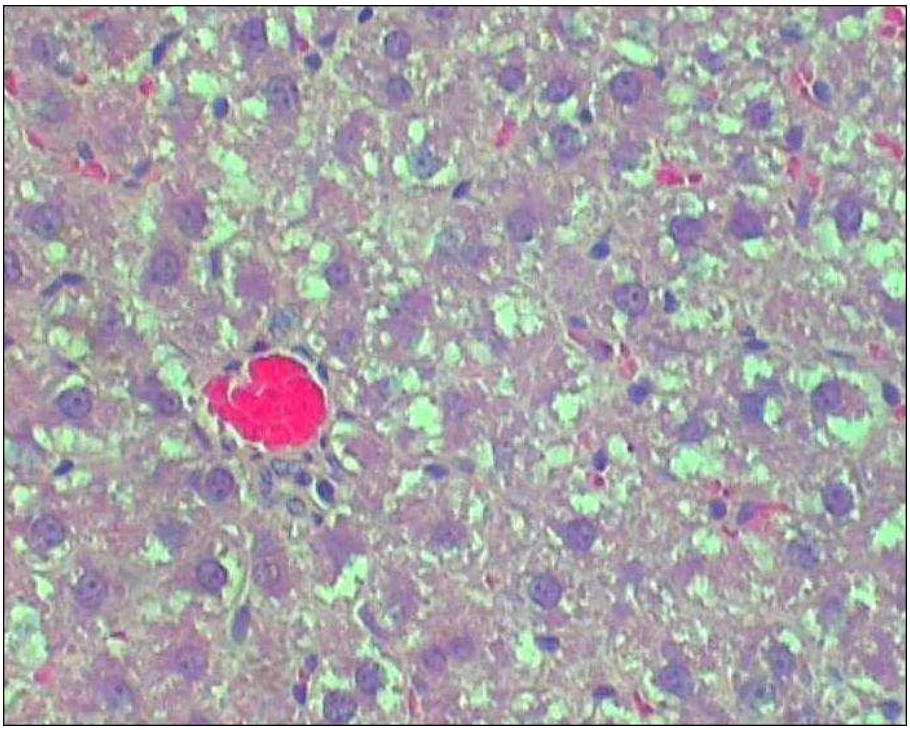

Figure 6.10 g): Liver section A from the S+LMS group after 28 days of treatment, showing minimal hepatic cytoplasmic swelling and vacuolar degeneration

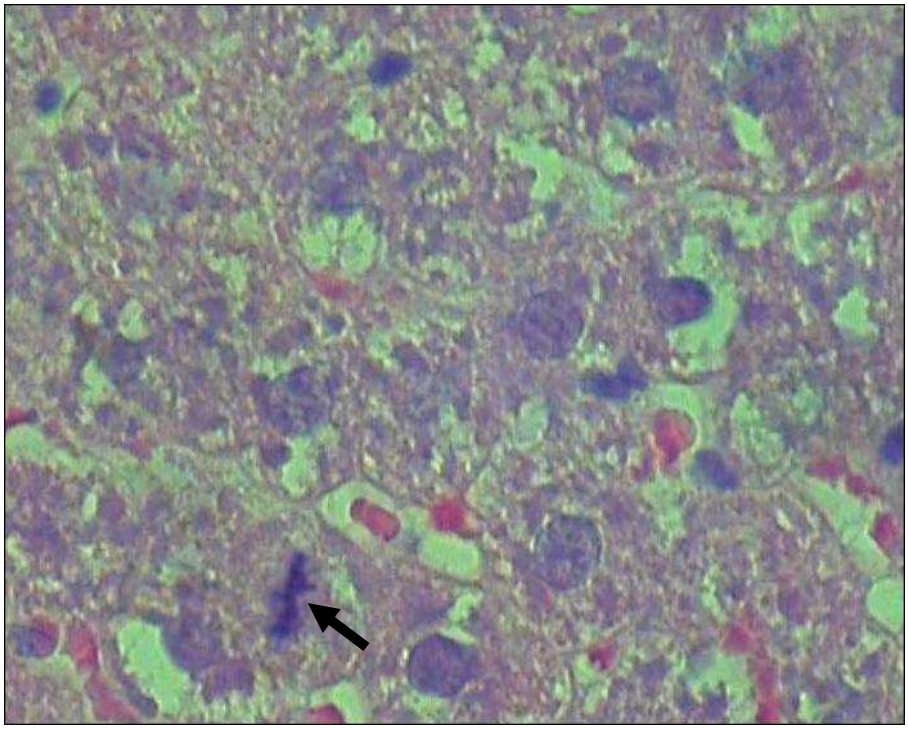

Figure 6.10 h): Liver section B from the S+LMS group after 28 days of treatment, showing minimal vacuolar degeneration, and a mitotic figure

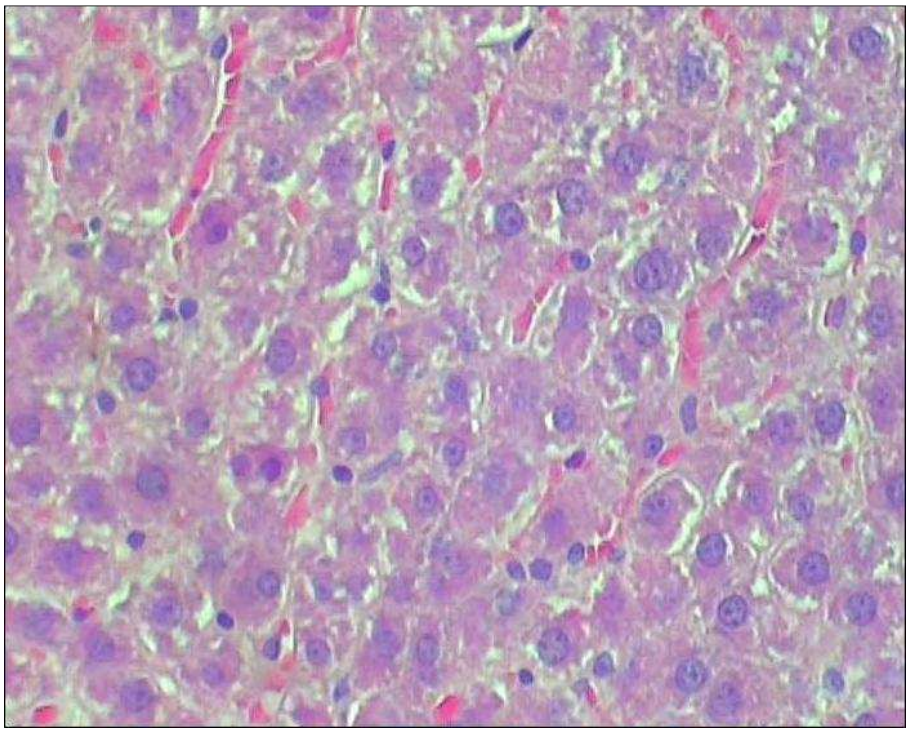

Figure 6.10 i): Liver section A from the S+LMS group after 42 days of treatment, showing normal hepatic cords and minimal vacuolar changes within the cytoplasm

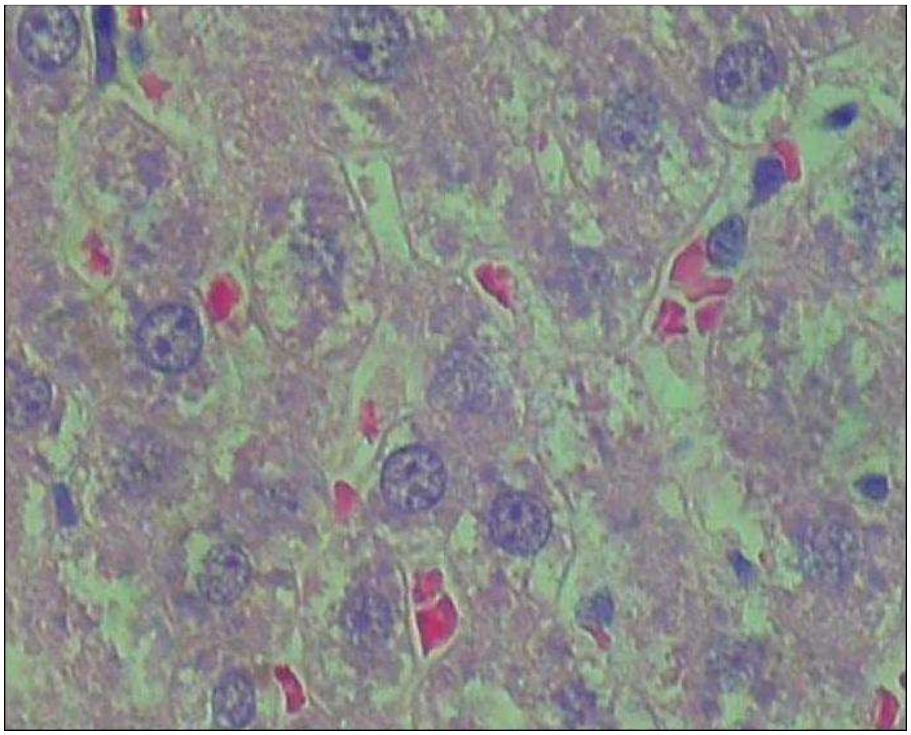

Figure 6.10 j): Liver section B from the S+LMS group after 42 days of treatment, showing minimal granular vacuolar degeneration and cell swelling

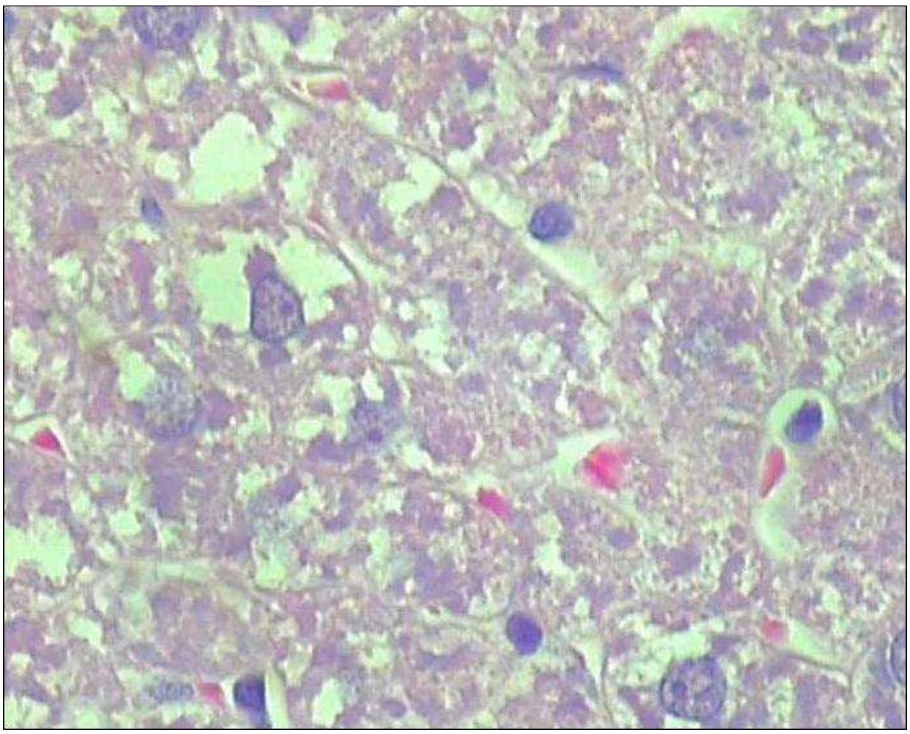

Figure 6.10 k): Liver section A from the INH+LMS group after 2 days of treatment, showing minimal degenerative changes

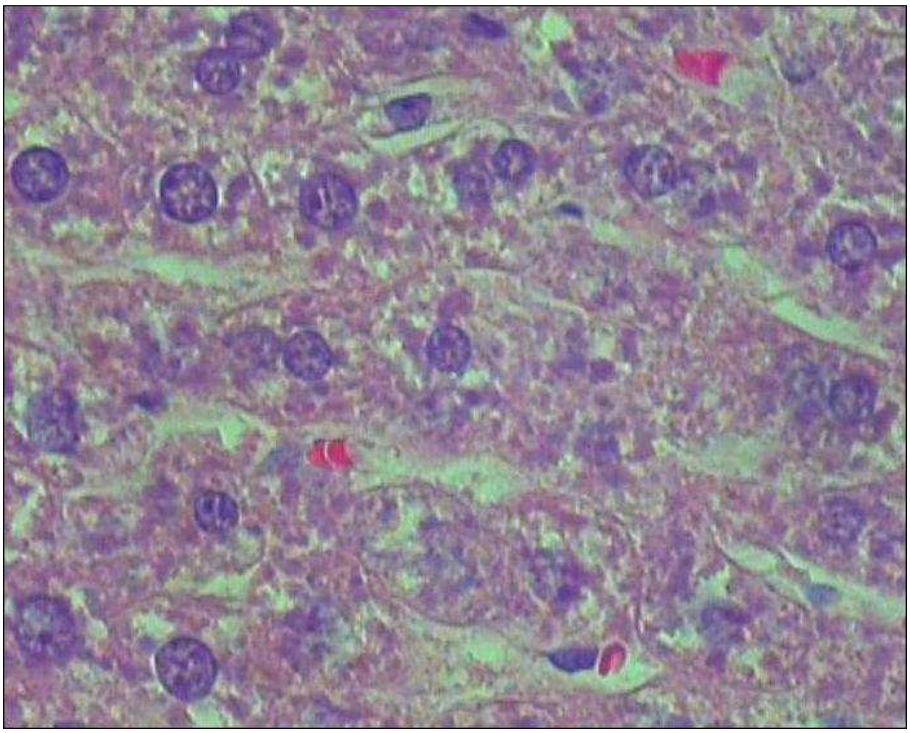

Figure 6.10 l): Liver section B from the INH+LMS group after 2 days of treatment, showing granular appearance of the hepatic cytoplasm, cytonecrosis and loss of nuclei

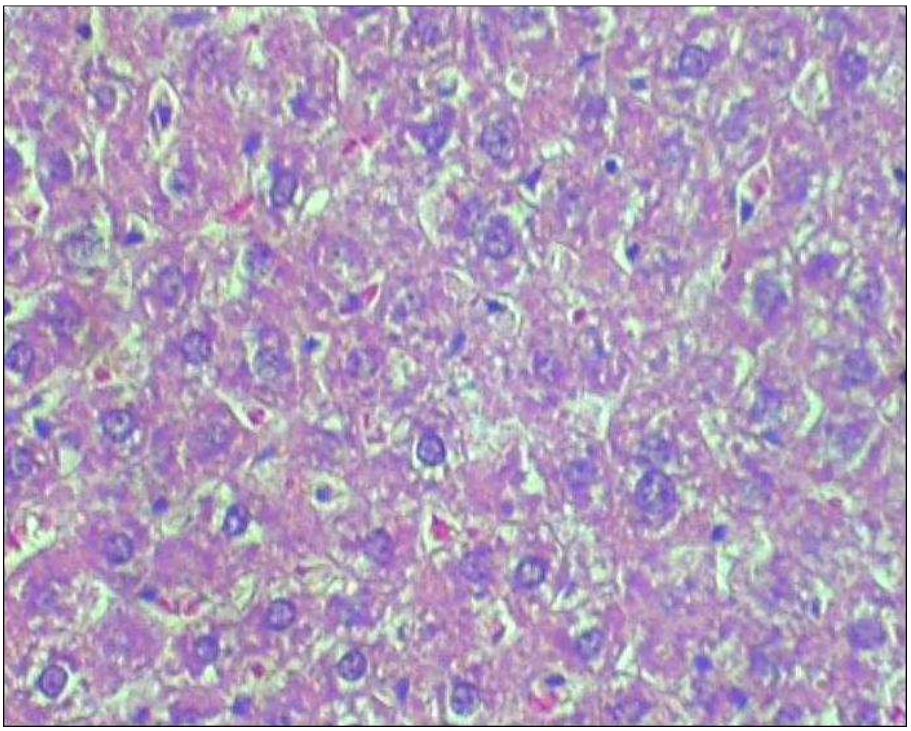

Figure 6.10 m): Liver section A from the INH+LMS group after 7 days of treatment, showing mild degeneration and single cell necrosis

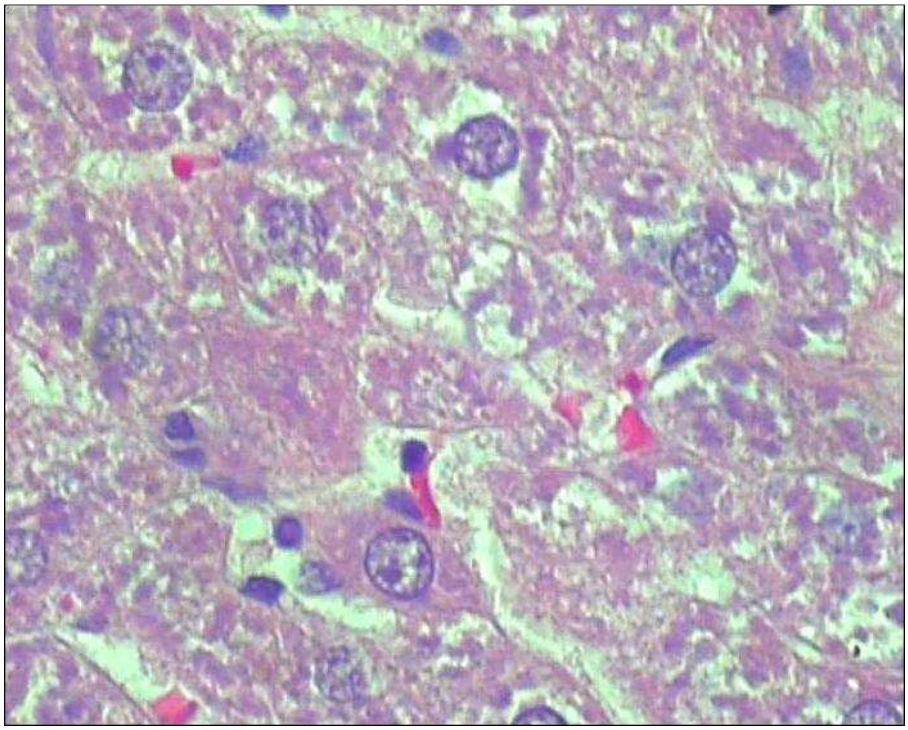

Figure 6.10 n): Liver section B from the INH+LMS group after 7 days of treatment, showing moderate degeneration and mild single cell necrosis

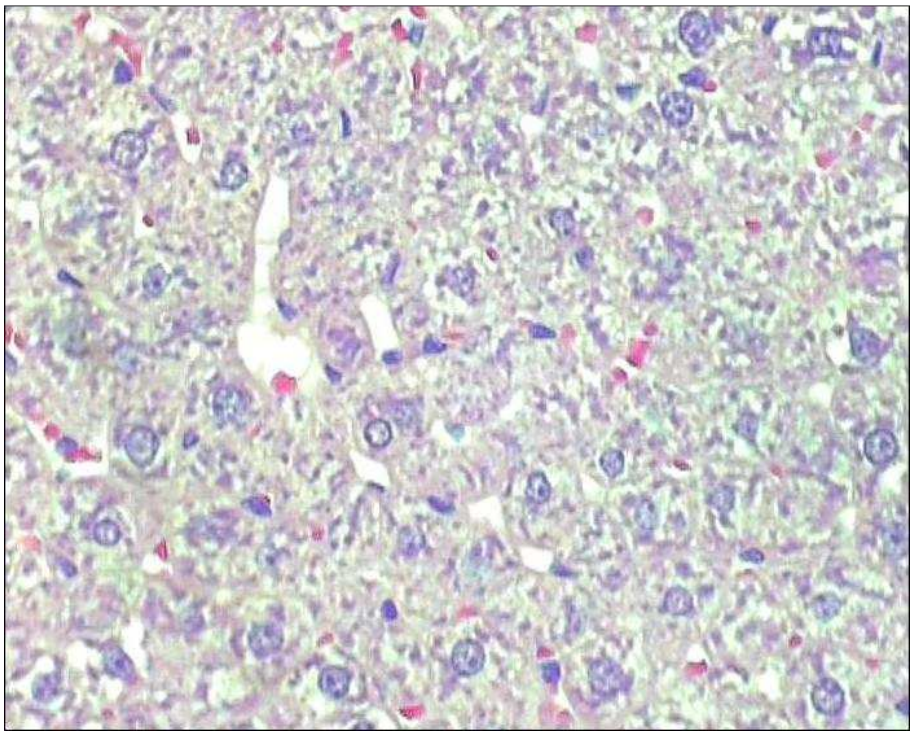

Figure 6.10 o): Liver section A from the INH+LMS group after 14 days of treatment, showing mild cytonecrosis with loss of cell boundaries, disruption of the cytoplasm and irregular appearance of the hepatic parenchyma

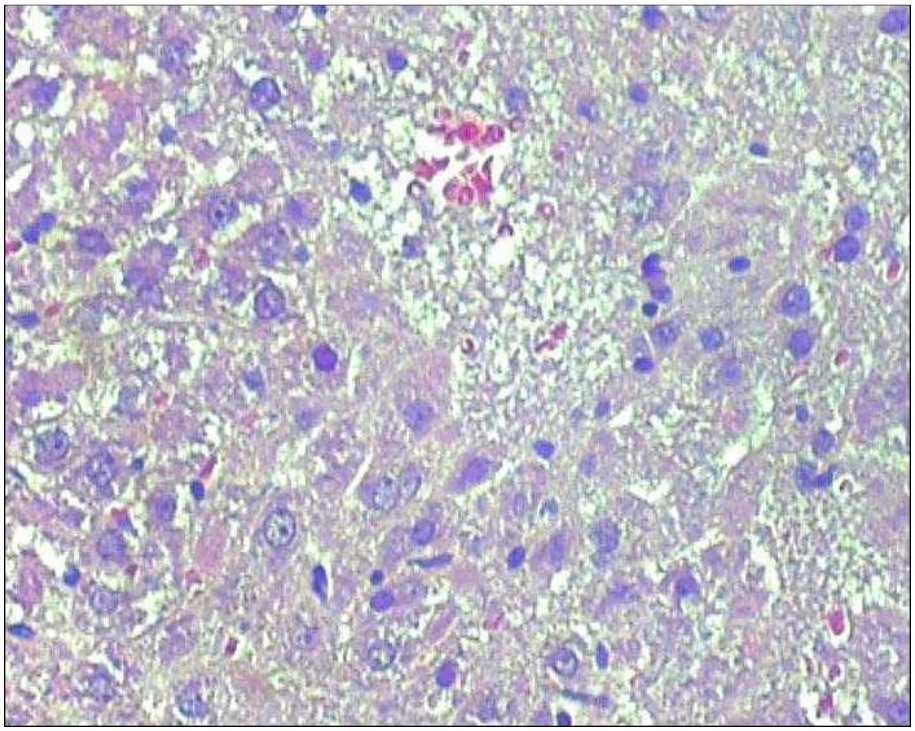

Figure 6.10 p): Liver section B from the INH+LMS group after 14 days of treatment, showing centrilobular zonal necrosis with breakdown of the reticular framework in the centrilobular area

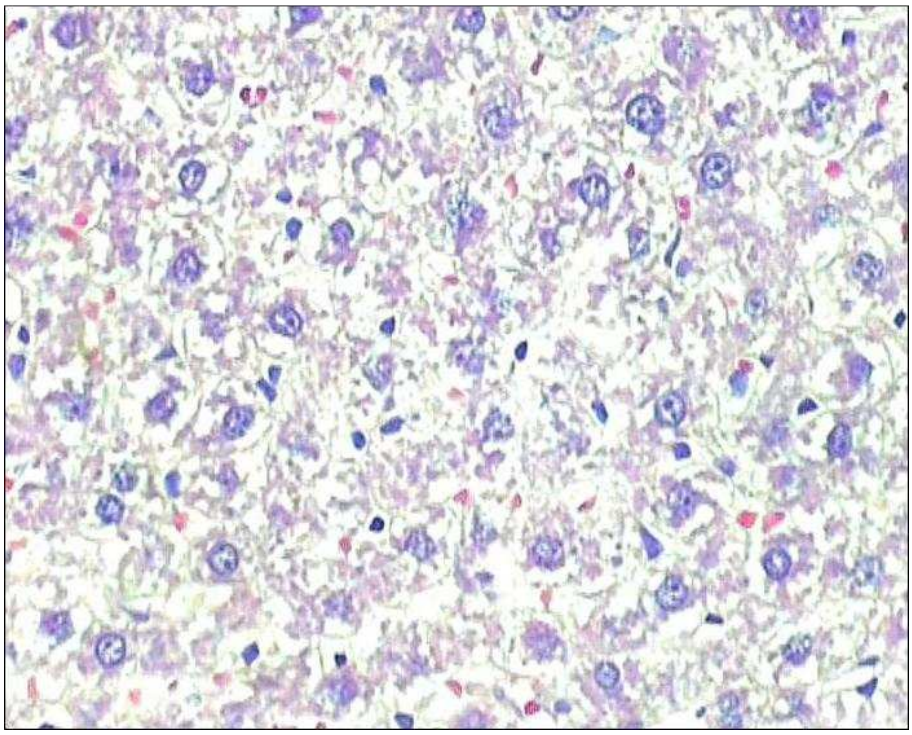

Figure 6.10 q): Liver section A from the INH+LMS group after 28 days of treatment, showing severe granular vacuolar degeneration and cell swelling with a cloudy and granular appearance of the hepatocytes

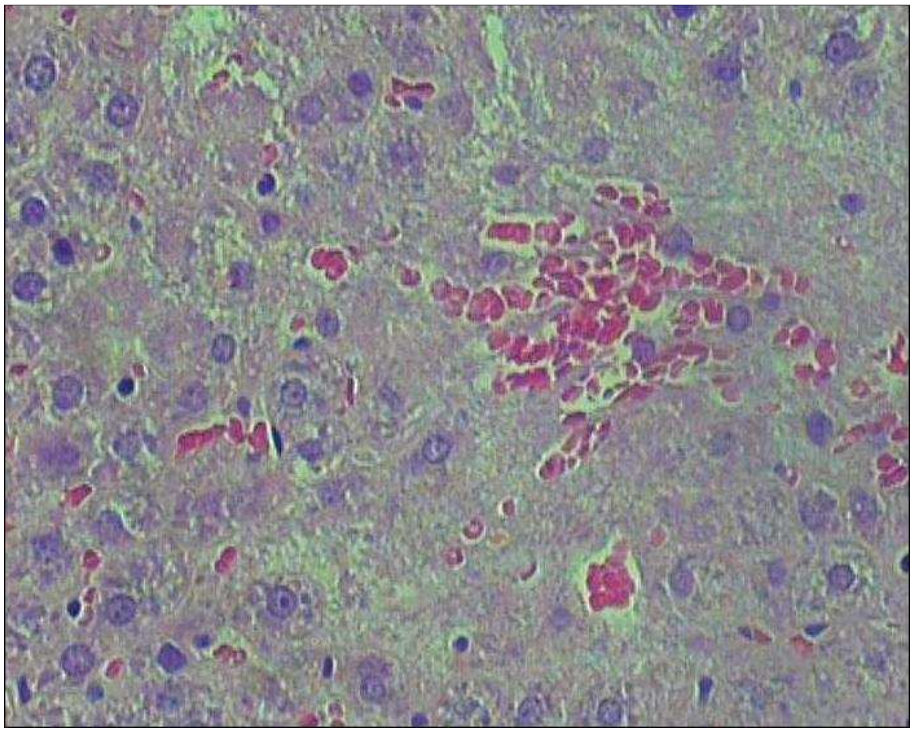

Figure 6.10 r): Liver section B from the INH+LMS group after 28 days of treatment, showing centrilobular zonal necrosis with loss of nuclei and disarrangement of the hepatocytic cords

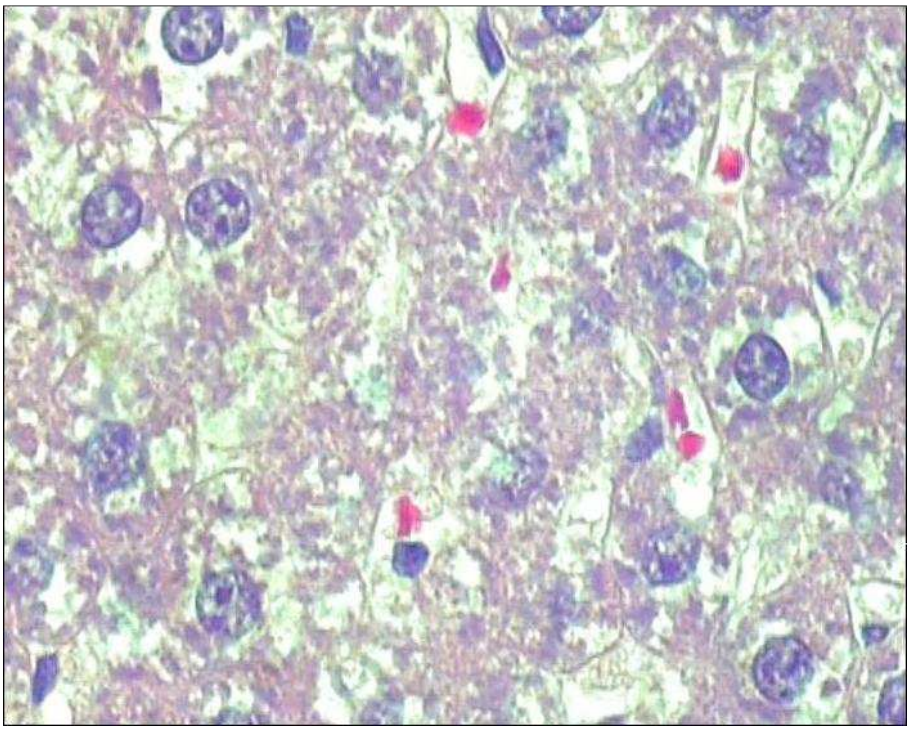

Figure 6.10 s): Liver section A from the INH+LMS group after 42 days of treatment, showing mild granular vacuolar degeneration and cytonecrosis

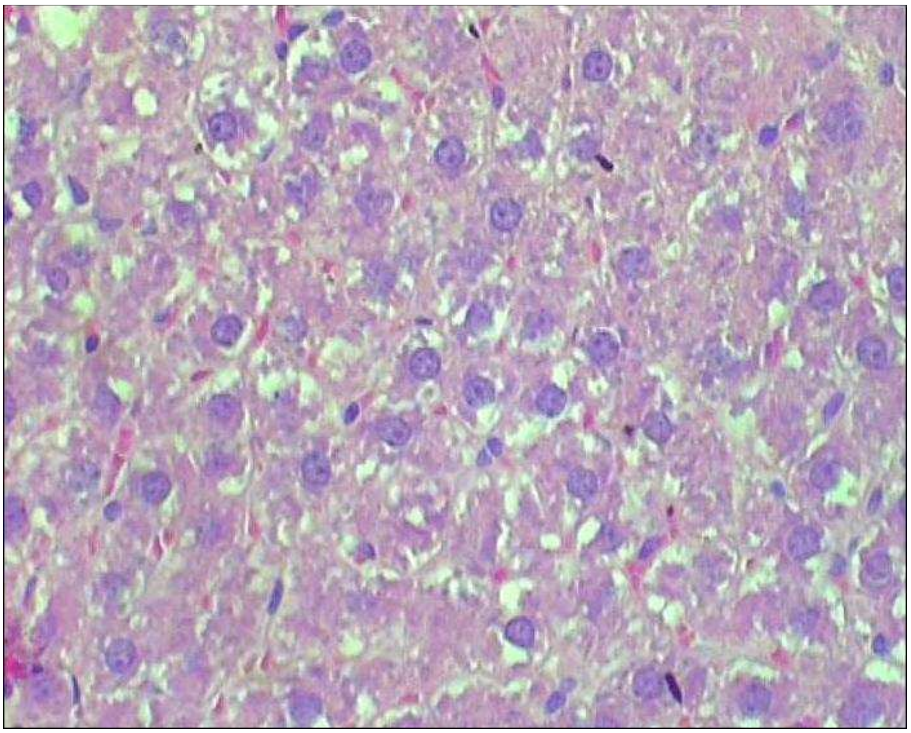

Figure 6.10 t): Liver section B from the INH+LMS group after 42 days of treatment, showing degeneration, and cytonecrosis graded as mild

Table 6.16 a): Tally of main pathology lesions (lesions score) in livers of untreated rats and the S+LMS group

Group	UnRx	S+LMS									
		2 Days		7 Days		14 Days		28 Days		42 Days	
(n = 2)	Fig.6.3a	Fig.6.10a	Fig.6.10b	Fig.6.10c	Fig.6.10d	Fig.6.10e	Fig.6.10f	Fig.6.10g	Fig.6.10h	Fig.6.10i	Fig.6.10j
Histopathological lesions											
Vacuolar degeneration	0	0	0	0	0	0	1+	1+	1+	1+	1+
Cell swelling	0	0	0	0	0	0	1+	1+	1+	1+	1+
Cytonecrosis	0	0	0	0	0	0	0	0	0	0	0
Centrilobular necrosis	0	0	0	0	0	0	0	0	0	0	0
Hepatocyte mitosis	0	0	0	1+	2+	0	0	0	1+	0	0
Average lesions score											
Vacuolar degeneration	0	0		0		0.5+		1+		1+	
Cell swelling	0	0		0		0.5+		1+		1+	
Cytonecrosis	0	0		0		0		0		0	
Centrilobular necrosis	0	0		0		0		0		0	
Hepatocyte mitosis	0	0		1.5+		0		0.5+		0	
Total lesion score	**0**	**0**		**1.5+**		**1+**		**2.5+**		**2+**	

UnRx = untreated; S = saline; LMS = levamisole

Table 6.16 b): Tally of main pathology lesions (lesions score) in livers of untreated rats and the INH+LMS group

Group	UnRx	INH+LMS									
		2 Days		7 Days		14 Days		28 Days		42 Days	
(n = 2)	Fig.6.3a	Fig.6.10k	Fig.6.10l	Fig.6.10m	Fig.6.10n	Fig.6.10o	Fig.6.10p	Fig.6.10q	Fig.6.10r	Fig.6.10s	Fig.6.10t
Histopathological lesions											
Vacuolar degeneration	0	1+	1+	2+	3+	3+	3+	4+	3+	2+	2+
Cell swelling	0	1+	1+	2+	3+	3+	3+	4+	3+	2+	2+
Cytonecrosis	0	0	1+	2+	2+	2+	2+	3+	2+	2+	2+
Centrilobular necrosis	0	0	0	0	0	0	1+	1+	1+	0	0
Hepatocyte mitosis	0	0	0	0	0	0	0	0	0	0	0
Average lesions score											
Vacuolar degeneration	0	1+		2.5+		3+		3.5+		2+	
Cell swelling	0	1+		2.5+		3+		3.5+		2+	
Cytonecrosis	0	0.5+		2+		2+		2.5+		2+	
Centrilobular necrosis	0	0		0		0.5+		1+		0	
Hepatocyte mitosis	0	0		0		0		0		0	
Total lesion score	**0**	**2.5+**		**7+**		**8.5+**		**10.5+**		**6+**	

UnRx = untreated; INH = isoniazid; LMS = levamisole

6.3.7 Isoniazid concentrations

Table 6.17 shows isoniazid concentrations of the INH and INH+LMS groups, while Figure 6.11 is a graphical illustration of the same. For the INH+LMS group, isoniazid concentrations increased up to day 28 ($p = 0.0159$) and had dropped by day 42 ($p = 0.0079$). Isoniazid levels were lower with concomitant isoniazid and levamisole administration than with isoniazid alone until day 14 ($p = 0.0556$), but this had reversed by day 28 ($p = 0.0079$).

Table 6.17: Average (mean ± SD) isoniazid concentrations of the INH and INH+LMS groups

Group	**INH**	**INH+LMS**
	INH concentration	**INH concentration**
(n = 5)	**(µg/ml)**	**(µg/ml)**
2 Days	1.891 ± 0.57	2.299 ± 0.71
7 Days	4.287 ± 1.50	2.360 ± 0.85
14 Days	8.628 ± 6.82	3.694 ± 2.71
28 Days	2.642 ± 0.81	9.618 ± 2.47
42 Days	1.607 ± 1.19	3.316 ± 1.05

INH = isoniazid; LMS = levamisole

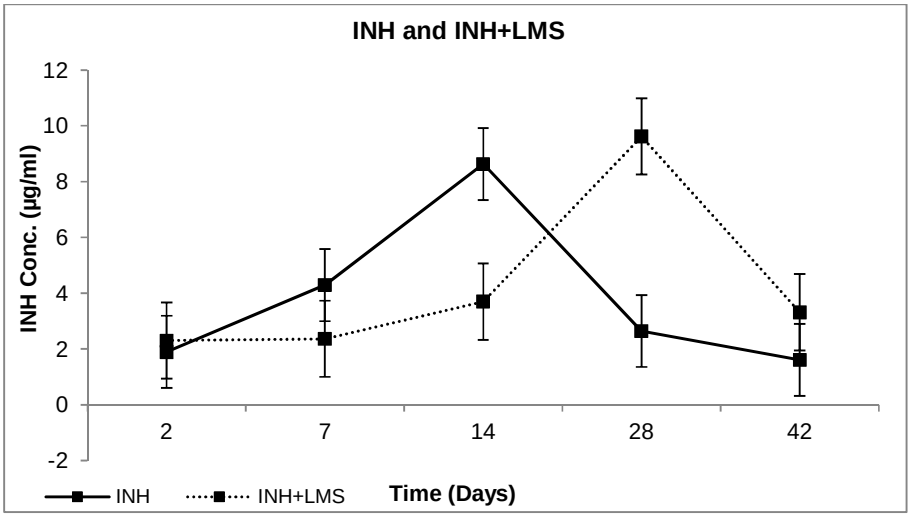

Figure 6.11: Isoniazid concentrations of the INH and INH+LMS groups over 42 days

6.3.8 Specific immunology tests

6.3.8.1 Direct observations

Table 6.18 shows changes in body weight of the S, S+LMS, INH and INH+LMS groups over the treatment period. All groups showed weight gain at all times, except for the S+LMS group after 2 days (Refer to Appendix H-1 and H-2 for baseline weights).

Table 6.18: Average (mean ± SD) change in rat weights of the S, S+LMS, INH and INH+LMS groups

Group (n = 5)	S change in weight (g)	S+LMS change in weight (g)	INH change in weight (g)	INH+LMS change in weight (g)
2 Days	9.2±4	-1.4±1	7.0±6	6.3±5
7 Days	35.7±8	19.4±10	31.0±19	21.2±6
14 Days	84.6±5	57.0±26	55.0±10	72.1±13
28 Days	107.8±10	96.1±13	111.9±12	106.4±30
42 Days	171.4±27	153.4±23	141.3±14	159.7±19

S = saline; LMS = levamisole; INH = isoniazid

6.3.8.2 Cytokines

Table 6.19 shows IL-2 and IL-10 concentrations of the S, S+LMS, INH and INH+LMS groups, while Figures 6.12 a – b are graphical illustrations of the same. IL-2 levels in the INH+LMS group fluctuated, and had decreased by day 42 ($p = 0.0500$). It was also statistically lower than in the INH group on days 7, 14 and 42 ($p = 0.0500$). IL-10 levels remained fairly constant in all groups. Throughout the 42 days, IL-10 concentrations were higher in the INH+LMS group compared to the INH group, but were not statistically significant.

Table 6.19: Average (mean ± SD) cytokine concentrations of the S, S+LMS, INH and INH+LMS groups

Group	Cytokine		Group	Cytokine	
(n = 3)	IL-2 (pg/ml)	IL-10 (pg/ml)	(n = 3)	IL-2 (pg/ml)	IL-10 (pg/ml)
Untreated					
0 Days	65.46 ± 2.0	31.08 ± 1.2			
S			**INH**		
2 Days	74.87 ± 6.5	29.96 ± 2.8	2 Days	70.56 ± 1.5	30.26 ± 6.0
7 Days	77.26 ± 5.8	34.57 ± 0.7	7 Days	83.80 ± 1.7	31.96 ± 0.7
14 Days	77.58 ± 6.6	35.69 ± 5.4	14 Days	78.38 ± 5.8	32.46 ± 4.8
28 Days	78.81 ± 4.6	32.46 ± 4.2	28 Days	77.42 ± 6.0	28.51 ± 5.2
42 Days	74.39 ± 5.7	32.03 ± 2.5	42 Days	72.32 ± 5.9	34.32 ± 6.8
S+LMS			**INH+LMS**		
2 Days	62.89 ± 2.2	30.57 ± 0.9	2 Days	75.67 ± 11.7	36.65 ± 3.5
7 Days	62.89 ± 0.8	31.31 ± 2.0	7 Days	49.33 ± 2.3	39.27 ± 5.3
14 Days	62.61 ± 9.4	32.44 ± 6.5	14 Days	47.00 ± 13.5	39.36 ± 4.4
28 Days	65.82 ± 3.7	29.82 ± 1.7	28 Days	69.67 ± 17.0	37.31 ± 5.3
42 Days	69.45 ± 3.6	29.26 ± 3.1	42 Days	45.67 ± 5.7	38.05 ± 3.2

IL = interleukin; S = saline; LMS = levamisole; INH = isoniazid

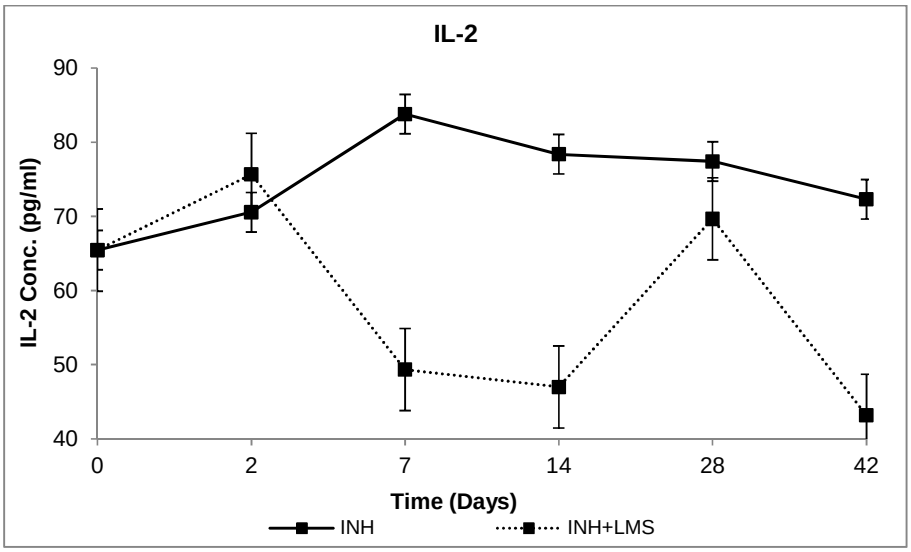

Figure 6.12 a): IL-2 concentrations of the INH and INH+LMS groups over 42 days

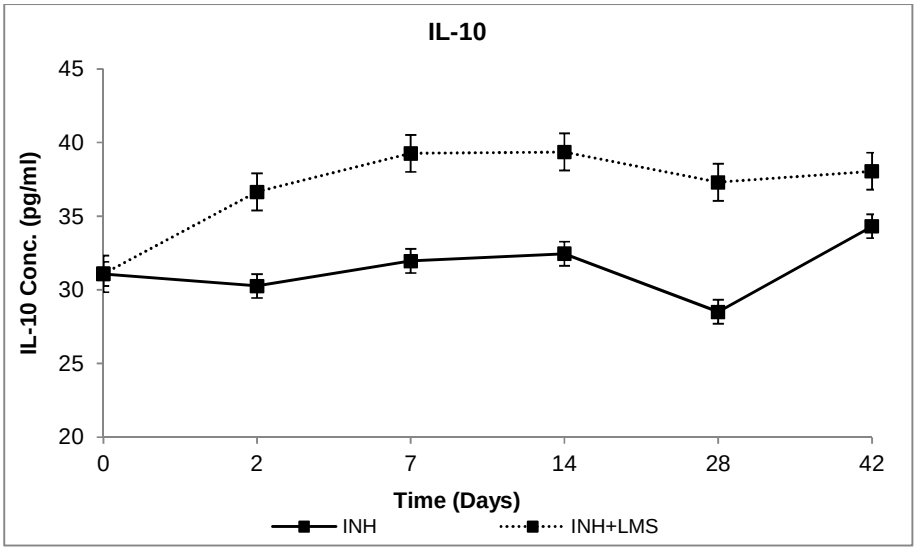

Figure 6.12 b): IL-10 concentrations of the INH and INH+LMS groups over 42 days

6.3.8.3 CD4 and CD8 counts

Table 6.20 shows CD4 and CD8 counts of the S, S+LMS, INH and INH+LMS groups, while Figures 6.13 a – b are graphical illustrations of the same. Over the 42 days, the CD4 count of the INH+LMS group declined gradually till the end of treatment (p = 0.0500), while the CD8 count dropped sharply between days 28 and 42 (p = 0.0500), and this was also lower than in the INH group (p = 0.0500).

Table 6.20: Average (mean ± SD) CD4 and CD8 counts of the S, S+LMS, INH and INH+LMS groups

Group	**Ly**	**T-Ly**		**Group**	**Ly**	**T-Ly**	
		CD4	**CD8**			**CD4**	**CD8**
(n = 3)	**(x10^9/l)**	**(x10^9/l)**	**(x10^9/l)**	**(n = 3)**	**(x10^9/l)**	**(x10^9/l)**	**(x10^9/l)**
Untreated							
0 Days	4.67±1.8	2.23±1.3	1.42±0.7				
S				**INH**			
2 Days	5.18±0.7	2.27±0.3	1.35±0.2	2 Days	5.02±1.1	1.95±0.4	1.27±0.2
7 Days	4.07±2.0	1.72±0.8	1.07±0.5	7 Days	5.10±0.8	2.20±0.5	1.28±0.3
14 Days	4.21±0.7	1.69±0.2	1.17±0.2	14 Days	4.91±0.9	2.28±0.4	1.08±0.5
28 Days	6.15±0.8	2.45±0.2	1.58±0.3	28 Days	4.56±0.5	1.76±0.4	1.24±0.1
42 Days	3.23±0.3	1.47±0.1	0.79±0.2	42 Days	4.05±0.6	1.50±0.2	1.06±0.2
S+LMS				**INH+LMS**			
2 Days	5.99±0.4	2.25±0.1	1.58±0.0	2 Days	6.35±0.9	2.47±0.5	1.58±0.4
7 Days	5.10±0.0	2.09±0.0	1.34±0.0	7 Days	5.64±1.2	2.36±0.2	1.21±0.4
14 Days	3.82±1.8	1.64±0.8	0.97±0.5	14 Days	5.26±1.0	2.28±0.5	1.24±0.3
28 Days	4.27±1.6	1.69±0.6	1.15±0.5	28 Days	4.81±0.3	1.86±0.1	1.33±0.2
42 Days	5.69±0.9	2.26±0.3	0.01±0.0	42 Days	5.00±0.6	1.70±0.2	0.01±0.0

Ly = lymphocytes; CD = cluster of differentiation; S = saline; LMS = levamisole; INH = isoniazid

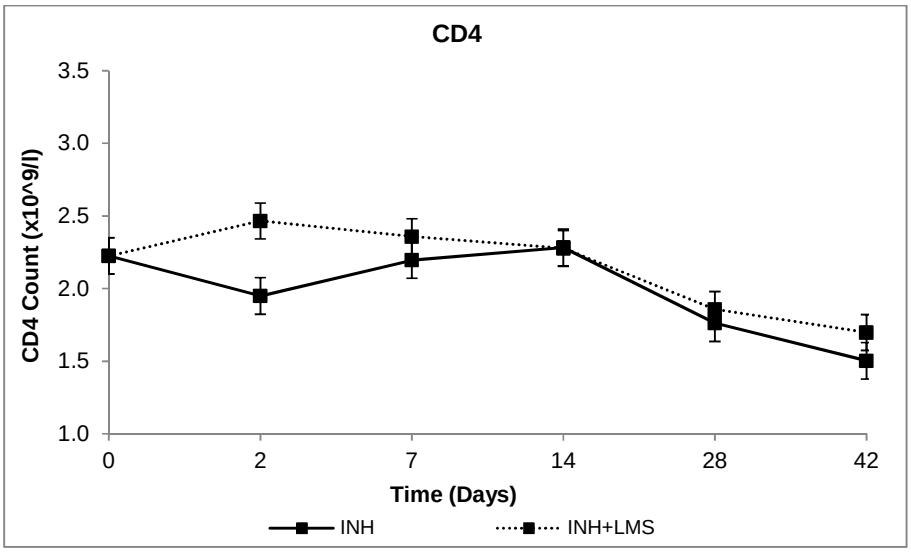

Figure 6.13 a): CD4 counts of the INH and INH+LMS groups over 42 days

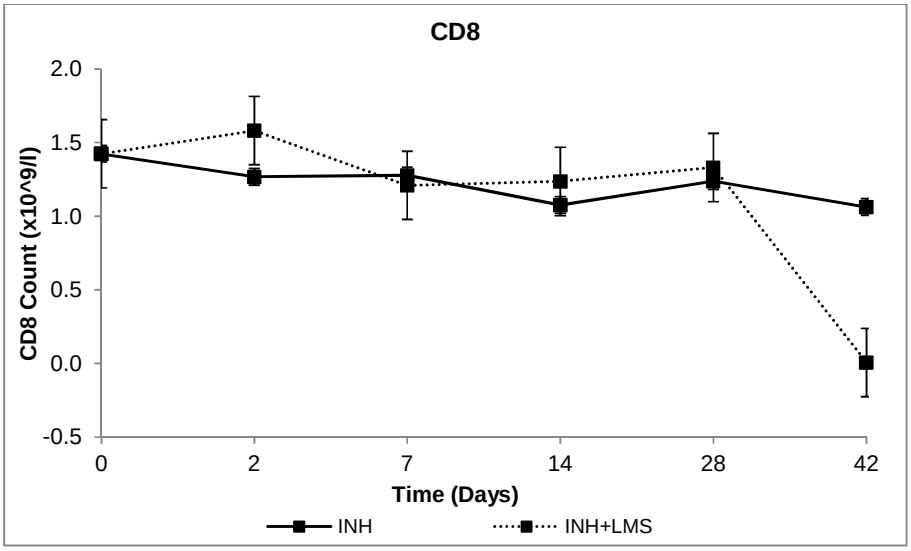

Figure 6.13 b): CD8 counts of the INH and INH+LMS groups over 42 days

6.3.8.4 Immunoglobulins

Table 6.21 shows concentrations of IgM and IgG of the S, S+LMS, INH and INH+LMS groups, while Figures 6.14 a – b are graphical illustrations of the same. IgM concentrations fluctuated in the INH+LMS group, and always higher than in the INH group ($p = 0.0500$). IgG concentrations showed some variations in the INH+LMS group, but were not statistically different. On day 42, IgG levels were higher in the INH group than in the INH+LMS group ($p = 0.0500$).

Table 6.21: Average (mean ± SD) immunoglobulin concentrations of the S, S+LMS, INH and INH+LMS groups

Group	Immunoglobulin		Group	Immunoglobulin	
	IgM	IgG		IgM	IgG
(n = 3)	(mg/ml)	(mg/ml)	(n = 3)	(mg/ml)	(mg/ml)
Untreated					
0 Days	0.109±0.02	14.434±1.10			
S			**INH**		
2 Days	0.104±0.04	14.137±0.91	2 Days	0.057±0.03	12.849±0.34
7 Days	0.110±0.04	14.302±0.70	7 Days	0.040±0.02	14.765±0.40
14 Days	0.110±0.03	12.617±0.29	14 Days	0.046±0.01	12.321±1.24
28 Days	0.075±0.03	16.350±1.00	28 Days	0.029±0.01	13.707±2.61
42 Days	0.046±0.01	17.109±0.26	42 Days	0.027±0.01	18.299±0.94
S+LMS			**INH+LMS**		
2 Days	0.040±0.00	9.486±0.95	2 Days	0.092±0.01	12.595±1.07
7 Days	0.045±0.02	12.487±1.21	7 Days	0.123±0.04	12.704±2.00
14 Days	0.059±0.02	11.961±1.72	14 Days	0.085±0.03	10.982±2.23
28 Days	0.053±0.02	12.095±1.15	28 Days	0.135±0.02	13.840±1.92
42 Days	0.046±0.01	13.013±1.30	42 Days	0.083±0.02	14.831±2.01

IgM = immunoglobulin M; IgG = immunoglobulin G; S = saline; LMS = levamisole; INH = isoniazid

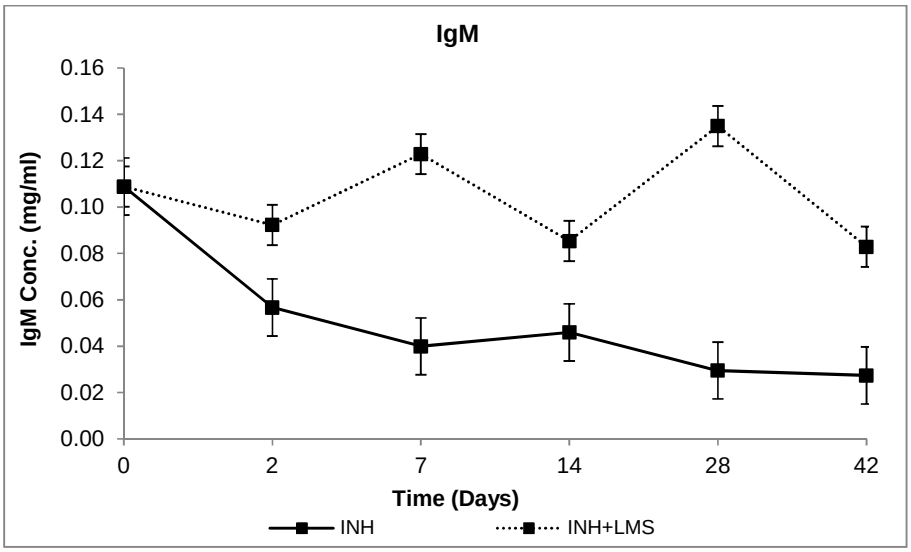

Figure 6.14 a): IgM concentrations of the INH and INH+LMS groups over 42 days

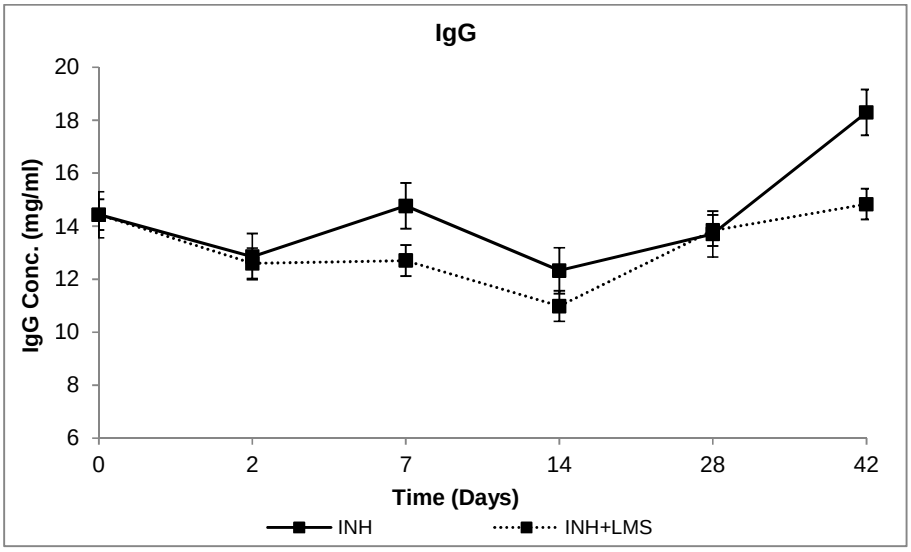

Figure 6.14 b): IgG concentrations of the INH and INH+LMS groups over 42 days

6.3.9 Main observations

- From the histopathology, co-treatment with levamisole caused liver injury up to 28 days, and this was more severe than with isoniazid alone.
- Isoniazid levels were lower with levamisole co-administration until day 14, after which it reversed.
- IL-2 levels had decreased by day 42 and were lower than with isoniazid alone. IL-10 levels were always higher than with isoniazid alone.
- IgM concentrations were always higher with levamisole co-administration than with isoniazid alone.

C. Phase III: Co-treatment with a CYP450 inducer

6.3.10 Physiological observations (function tests)

6.3.10.1 Full blood count

Table 6.22 shows results of the full blood count of the S, S+CBZ, INH and INH+CBZ groups. The changes of red blood count parameters as observed for concomitant isoniazid and carbamazepine treatment, are similar to that of isoniazid alone (Section 6.3.1.1). By the end of the treatment period, haemoglobin, haematocrit, MCV, MCH and MCHC were statistically different between the two groups ($p = 0.0500$). For white blood count parameters no statistical differences were seen in the INH+CBZ group, nor between the two groups.

Table 6.22: Average (mean ± SD) full blood count and platelets results of the S, S+CBZ, INH and INH+CBZ groups

Group (n = 3)	RCC ($x10^{12}$/l)	Hb (g/dl)	Hct (l/l)	MCV (fl)	MCH (pg)	MCHC (g/dl)	Plt ($x10^9$/l)	WCC ($x10^9$/l)	Neu ($x10^9$/l)	Ly ($x10^9$/l)	Mo ($x10^9$/l)	Eos ($x10^9$/l)	Bas ($x10^9$/l)
Untreated													
0 Days	6.28±0.2	12.9±0.3	0.398±0.01	63.5±2.5	20.5±0.4	32.3±0.8	860±221.1	6.95±2.7	0.77±0.2	4.67±1.8	0.19±0.1	0.02±0.0	0.00±0.0
S													
2 Days	6.67±0.2	13.7±0.1	0.422±0.01	63.3±2.3	20.6±0.6	32.4±0.6	849±81.6	6.50±0.9	0.60±0.2	5.18±0.7	0.21±0.0	0.50±0.2	0.01±0.0
7 Days	7.53±0.9	15.3±1.7	0.451±0.04	60.1±2.5	20.3±0.2	33.9±1.2	1033±79.8	5.44±2.4	1.03±0.8	4.07±2.0	0.30±0.3	0.04±0.0	0.01±0.0
14 Days	6.77±0.6	13.9±1.1	0.417±0.03	61.8±2.8	20.5±0.6	33.2±0.6	721±196.4	5.22±1.2	0.63±0.5	4.21±0.7	0.18±0.1	0.18±0.1	0.05±0.0
28 Days	7.07±0.7	13.9±1.3	0.390±0.04	55.1±1.0	19.7±0.1	35.8±0.6	961±172.5	7.38±1.0	0.91±0.2	6.15±0.8	0.24±0.1	0.07±0.0	0.01±0.0
42 Days	6.93±0.8	13.4±1.8	0.374±0.05	53.9±1.0	19.3±0.4	35.8±0.2	839±166.0	3.93±0.3	0.54±0.1	3.23±0.3	0.11±0.0	0.04±0.0	0.01±0.0
S+CBZ													
2 Days	6.74±0.2	13.2±0.1	0.406±0.00	60.4±2.0	19.6±0.6	28.8±6.5	953±95.9	6.48±1.0	0.51±0.0	5.46±1.1	0.24±0.3	0.02±0.0	0.01±0.0
7 Days	6.62±0.4	13.1±0.6	0.414±0.02	62.5±1.3	19.8±0.4	31.7±0.2	779±187.7	7.32±2.4	0.59±0.2	6.20±2.1	0.27±0.1	0.27±0.3	0.01±0.0
14 Days	7.32±0.1	14.7±0.3	0.442±0.01	60.4±2.8	20.1±0.5	33.3±0.8	861±149.2	8.41±0.7	0.76±0.0	7.28±0.5	0.33±0.2	0.05±0.0	0.01±0.0
28 Days	7.27±0.9	14.5±1.7	0.424±0.05	58.4±0.8	19.9±0.3	34.1±0.9	637±79.9	5.48±1.3	0.84±0.3	4.32±1.0	0.23±0.3	0.08±0.0	0.00±0.0
42 Days	7.90±0.1	15.4±0.4	0.451±0.01	57.1±1.4	19.5±0.4	34.2±0.2	727±48.7	6.47±0.6	0.66±0.1	5.55±0.5	0.21±0.0	0.05±0.0	0.01±0.0
INH													
2 Days	6.57±0.3	13.4±0.4	0.416±0.01	63.4±2.0	20.5±0.3	32.3±0.7	880±54.5	5.86±1.1	0.59±0.0	5.02±1.1	0.21±0.1	0.03±0.0	0.00±0.0
7 Days	6.05±0.6	12.7±1.1	0.381±0.04	63.0±0.6	21.0±0.2	33.3±0.2	478±19.1	5.98±0.8	0.37±0.3	5.10±0.8	0.19±0.1	0.02±0.0	0.01±0.0
14 Days	6.65±0.6	13.1±1.2	0.394±0.04	59.2±2.7	19.8±0.6	33.4±0.7	494±50.2	5.78±1.1	0.61±0.1	4.91±0.9	0.23±0.1	0.03±0.0	0.00±0.0
28 Days	7.27±0.4	14.3±0.8	0.406±0.02	55.9±0.6	19.7±0.1	35.3±0.4	845±116.0	5.44±0.4	0.66±0.1	4.56±0.4	0.17±0.1	0.04±0.0	0.01±0.0
42 Days	7.25±0.7	13.9±1.2	0.390±0.04	53.8±1.9	19.2±0.4	35.6±0.8	785±152.7	5.11±0.9	0.84±0.1	4.05±0.6	0.15±0.1	0.07±0.0	0.01±0.0
INH+CBZ													
2 Days	6.78±0.4	13.7±0.3	0.411±0.01	60.6±3.4	20.3±1.0	33.4±0.2	788±404.0	7.31±1.5	0.89±0.6	5.98±0.8	0.41±0.2	0.03±0.0	0.01±0.0
7 Days	6.52±0.6	13.1±1.1	0.400±0.03	61.4±2.5	20.2±0.6	32.8±0.5	762±83.8	7.18±1.6	0.73±0.2	5.95±1.8	0.31±0.0	0.18±0.2	0.01±0.0
14 Days	6.58±0.3	13.1±0.4	0.404±0.01	61.5±1.4	20.0±0.5	32.5±0.1	778±117.4	5.88±0.5	0.60±0.2	4.88±0.3	0.26±0.1	0.13±0.2	0.01±0.0
28 Days	7.59±0.2	14.8±0.3	0.440±0.01	57.9±1.1	19.5±0.3	33.6±0.2	613±28.6	5.18±1.4	0.67±0.1	4.30±1.3	0.17±0.1	0.04±0.0	0.00±0.0
42 Days	7.49±0.2	14.9±0.3	0.438±0.01	58.4±1.5	19.9±0.3	34.1±0.4	578±41.6	6.48±1.0	0.63±0.1	5.60±1.1	0.19±0.1	0.04±0.0	0.01±0.0

RCC = red cell count; Hb = haemoglobin; Hct = haematocrit; MCV = mean corpuscular volume; MCH = mean corpuscular haemoglobin; MCHC = mean corpuscular haemoglobin concentration; Plt = platelets; WCC = white cell count; Neu = neutrophils; Ly = lymphocytes; Mo = monocytes; Eos = eosinophils; Bas = basophils; S = saline; CBZ = carbamazepine; INH = isoniazid

6.3.10.2 Renal function tests

Table 6.23 shows the changes of BUN and Cr of the S, S+CBZ, INH and INH+CBZ groups. In all groups BUN and Cr levels were normal. Here, Cr levels also spiked on day 28, but were still within the normal range (p = 0.0500).

Table 6.23: Average (mean ± SD) renal function test results of the S, S+CBZ, INH and INH+CBZ groups

Group	**RFT**		**Group**	**RFT**	
(n = 3)	**BUN (mmol/l)**	**Cr (µmol/l)**	**(n = 3)**	**BUN (mmol/l)**	**Cr (µmol/l)**
Untreated					
0 Days	7.2 ± 1	37 ± 8			
S			**INH**		
2 Days	7.3 ± 1	39 ± 2	2 Days	6.7 ± 0	34 ± 6
7 Days	8.1 ± 0	46 ± 7	7 Days	6.6 ± 0	36 ± 2
14 Days	7.5 ± 1	39 ± 3	14 Days	6.2 ± 1	43 ± 8
28 Days	10.6 ± 2	73 ± 17	28 Days	7.3 ± 1	69 ± 4
42 Days	5.8 ± 1	38 ± 9	42 Days	5.8 ± 0	34 ± 21
S+CBZ			**INH+CBZ**		
2 Days	6.5 ± 0	36 ± 2	2 Days	6.5 ± 0	40 ± 10
7 Days	6.8 ± 1	33 ± 6	7 Days	6.4 ± 1	33 ± 5
14 Days	6.4 ± 1	36 ± 6	14 Days	6.1 ± 1	32 ± 3
28 Days	7.2 ± 1	64 ± 12	28 Days	5.7 ± 1	64 ± 1
42 Days	7.2 ± 1	8 ± 6	42 Days	6.6 ± 0	11 ± 6

RFT = renal function test; BUN = blood urea nitrogen; Cr = creatinine; S = saline; CBZ = carbamazepine; INH = isoniazid

6.3.10.3 Liver function tests

Table 6.24 shows the changes of ALT, AST and ALP of the S, S+CBZ, INH and INH+CBZ groups. Over the 42 days, the results were similar in all groups.

Table 6.24: Average (mean ± SD) liver function test results of the S, S+CBZ, INH and INH+CBZ groups

Group	LFT			Group	LFT		
	ALT	AST	ALP		ALT	AST	ALP
(n = 3)	(U/l)	(U/l)	(U/l)	(n = 3)	(U/l)	(U/l)	(U/l)
Untreated							
0 Days	50±5	88±14	352±76				
S				**INH**			
2 Days	46±2	90±7	400±7	2 Days	46±4	104±7	335±32
7 Days	49±10	103±25	304±13	7 Days	53±13	233±223	369±10
14 Days	58±4	127±37	508±37	14 Days	43±4	98±29	364±38
28 Days	47±2	115±44	216±19	28 Days	46±4	143±36	220±29
42 Days	46±6	76±28	109±76	42 Days	51±2	86±14	127±78
S+CBZ				**INH+CBZ**			
2 Days	52±4	90±12	332±18	2 Days	56±3	142±19	369±65
7 Days	43±6	86±8	341±28	7 Days	47±4	160±36	359±16
14 Days	47±2	85±2	356±1	14 Days	49±5	182±79	345±20
28 Days	52±5	97±7	152±78	28 Days	48±10	69±4	142±23
42 Days	45±2	91±20	104±74	42 Days	47±4	68±2	55±22

LFT = liver function test; ALT = alanine aminotransferase; AST = aspartate aminotransferase; ALP = alkaline phosphatase; S = saline; CBZ = carbamazepine; INH = isoniazid

6.3.10.4 Liver histopathology

(a) Liver histopathology reports

Liver sections for histopathology (Figures 6.15 a – t) were randomly selected and the main histopathology lesions are summarised in the tally tables (Tables 6.25 a and b). The following report is a summary of the features of the lesion:

i. Figures 6.15 a and b: Liver sections A and B from the S+CBZ group after 2 days of saline and carbamazepine co-treatment

Representative photographs of rat livers, after 2 days of daily saline and carbamazepine co-treatment. The report: “Liver section A demonstrated normal hepatic parenchymal cells, while section B showed minimal degeneration.”

ii. Figures 6.15 c and d: Liver sections A and B from the S+CBZ group after 7 days of saline and carbamazepine co-treatment

Representative photographs of rat livers, after 7 days of daily saline and carbamazepine co-treatment. The report: “In liver sections A and B, vacuolar changes of minimal degree are observed within the liver parenchymal cells.”

iii. Figures 6.15 e and f: Liver sections A and B from the S+CBZ group after 14 days of saline and carbamazepine co-treatment

Representative photographs of rat livers, after 14 days of daily saline and carbamazepine co-treatment. The report: “The degeneration and vacuolar cell swelling is graded as mild, while mild single cell necrosis is also observed in liver sections A and B.”

iv. Figures 6.15 g and h: Liver sections A and B from the S+CBZ group after 28 days of saline and carbamazepine co-treatment

Representative photographs of rat livers, after 28 days of daily saline and carbamazepine co-treatment. The report: “The vacuolar granular degeneration is classified as minimal in liver sections A and B. Cytonecrosis of minimal degree is present in liver section B.”

v. Figures 6.15 i and j: Liver sections A and B from the S+CBZ group after 42 days of saline and carbamazepine co-treatment

Representative photographs of rat livers, after 42 days of daily saline and carbamazepine co-treatment. The report: “Minimal hepatocyte degeneration, as well as minimal single cell necrosis and loss of hepatic nuclei could be demonstrated in liver sections A and B.”

vi. Figures 6.15 k and l: Liver sections A and B from the INH+CBZ group after 2 days of isoniazid and carbamazepine co-treatment

Representative photographs of rat livers, after 2 days of daily isoniazid and carbamazepine co-treatment. The report: “The hepatocyte degenerative changes are mild, while similar single cell necrosis could be demonstrated in liver sections A and B. Mild nuclear loss could be confirmed in section A.”

vii. Figures 6.15 m and n: Liver sections A and B from the INH+CBZ group after 7 days of isoniazid and carbamazepine co-treatment

Representative photographs of rat livers, after 7 days of daily isoniazid and carbamazepine co-treatment. The report: “Vacuolar changes and cytonecrosis of mild degree are observed within hepatocytes in liver sections A and B.”

viii. Figures 6.15 o and p: Liver sections A and B from the INH+CBZ group after 14 days of isoniazid and carbamazepine co-treatment

Representative photographs of rat livers, after 14 days of daily isoniazid and carbamazepine co-treatment. The report: “Mild granular vacuolar degeneration and cell swelling were observed in liver sections A and B. Cytonecrosis was minimal, and no centrilobular zonal necrosis or hepatocyte mitosis was recorded. Some lymphocytic infiltrates were visible in the periportal parenchyma.”

ix. Figures 6.15 q and r: Liver sections A and B from the INH+CBZ group after 28 days of isoniazid and carbamazepine co-treatment

Representative photographs of rat livers, after 28 days of daily isoniazid and carbamazepine co-treatment. The report: “In section A, granular vacuolar degeneration and cell swelling was mild, while in section B it was only minimal. Cytonecrosis was minimal in both sections, with no centrilobular zonal necrosis. Hepatocyte mitosis, suggestive of cell regeneration, was only minimally present in section A.”

x. Figures 6.15 s and t: Liver sections A and B from the INH+CBZ group after 42 days of isoniazid and carbamazepine co-treatment

Representative photographs of rat livers, after 42 days of daily isoniazid and carbamazepine co-treatment. The report: “Granular vacuolar changes are moderate, while mild cytonecrosis and minimal centrilobular necrosis are detected in liver section A. The findings were similar in section B, with the exception of mild vacuolar degeneration.”

In view of the histopathology photographs (Figures 6.15 a – t), reports and tally tables (Tables 6.25 a and b), it was concluded that treatment with isoniazid alone caused a greater degree of liver injury than the co-administration of isoniazid and carbamazepine.

(b) Liver histopathology photographs

Figures 6.15 a – t are representative of randomly selected liver sections of the S+CBZ and INH+CBZ groups.

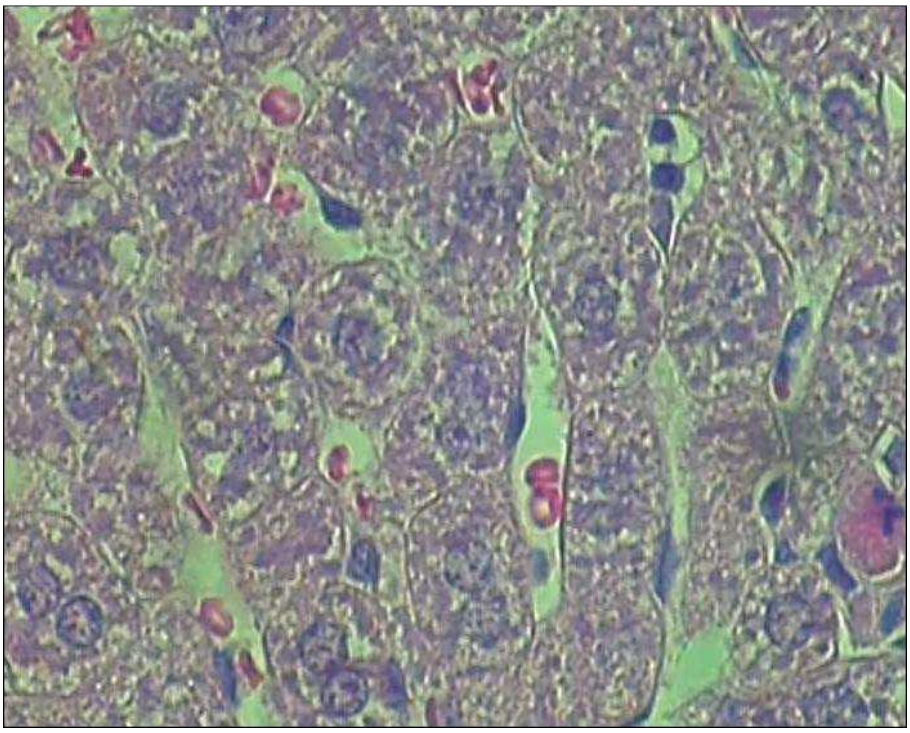

Figure 6.15 a): Liver section A from the S+CBZ group after 2 days of treatment, showing normal hepatic parenchymal cells

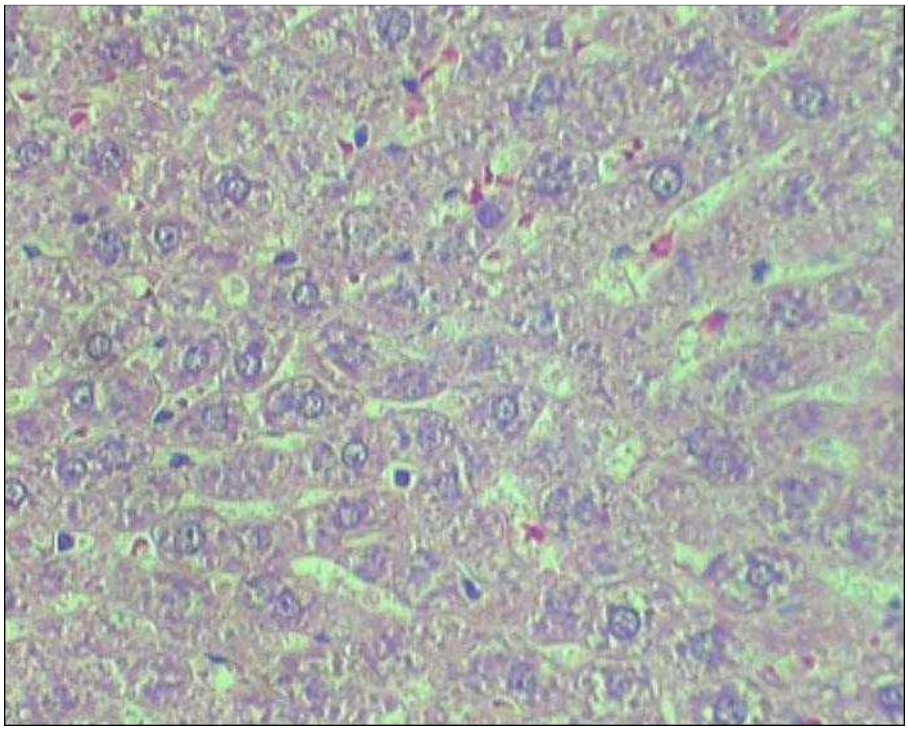

Figure 6.15 b): Liver section B from the S+CBZ group after 2 days of treatment, showing minimal degeneration within the hepatic parenchymal cells

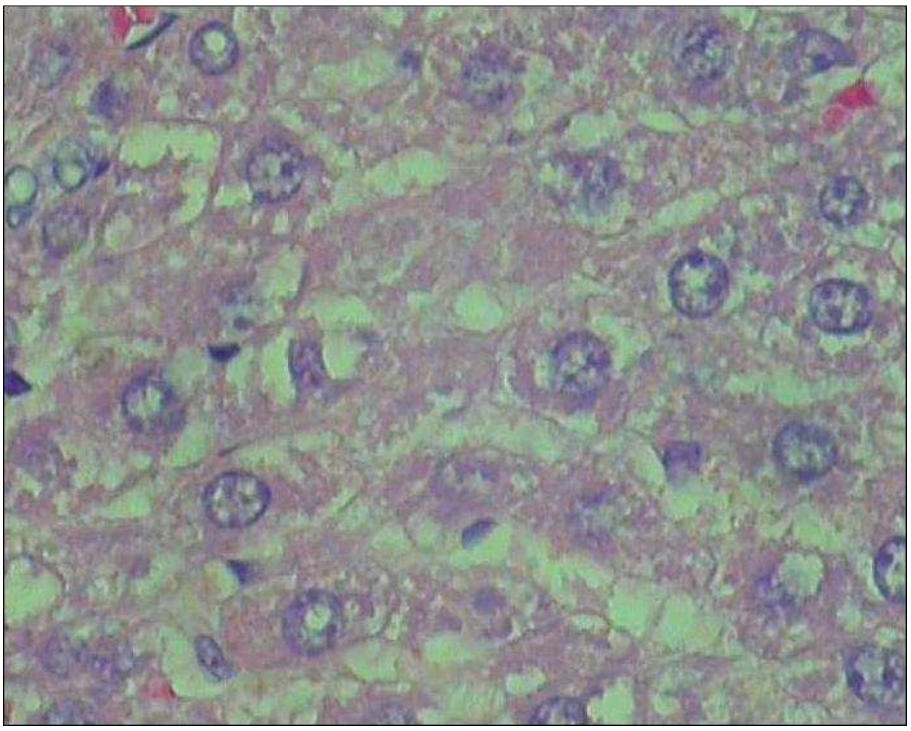

Figure 6.15 c): Liver section A from the S+CBZ group after 7 days of treatment, showing vacuolar changes of minimal degree within the liver parenchymal cells

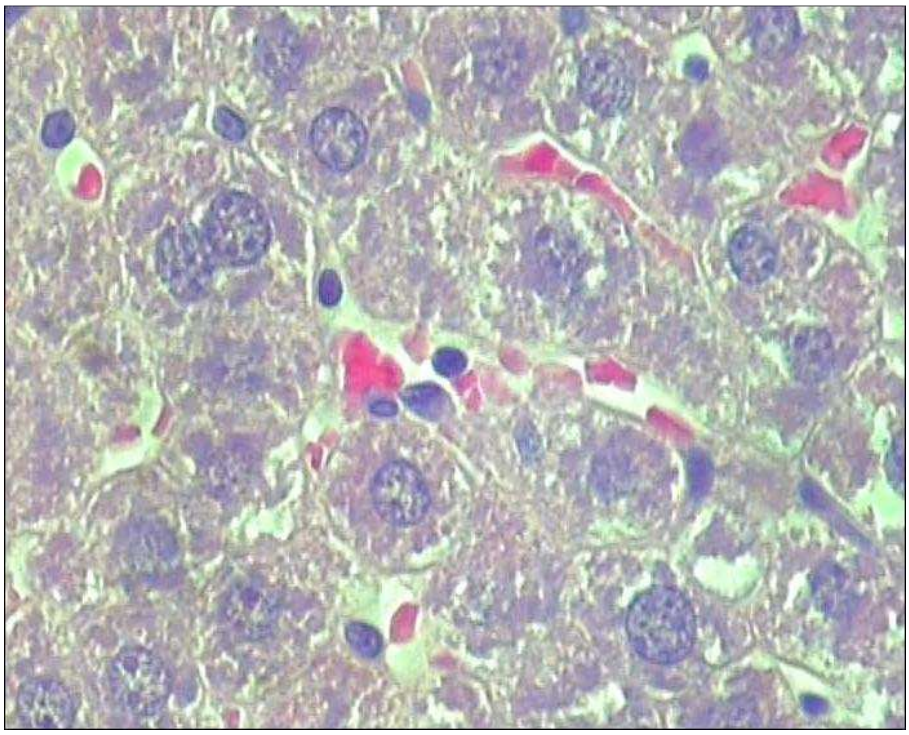

Figure 6.15 d): Liver section B from the S+CBZ group after 7 days of treatment, showing minimal vacuolar degeneration in the hepatocytes

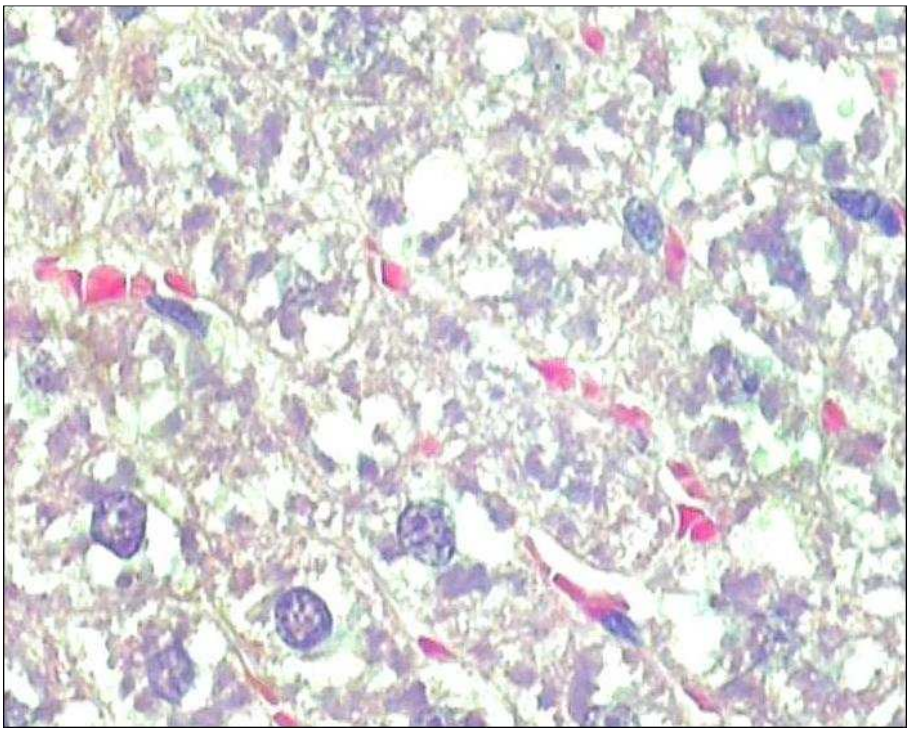

Figure 6.15 e): Liver section A from the S+CBZ group after 14 days of treatment, showing mild degeneration and vacuolar cell swelling, and single cell necrosis

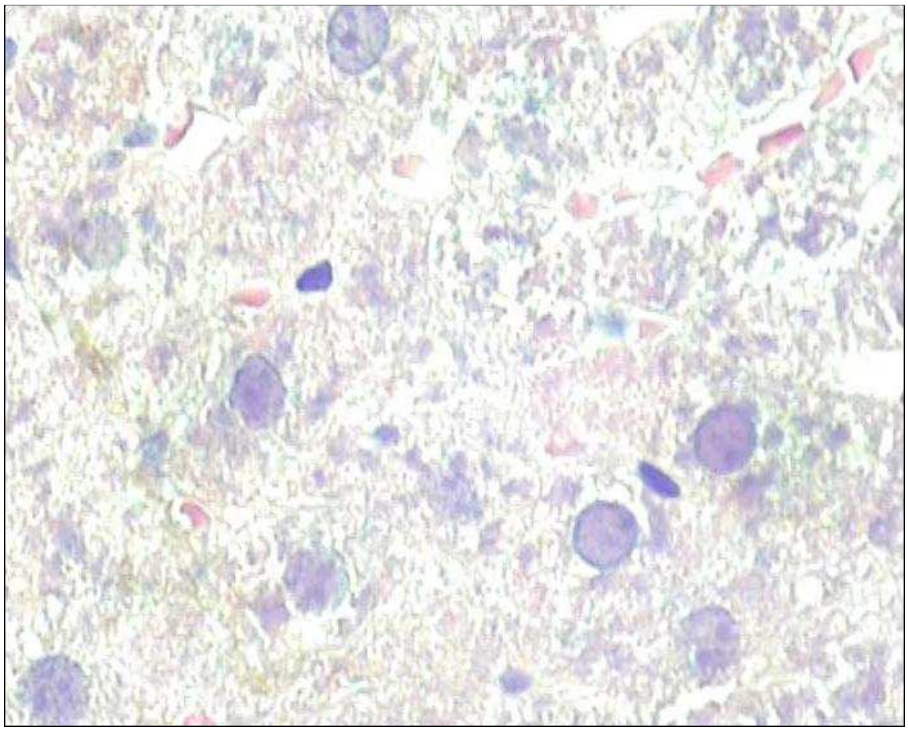

Figure 6.15 f): Liver section B from the S+CBZ group after 14 days of treatment, showing mild hepatocyte degeneration and cytonecrosis

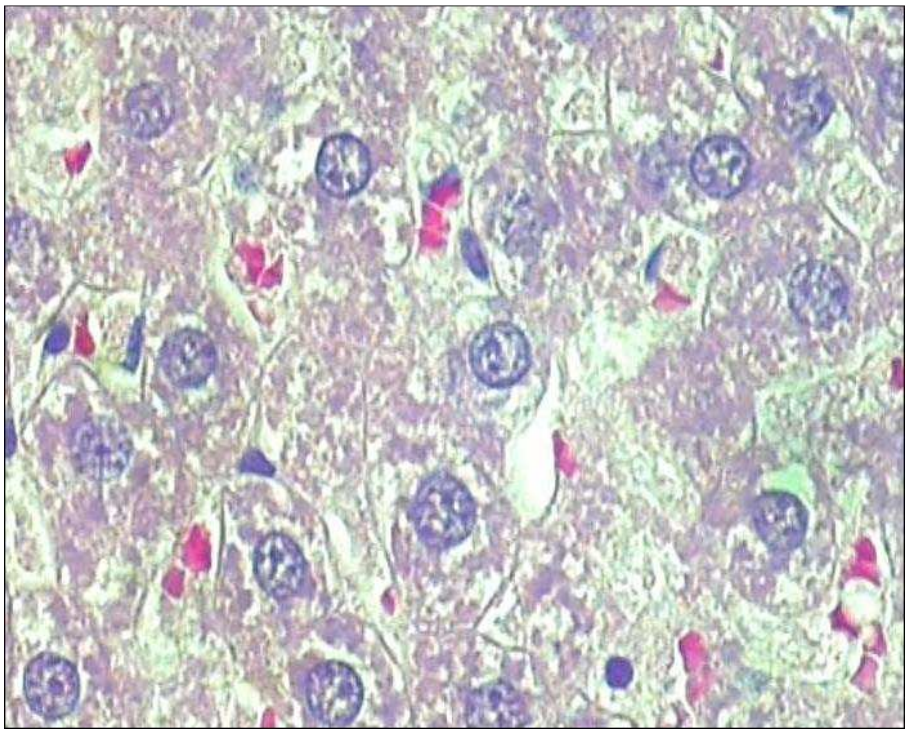

Figure 6.15 g): Liver section A from the S+CBZ group after 28 days of treatment, showing vacuolar granular degeneration classified as minimal

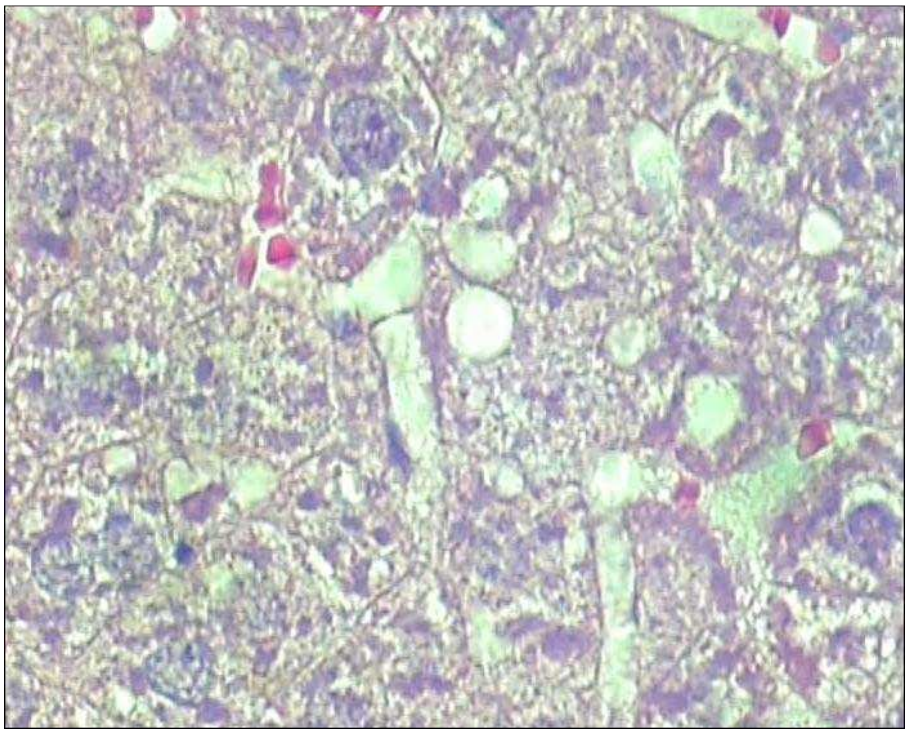

Figure 6.15 h): Liver section B from the S+CBZ group after 28 days of treatment, showing minimal degeneration, as well as cytonecrosis

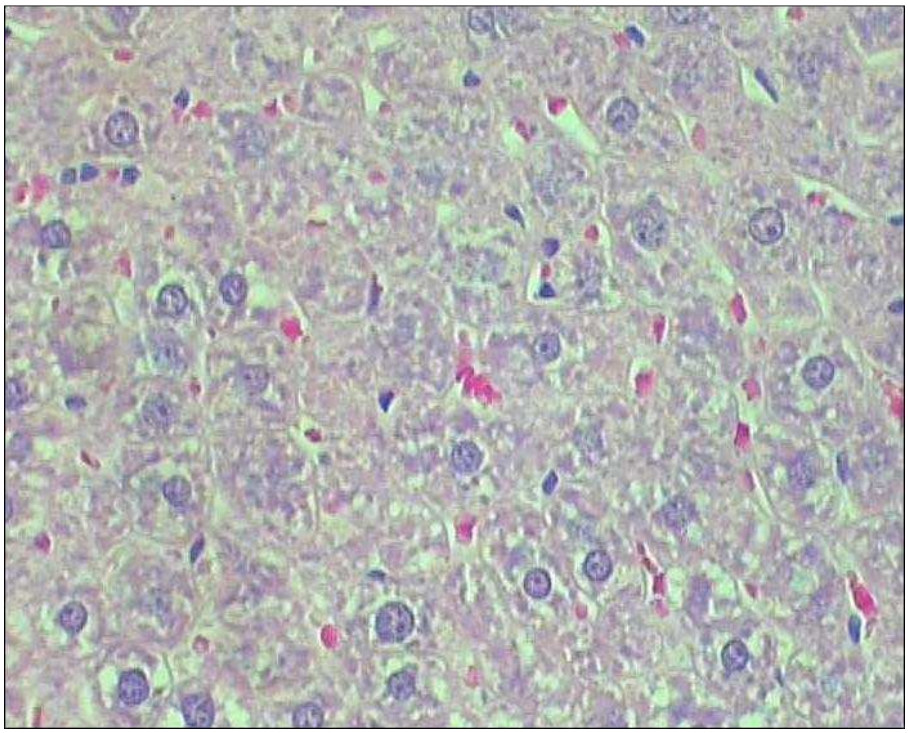

Figure 6.15 i): Liver section A from the S+CBZ group after 42 days of treatment, showing minimal hepatocyte degeneration, as well as single cell necrosis and loss of hepatic nuclei

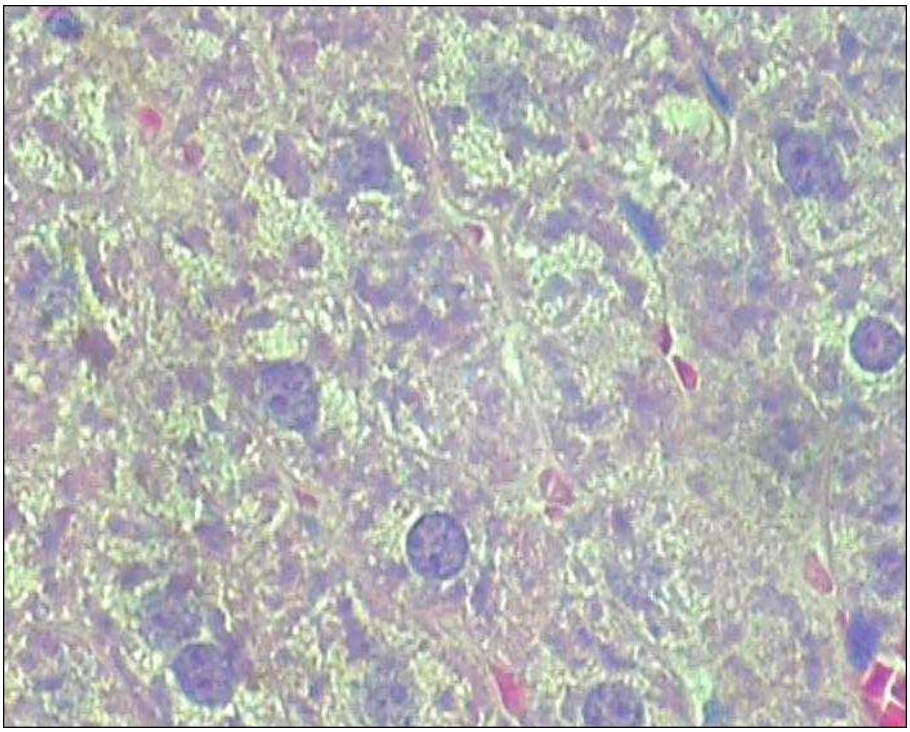

Figure 6.15 j): Liver section B from the S+CBZ group after 42 days of treatment, showing minimal hepatocyte degeneration, single cell necrosis and loss of hepatic nuclei

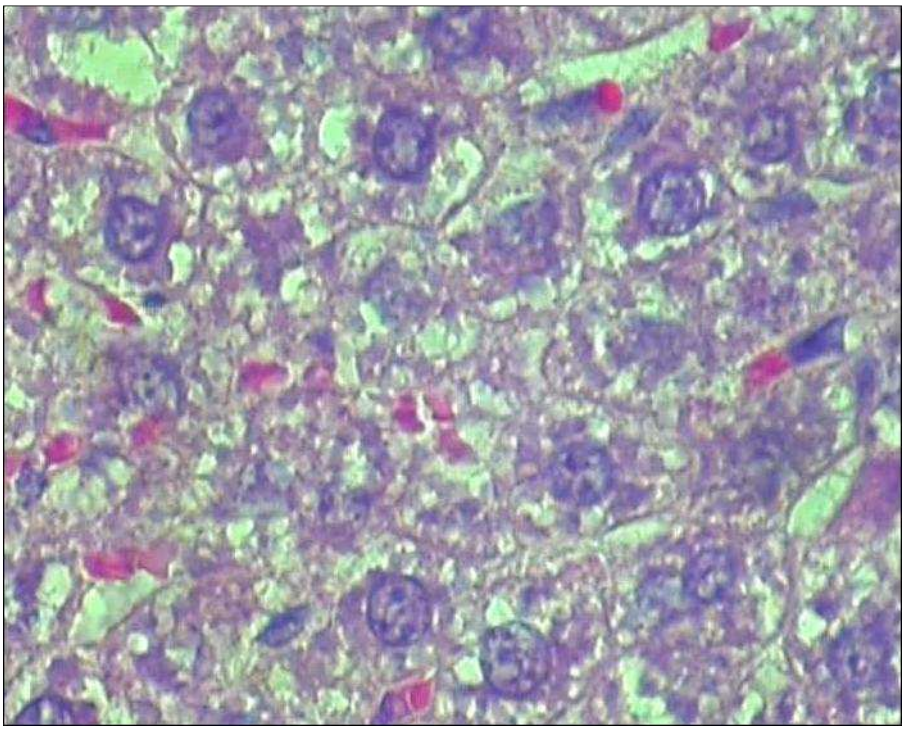

Figure 6.15 k): Liver section A from the INH+CBZ group after 2 days of treatment, showing mild degeneration and nuclear loss, suggesting single cell necrosis

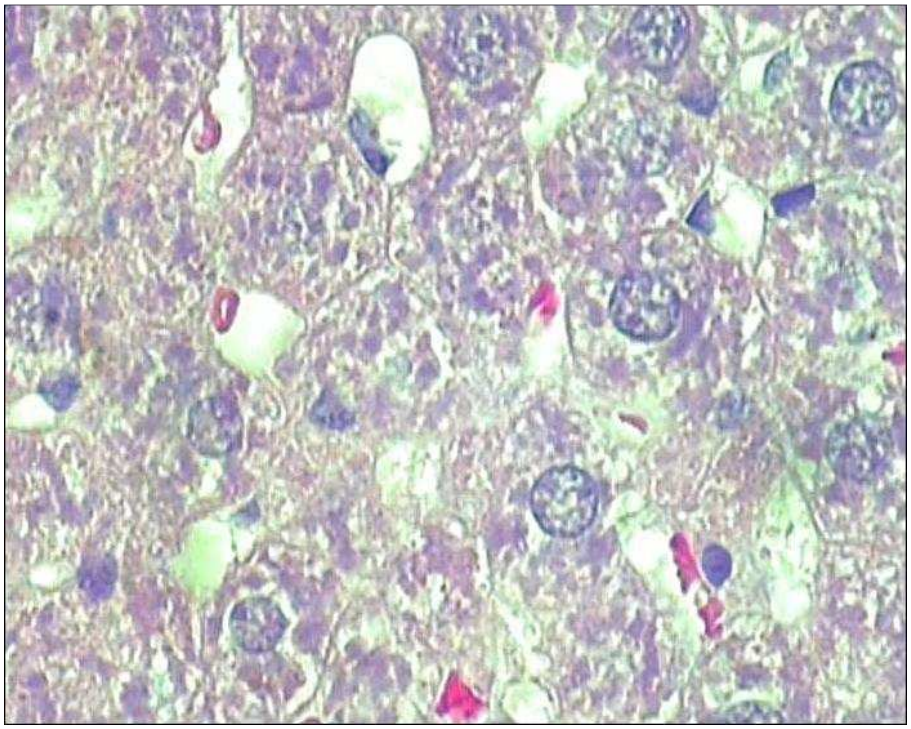

Figure 6.15 l): Liver section B from the INH+CBZ group after 2 days of treatment, showing mild granular vacuolar degeneration and single cell necrosis within the liver parenchyma

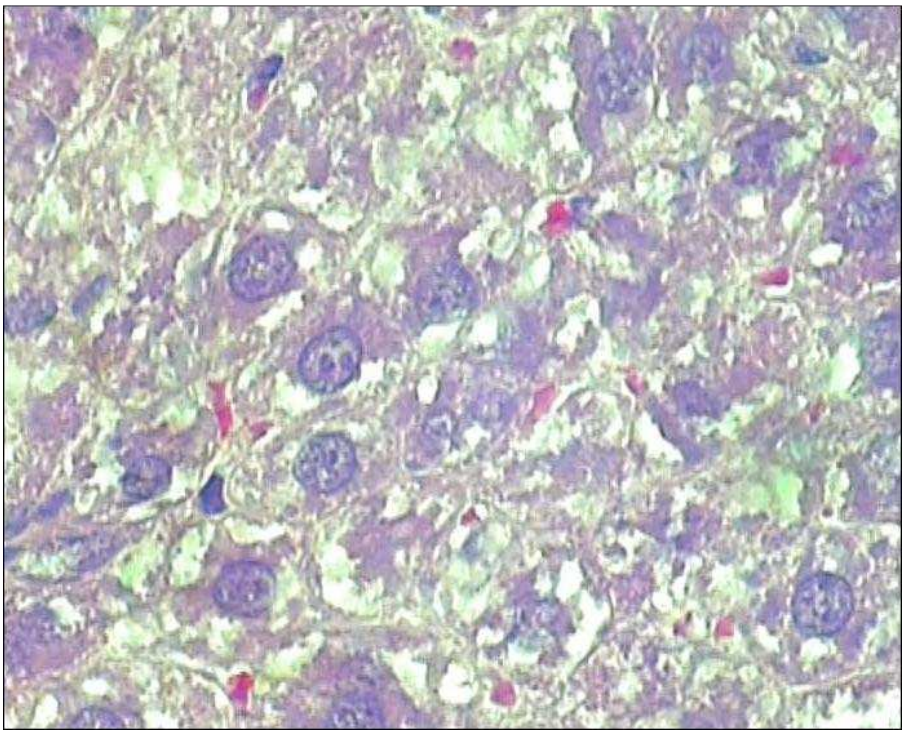

Figure 6.15 m): Liver section A from the INH+CBZ group after 7 days of treatment, showing vacuolar changes and cytonecrosis of mild degree

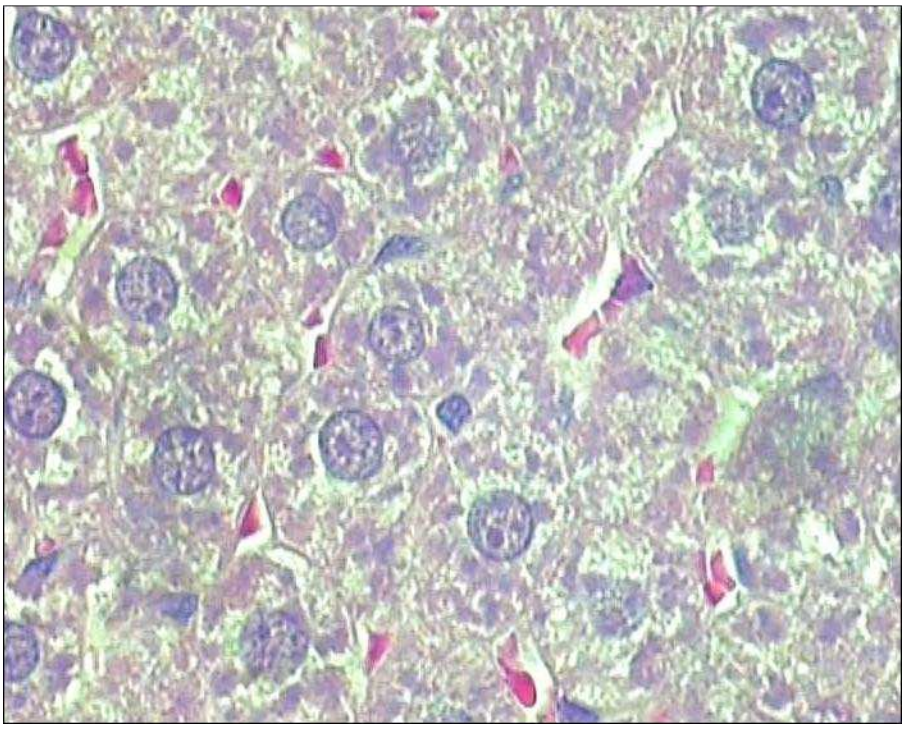

Figure 6.15 n): Liver section B from the INH+CBZ group after 7 days of treatment, showing mild degeneration and minimal cytonecrosis

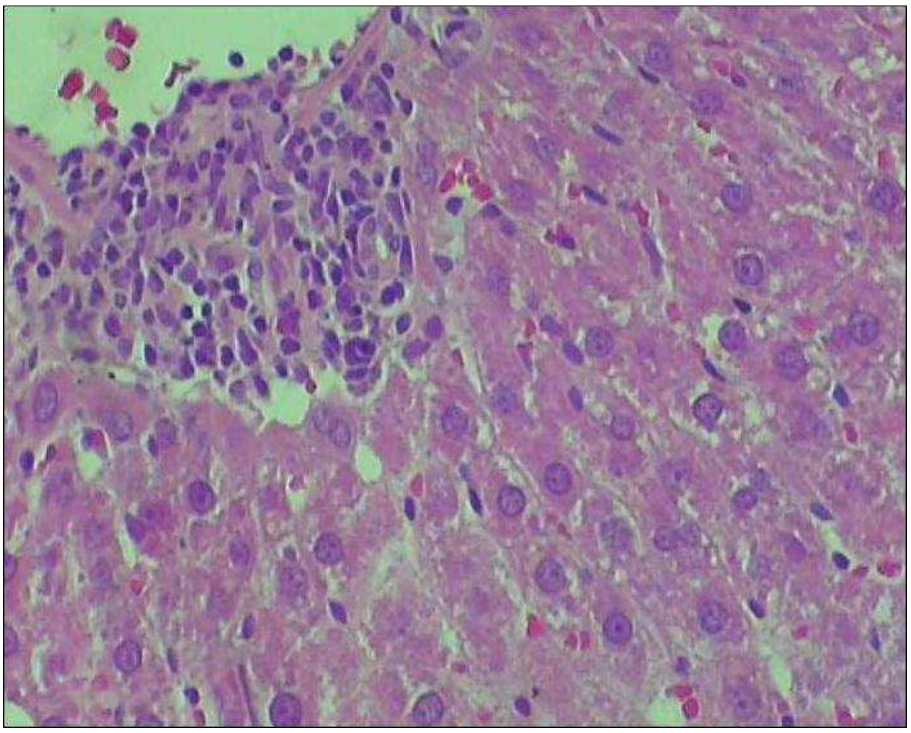

Figure 6.15 o): Liver section A from the INH+CBZ group after 14 days of treatment, showing a portal area with minimal lymphocytic infiltrates in the periportal parenchyma

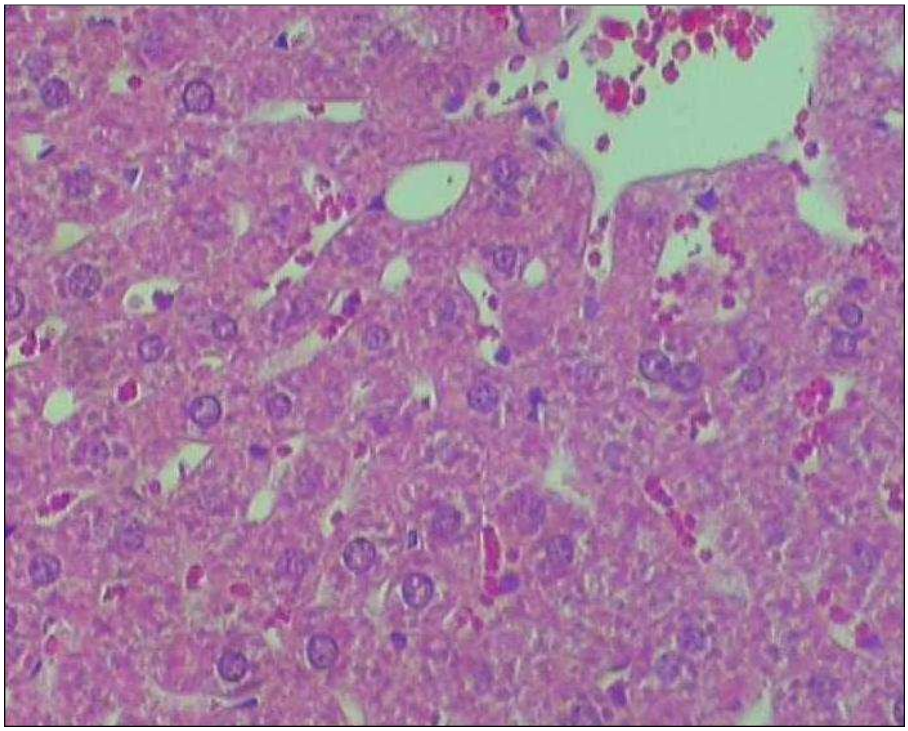

Figure 6.15 p): Liver section B from the INH+CBZ group after 14 days of treatment, showing centrilobular hepatocytes with mild degeneration and swelling

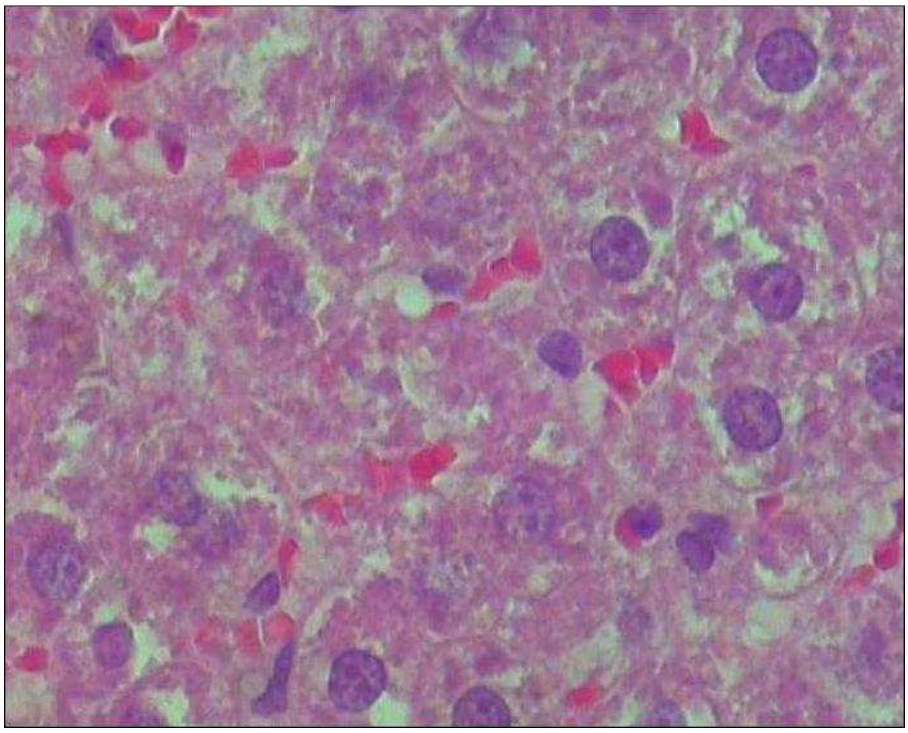

Figure 6.15 q): Liver section A from the INH+CBZ group after 28 days of treatment, showing hepatocytes with mild vacuolar degeneration

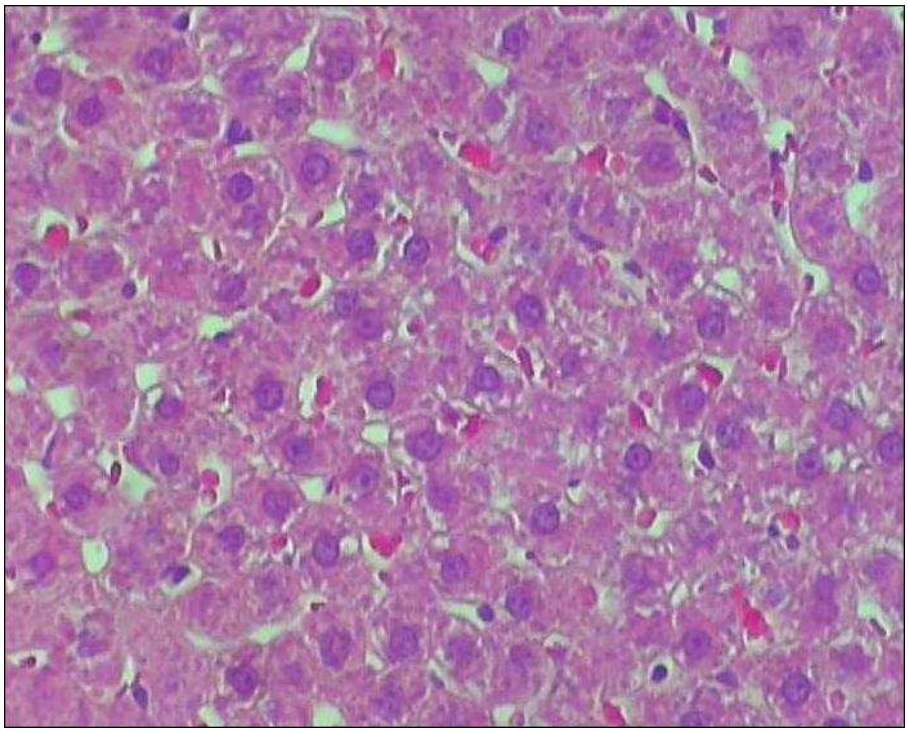

Figure 6.15 r): Liver section B from the INH+CBZ group after 28 days of treatment, showing hepatic cords with minimal granular vacuolar degeneration, and cytonecrosis in the parenchyma

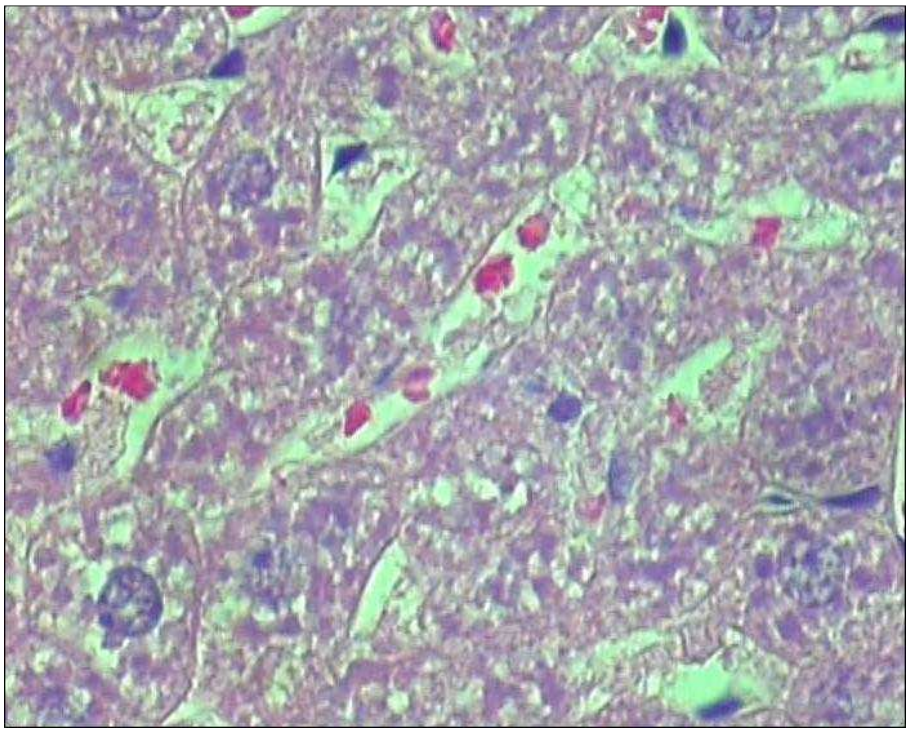

Figure 6.15 s): Liver section A from the INH+CBZ group after 42 days of treatment, showing degeneration, as well as cytonecrosis

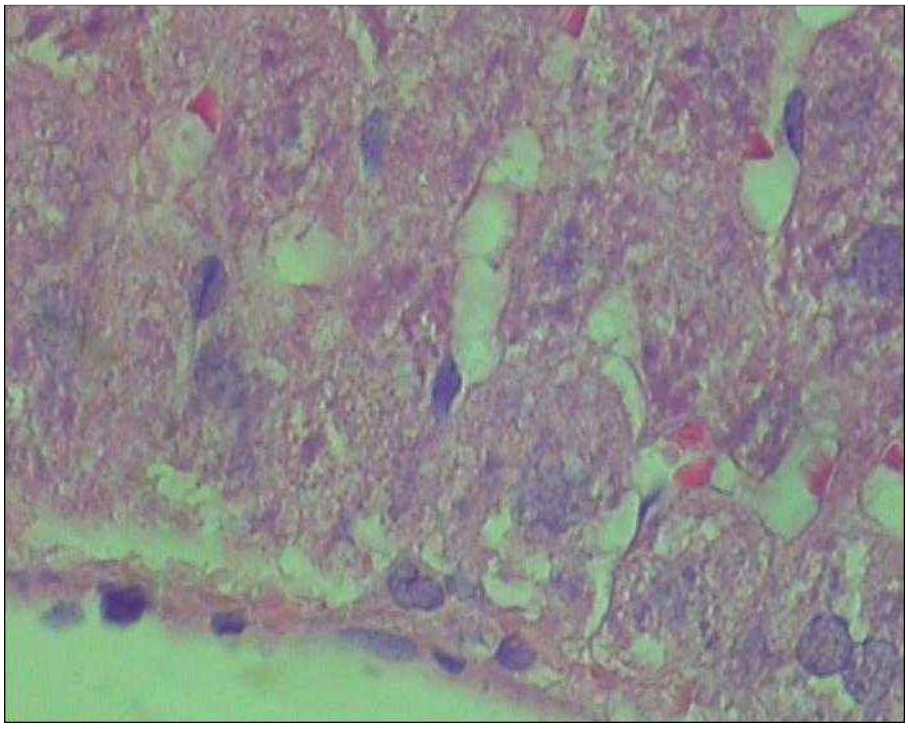

Figure 6.15 t): Liver section B from the INH+CBZ group after 42 days of treatment, showing a centrilobular area with necrosis of the hepatocytes

Table 6.25 a): Tally of main pathology lesions (lesions score) in livers of untreated rats and the S+CBZ group

Group	UnRx	S+CBZ									
		2 Days		7 Days		14 Days		28 Days		42 Days	
(n = 2)	Fig.6.3a	Fig.6.15a	Fig.6.15b	Fig.6.15c	Fig.6.15d	Fig.6.15e	Fig.6.15f	Fig.6.15g	Fig.6.15h	Fig.6.15i	Fig.6.15j
Histopathological lesions											
Vacuolar degeneration	0	0	1+	1+	1+	2+	2+	1+	1+	1+	1+
Cell swelling	0	0	1+	1+	1+	2+	2+	1+	1+	1+	1+
Cytonecrosis	0	0	0	0	0	2+	2+	0	1+	1+	1+
Centrilobular necrosis	0	0	0	0	0	0	0	0	0	0	0
Hepatocyte mitosis	0	0	0	0	0	0	0	0	0	0	0
Average lesions score											
Vacuolar degeneration	0	0.5+		1+		2+		1+		1+	
Cell swelling	0	0.5+		1+		2+		1+		1+	
Cytonecrosis	0	0		0		2+		0.5+		1+	
Centrilobular necrosis	0	0		0		0		0		0	
Hepatocyte mitosis	0	0		0		0		0		0	
Total lesion score	**0**	**1+**		**2+**		**6+**		**2.5+**		**3+**	

UnRx = untreated; S = saline; CBZ = carbamazepine

Table 6.25 b): Tally of main pathology lesions (lesions score) in livers of untreated rats and the INH+CBZ group

Group	UnRx	INH+CBZ									
		2 Days		7 Days		14 Days		28 Days		42 Days	
(n = 2)	Fig.6.3a	Fig.6.15k	Fig.6.15l	Fig.6.15m	Fig.6.15n	Fig.6.15o	Fig.6.15p	Fig.6.15q	Fig.6.15r	Fig.6.15s	Fig.6.15t
Histopathological lesions											
Vacuolar degeneration	0	2+	2+	2+	2+	2+	2+	2+	1+	3+	2+
Cell swelling	0	2+	2+	2+	2+	2+	2+	2+	1+	3+	2+
Cytonecrosis	0	2+	2+	2+	1+	1+	1+	1+	1+	2+	2+
Centrilobular necrosis	0	0	0	0	0	0	0	0	0	1+	1+
Hepatocyte mitosis	0	0	0	0	0	0	0	1+	0	0	0
Average lesions score											
Vacuolar degeneration	0	2+		2+		2+		1.5+		2.5+	
Cell swelling	0	2+		2+		2+		1.5+		2.5+	
Cytonecrosis	0	2+		1.5+		1+		1+		2+	
Centrilobular necrosis	0	0		0		0		0		1+	
Hepatocyte mitosis	0	0		0		0		0.5+		0	
Total lesion score	**0**	**6+**		**5.5+**		**5+**		**4.5+**		**8+**	

UnRx = untreated; INH = isoniazid; CBZ = carbamazepine

6.3.11 Isoniazid concentrations

Table 6.26 shows isoniazid concentrations of the INH and INH+CBZ groups, while Figure 6.16 is a graphical illustration of the same. For the INH+CBZ group, isoniazid concentrations increased slightly up to 14 days, and dropped by 28 days ($p = 0.0040$). Throughout, isoniazid levels were lower with isoniazid and carbamazepine co-administration, especially on day 28 ($p = 0.0040$).

Table 6.26: Average (mean ± SD) isoniazid concentrations of the INH and INH+CBZ groups

Group (n = 5)	INH INH concentration (µg/ml)	INH+CBZ INH concentration (µg/ml)
2 Days	1.891 ± 0.57	1.701 ± 1.39
7 Days	4.287 ± 1.50	3.596 ± 1.57
14 Days	8.628 ± 6.82	4.347 ± 3.07
28 Days	2.642 ± 0.81	0.169 ± 0.38
42 Days	1.607 ± 1.19	2.678 ± 5.36

INH = isoniazid; CBZ = carbamazepine

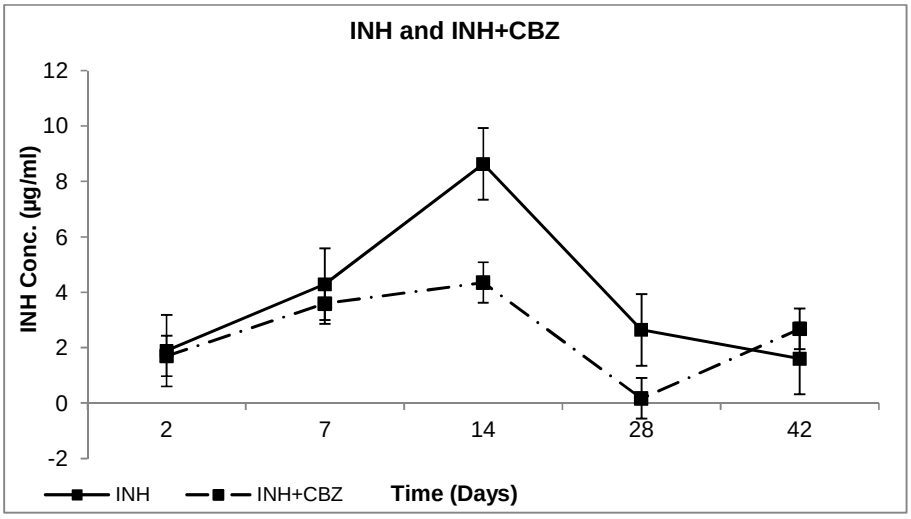

Figure 6.16: Isoniazid concentrations of the INH and INH+CBZ groups over 42 days

6.3.12 Specific immunology tests

6.3.12.1 Direct observations

Table 6.27 shows changes in body weight of the S, S+CBZ, INH and INH+CBZ groups over the 42 day treatment period. All groups showed weight gain at all times, except for the S+CBZ group after 2 days (Refer to Appendix H-1 and H-2 for baseline weights).

Table 6.27: Average (mean ± SD) change in rat weights of the S, S+CBZ, INH and INH+CBZ groups

Group (n = 5)	S change in weight (g)	S+CBZ change in weight (g)	INH change in weight (g)	INH+CBZ change in weight (g)
2 Days	9.2±4	-3.9±2	7.0±6	11.2±3
7 Days	35.7±8	34.0±4	31.0±19	22.8±3
14 Days	84.6±5	66.2±4	55.0±10	66.8±7
28 Days	107.8±10	97.3±30	111.9±12	109.7±34
42 Days	171.4±27	142.5±18	141.3±14	129.8±7

S = saline; CBZ = carbamazepine; INH = isoniazid

6.3.12.2 Cytokines

Table 6.28 shows IL-2 and IL-10 concentrations of the S, S+CBZ, INH and INH+CBZ groups, while Figures 6.17 a – b are graphical illustrations of the same. For the INH+CBZ group, IL-2 levels increased up to day 14 ($p = 0.0500$) and then declined. It was also higher than in the INH group until day 14 ($p = 0.0500$). IL-10 concentrations in the INH+CBZ group had declined by day 42 ($p = 0.0500$), and were higher than in the INH group ($p = 0.0500$).

Table 6.28: Average (mean ± SD) cytokine concentrations of the S, S+CBZ, INH and INH+CBZ groups

Group	Cytokine		Group	Cytokine	
(n = 3)	IL-2 (pg/ml)	IL-10 (pg/ml)	(n = 3)	IL-2 (pg/ml)	IL-10 (pg/ml)
Untreated					
0 Days	65.46 ± 2.0	31.08 ± 1.2			
S			**INH**		
2 Days	74.87 ± 6.5	29.96 ± 2.8	2 Days	70.56 ± 1.5	30.26 ± 6.0
7 Days	77.26 ± 5.8	34.57 ± 0.7	7 Days	83.80 ± 1.7	31.96 ± 0.7
14 Days	77.58 ± 6.6	35.69 ± 5.4	14 Days	78.38 ± 5.8	32.46 ± 4.8
28 Days	78.81 ± 4.6	32.46 ± 4.2	28 Days	77.42 ± 6.0	28.51 ± 5.2
42 Days	74.39 ± 5.7	32.03 ± 2.5	42 Days	72.32 ± 5.9	34.32 ± 6.8
S+CBZ			**INH+CBZ**		
2 Days	52.33 ± 14.6	37.31 ± 2.3	2 Days	86.00 ± 2.8	41.80 ± 3.1
7 Days	112.50 ± 7.8	40.11 ± 4.7	7 Days	104.00 ± 72.8	35.25 ± 3.1
14 Days	120.83 ± 12.8	40.11 ± 3.1	14 Days	169.00 ± 22.6	37.68 ± 0.6
28 Days	117.75 ± 23.7	40.67 ± 6.0	28 Days	62.00 ± 16.5	38.61 ± 1.8
42 Days	92.50 ± 46.0	36.00 ± 4.9	42 Days	44.33 ± 9.0	31.69 ± 2.5

IL = interleukin; S = saline; INH = isoniazid; CBZ = carbamazepine

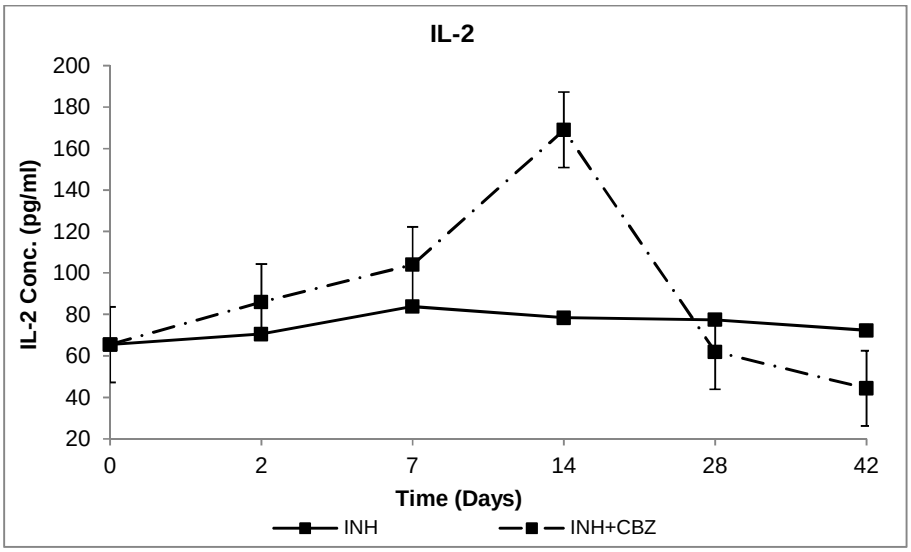

Figure 6.17 a): IL-2 concentrations of the INH and INH+CBZ groups over 42 days

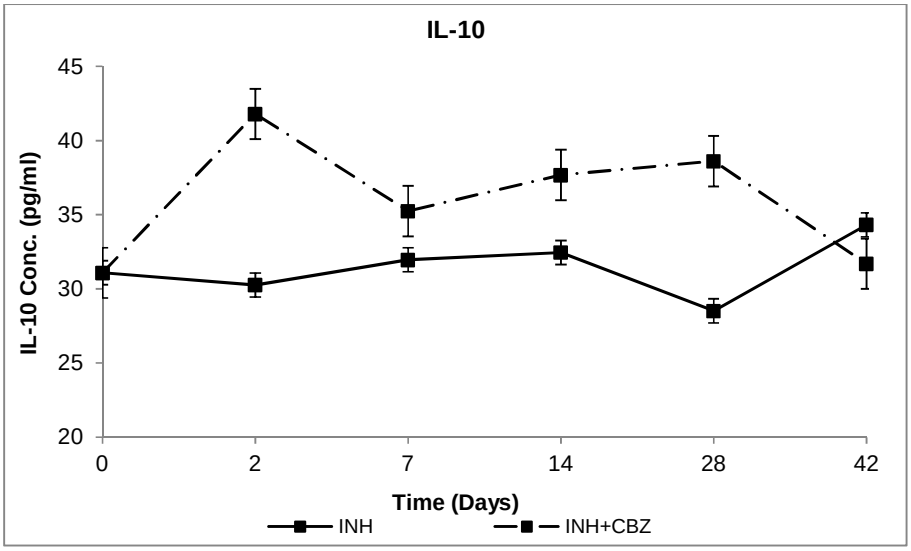

Figure 6.17 b): IL-10 concentrations of the INH and INH+CBZ groups over 42 days

6.3.12.3 CD4 and CD8 counts

Table 6.29 shows CD4 and CD8 counts of the S, S+CBZ, INH and INH+CBZ groups, while Figures 6.18 a – b are graphical illustrations of the same. Over the 42 days, CD4 and CD8 counts of the INH+CBZ group were normal, despite the drop in CD8 count on day 28 ($p = 0.0500$).

Table 6.29: Average (mean ± SD) CD4 and CD8 counts of the S, S+CBZ, INH and INH+CBZ groups

Group	**Ly**	**T-Ly**		**Group**	**Ly**	**T-Ly**	
		CD4	**CD8**			**CD4**	**CD8**
(n = 3)	**(x10^9/l)**	**(x10^9/l)**	**(x10^9/l)**	**(n = 3)**	**(x10^9/l)**	**(x10^9/l)**	**(x10^9/l)**
Untreated							
0 Days	4.67±1.8	2.23±1.3	1.42±0.7				
S				**INH**			
2 Days	5.18±0.7	2.27±0.3	1.35±0.2	2 Days	5.02±1.1	1.95±0.4	1.27±0.2
7 Days	4.07±2.0	1.72±0.8	1.07±0.5	7 Days	5.10±0.8	2.20±0.5	1.28±0.3
14 Days	4.21±0.7	1.69±0.2	1.17±0.2	14 Days	4.91±0.9	2.28±0.4	1.08±0.5
28 Days	6.15±0.8	2.45±0.2	1.58±0.3	28 Days	4.56±0.5	1.76±0.4	1.24±0.1
42 Days	3.23±0.3	1.47±0.1	0.79±0.2	42 Days	4.05±0.6	1.50±0.2	1.06±0.2
S+CBZ				**INH+CBZ**			
2 Days	5.46±1.1	1.96±0.3	1.39±0.1	2 Days	5.98±0.8	2.45±0.2	1.49±0.3
7 Days	6.20±2.1	2.33±0.8	1.41±0.4	7 Days	5.95±1.8	2.26±0.6	1.39±0.2
14 Days	7.28±0.5	2.70±0.2	1.59±0.2	14 Days	4.88±0.3	2.06±0.5	1.07±0.1
28 Days	4.32±1.0	1.79±0.4	0.00±0.0	28 Days	4.30±1.3	2.04±0.7	0.00±0.0
42 Days	5.55±0.5	1.73±0.2	1.54±0.2	42 Days	5.60±1.1	2.19±0.4	1.47±0.3

Ly = lymphocytes; CD = cluster of differentiation; S = saline; INH = isoniazid; CBZ = carbamazepine

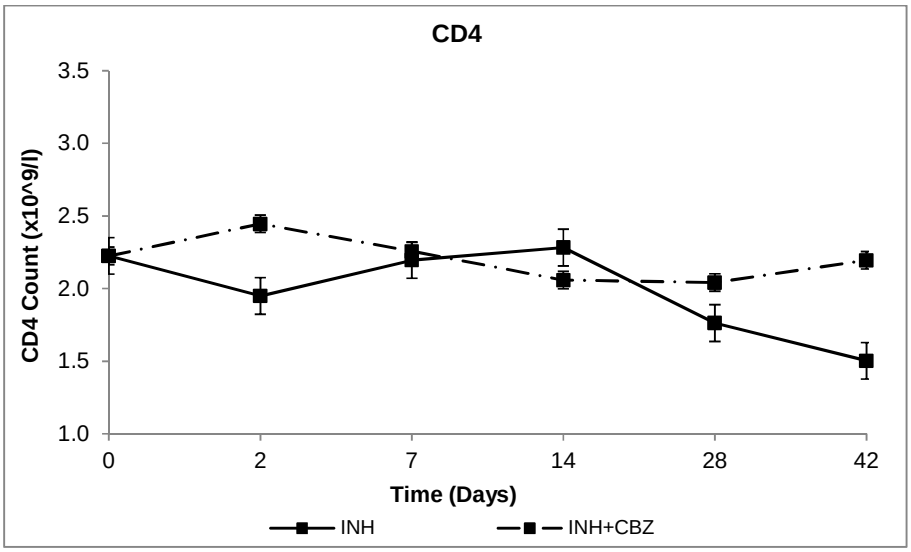

Figure 6.18 a): CD4 counts of the INH and INH+CBZ groups over 42 days

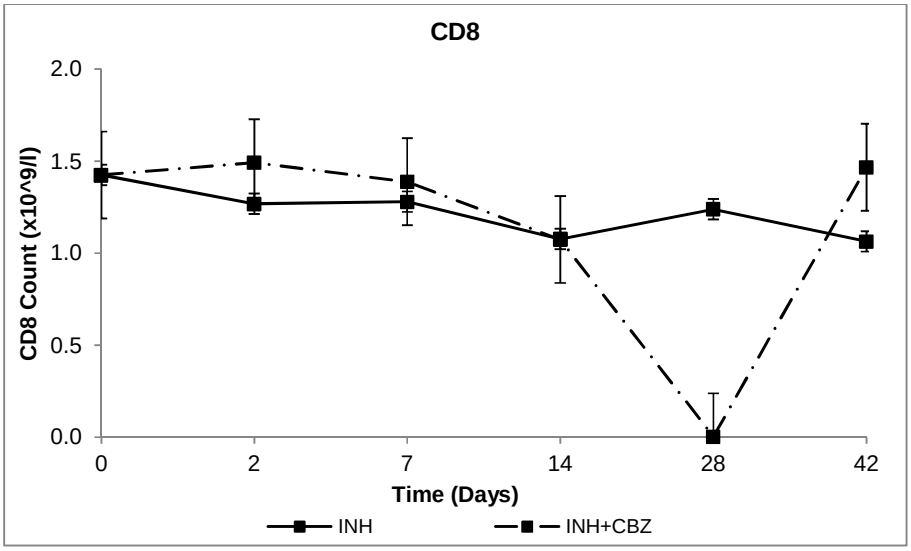

Figure 6.18 b): CD8 counts of the INH and INH+CBZ groups over 42 days

6.3.12.4 Immunoglobulins

Table 6.30 shows concentrations of IgM and IgG of the S, S+CBZ, INH and INH+CBZ groups, while Figures 6.19 a – b are graphical illustrations of the same. For the INH+CBZ group, IgM and IgG concentrations were normal over the 42 days treatment period. IgG levels were lower in the INH+CBZ group than in the INH group ($p = 0.0500$).

Table 6.30: Average (mean ± SD) immunoglobulin concentrations of the S, S+CBZ, INH and INH+CBZ groups

Group	**Immunoglobulin**		**Group**	**Immunoglobulin**	
	IgM	**IgG**		**IgM**	**IgG**
(n = 3)	**(mg/ml)**	**(mg/ml)**	**(n = 3)**	**(mg/ml)**	**(mg/ml)**
Untreated					
0 Days	0.109±0.02	14.434±1.10			
S			**INH**		
2 Days	0.104±0.04	14.137±0.91	2 Days	0.057±0.03	12.849±0.34
7 Days	0.110±0.04	14.302±0.70	7 Days	0.040±0.02	14.765±0.40
14 Days	0.110±0.03	12.617±0.29	14 Days	0.046±0.01	12.321±1.24
28 Days	0.075±0.03	16.350±1.00	28 Days	0.029±0.01	13.707±2.61
42 Days	0.046±0.01	17.109±0.26	42 Days	0.027±0.01	18.299±0.94
S+CBZ			**INH+CBZ**		
2 Days	0.107±0.01	9.823±1.19	2 Days	0.091±0.03	8.156±0.60
7 Days	0.108±0.00	8.483±0.63	7 Days	0.066±0.02	8.228±0.11
14 Days	0.083±0.01	9.751±2.10	14 Days	0.044±0.02	8.735±0.98
28 Days	0.084±0.01	11.525±2.12	28 Days	0.039±0.03	9.931±0.88
42 Days	0.071±0.01	12.140±1.83	42 Days	0.049±0.03	9.128±1.29

IgM = immunoglobulin M; IgG = immunoglobulin G; S = saline; INH = isoniazid; CBZ = carbamazepine

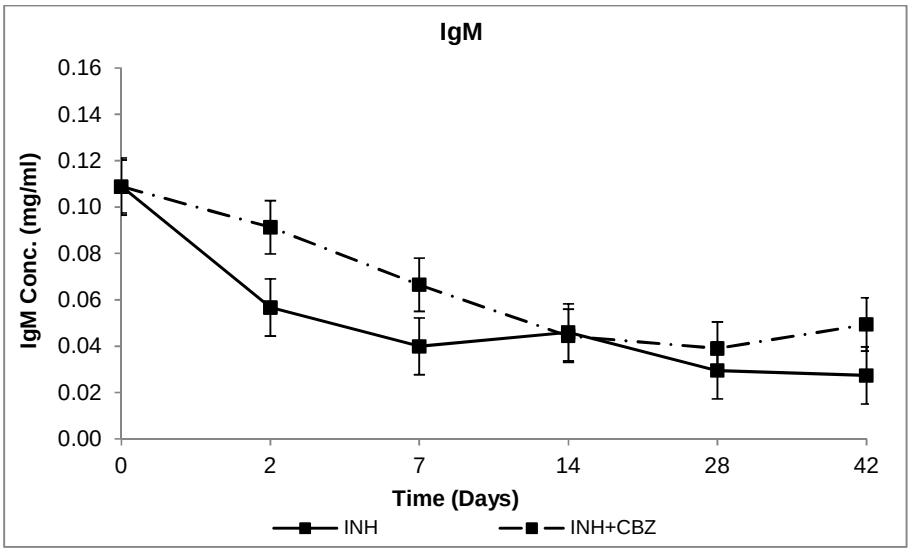

Figure 6.19 a): IgM concentrations of the INH and INH+CBZ groups over 42 days

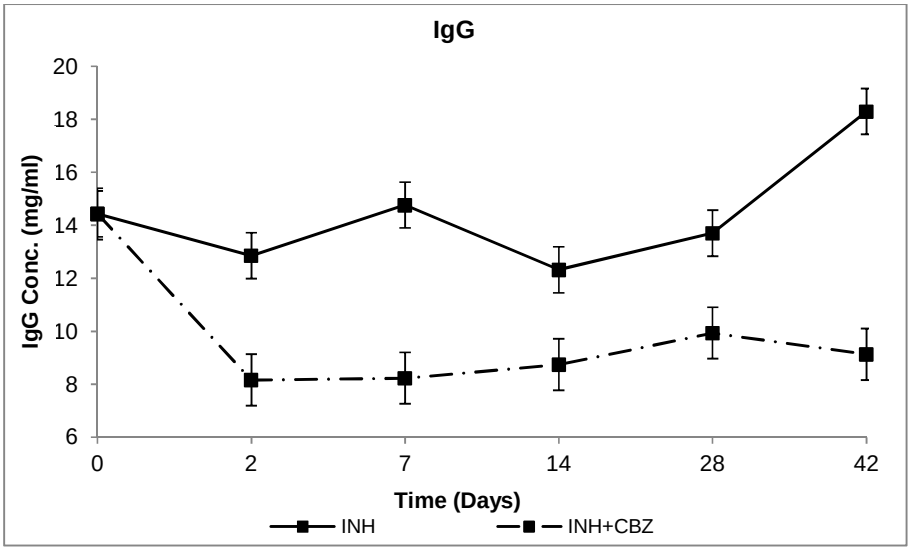

Figure 6.19 b): IgG concentrations of the INH and INH+CBZ groups over 42 days

6.3.13 Activity of rat CYP1A2, CYP2E1 and CYP3A2 *in vivo*

6.3.13.1 Protein concentrations

The results of BSA calibration samples are the same as shown in Section 6.3.4.1 (Table 6.10 and Figure 6.8). Table 6.31 shows microsomal protein concentrations of untreated rats, and the INH and INH+CBZ groups.

Table 6.31: Average (mean ± SD) microsomal protein concentrations of the untreated, INH and INH+CBZ groups

Selected liver (n = 3)	Prot. conc. (mg/ml)	Abs. (nm)	Selected liver (n = 3)	Prot. conc. (mg/ml)	Abs. (nm)
Untreated					
Rat 1	49.40 ± 6.8	0.132 ± 0.02			
Rat 2	55.02 ± 1.1	0.146 ± 0.00			
Rat 4	51.00 ± 5.1	0.136 ± 0.01			
INH-2D			**INH+CBZ-2D**		
Rat 1	31.05 ± 1.8	0.059 ± 0.00	Rat 2	17.70 ± 2.1	0.035 ± 0.00
Rat 2	38.52 ± 0.3	0.077 ± 0.00	Rat 3	30.63 ± 1.2	0.058 ± 0.00
Rat 3	51.76 ± 3.3	0.108 ± 0.01	Rat 4	31.27 ± 0.3	0.060 ± 0.00
INH-7D			**INH+CBZ-7D**		
Rat 1	31.50 ± 0.3	0.078 ± 0.00	Rat 2	38.12 ± 0.3	0.095 ± 0.00
Rat 3	42.21 ± 2.2	0.105 ± 0.01	Rat 4	31.78 ± 6.0	0.068 ± 0.01
Rat 5	40.07 ± 2.5	0.100 ± 0.01	Rat 5	40.07 ± 5.8	0.100 ± 0.01
INH-14D			**INH+CBZ-14D**		
Rat 1	20.00 ± 0.3	0.051 ± 0.00	Rat 2	57.99 ± 4.6	0.133 ± 0.01
Rat 4	23.91 ± 0.7	0.059 ± 0.00	Rat 3	49.24 ± 2.6	0.114 ± 0.01
Rat 5	34.73 ± 2.9	0.083 ± 0.01	Rat 4	46.70 ± 0.3	0.104 ± 0.00

Prot. conc. = protein concentration; Abs. = absorption; INH = isoniazid; CBZ = carbamazepine; D = days

6.3.13.2 CYP1A2, CYP2E1 and CYP3A2 activity in vivo

Table 6.32 shows CYP1A2, CYP2E1 and CYP3A2 activity after 2, 7 and 14 days of isoniazid alone (INH) and concomitant isoniazid and carbamazepine (INH+CBZ) treatment, while Figures 6.20 a – c are graphical illustrations of the same. Co-treatment with isoniazid and carbamazepine increased CYP1A2 activity up to 14 days ($p = 0.0286$), and this was similar with isoniazid alone. CYP2E1 reaction rate was slightly elevated until day 2, and was mostly lower than with isoniazid alone ($p = 0.0500$). Concomitant administration of isoniazid and carbamazepine had a minimal effect on CYP3A2 activity, which was similar to treatment with isoniazid alone.

Table 6.32: Average (mean ± SD) CYP1A2, CYP2E1 and CYP3A2 activity

Group (n = 3)	**CYP1A2 (pmol/min*mg)**	**CYP2E1 (nmol/min*mg)**	**CYP3A2 (pmol/min*mg)**
Untreated			
0 Days	4.40 ± 0.8	0.77 ± 0.1	84.63 ± 6.9
INH			
2 Days	5.00 ± 0.3	1.08 ± 0.2	75.94 ± 6.6
7 Days	8.25 ± 0.4	1.30 ± 0.3	87.91 ± 20.0
14 Days	10.12 ± 1.1	2.40 ± 1.4	74.09 ± 5.8
INH+CBZ			
2 Days	4.37 ± 0.7	1.22 ± 0.5	71.04 ± 17.4
7 Days	9.78 ± 0.4	1.22 ± 0.2	80.47 ± 23.1
14 Days	10.20 ± 0.9	1.26 ± 0.3	61.10 ± 2.1

RR = reaction rate; INH = isoniazid; CBZ = carbamazepine

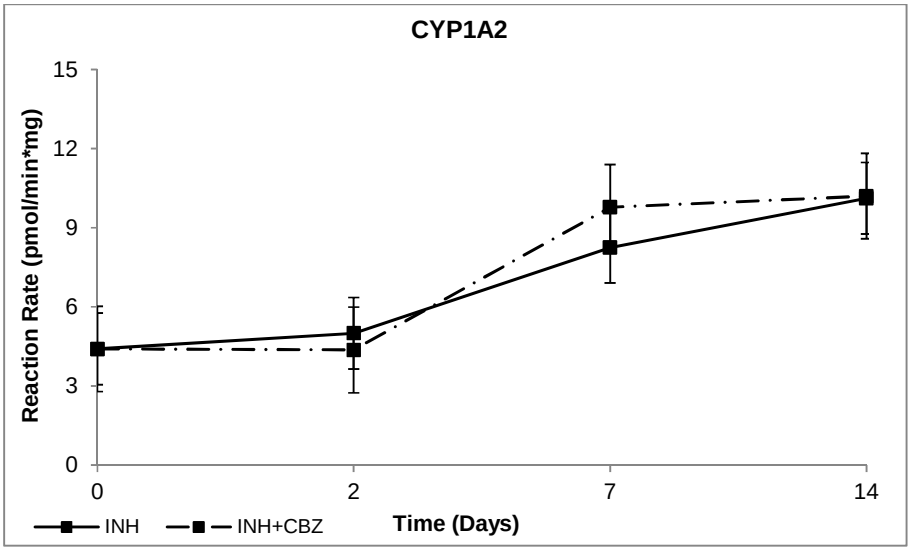

Figure 6.20 a): CYP1A2 activity after isoniazid alone, and carbamazepine co-treatment

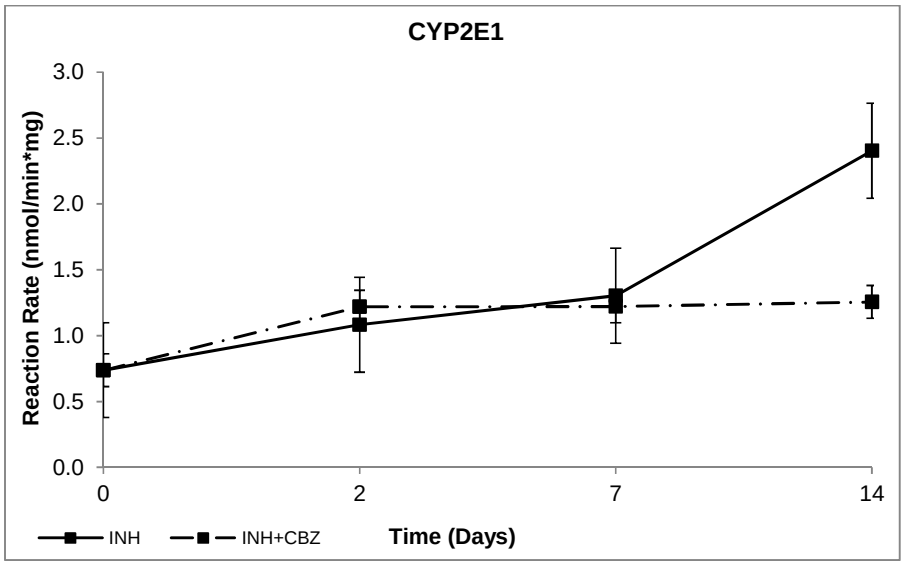

Figure 6.20 b): CYP2E1 activity after isoniazid alone, and carbamazepine co-treatment

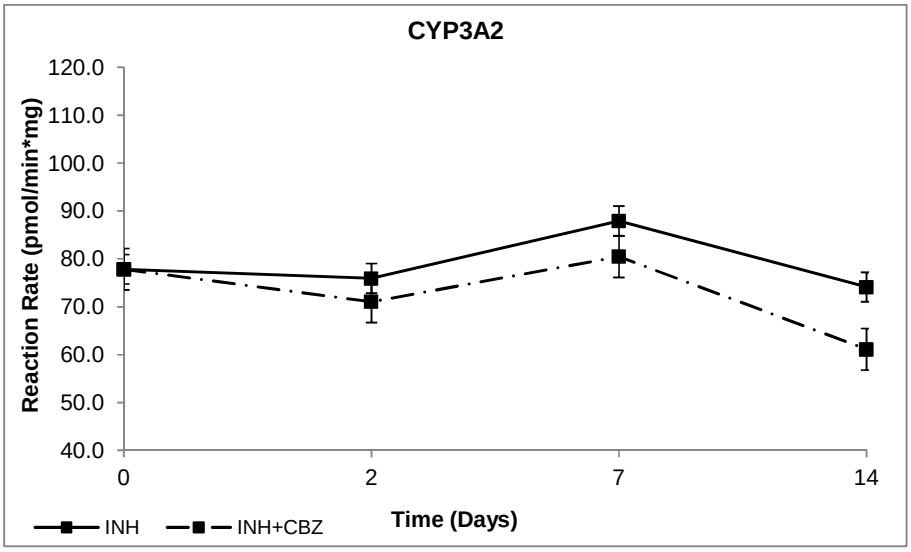

Figure 6.20 c): CYP3A2 activity after isoniazid alone, and carbamazepine co-treatment

6.3.14 Main observations

- Co-administration with carbamazepine caused less liver injury up to day 28.
- Throughout, isoniazid levels were lower with carbamazepine co-administration than with isoniazid alone.
- Carbamazepine co-treatment increased IL-2 levels till day 14 and this was higher than with isoniazid alone. IL-10 concentrations had declined by day 42, but were still higher than with isoniazid alone.
- Co-administration with carbamazepine did not stimulate the same IgG response as seen with isoniazid alone.
- Co-treatment with carbamazepine induced CYP1A2 and CYP2E1 activity, while the effect on CYP3A2 activity was minimal.

6.4 SUMMARY OF THE RESULTS

Based on the postulated mechanism of isoniazid-induced hepatotoxicity in which the immune system and CYP450 were implicated, this study aimed at demonstrating the changes in the different processes (physiological and pathological) as monitored by the relevant biomarkers during daily isoniazid administration at normal doses in animals that did not develop clinical hepatotoxicity. It was observed that the changes described for the different parameters occurred within their normal concentration ranges. Specifically, the liver injury was subclinical (*i.e.*, normal liver function tests) and the concentrations for isoniazid and chemokines were within their normal ranges.

These early changes were studied on the premise that they are the premonitory symptoms of the major pathological process; that during normal dosing, the body systems, which include the immune system and CYP450 enzymes, are able to respond to drug insults, indicated by increased or decreased chemokines or enzyme activity but within their normal range, and that understanding these early changes is vital to unveiling not only the mechanism of isoniazid hepatotoxicity, but also the development of strategies to prevent isoniazid hepatotoxicity. In the same perspective for this subclinical evaluation, the changes or responses in the test group relative to those in the control or INH group, may not need to be statistically significant to make sense.

Table 6.33 is a summary of the changes or responses in the test groups relative to those in the control or INH group, while Table 6.34 shows the relation between lesions scores and isoniazid concentrations, and Figure 6.21 is a graphical illustration of the same. Chronic treatment with isoniazid caused subclinical liver injury in the first 2 – 3 weeks followed by healing by week 6 (Table 6.33; INH alone). This was associated with a moderate increase in IL-2 up to day 7 and CD4 count up to day 28 (Th1 response) followed by an increase in IL-10 from day 7 onwards (Th2 response), high isoniazid concentrations by day 14 (Table 6.34) and a progressive increase in CYP2E1 activity (Table 6.33; INH alone). In effect, the isoniazid-induced subclinical liver injury was associated with high isoniazid concentrations, increased CYP2E1 activity and immune activation whereby the Th1 response correlated with

the toxic phase, and the Th2 response with the healing phase (Table 6.33; INH alone).

Co-treatment with an immune stimulant, levamisole, led to a more severe liver injury, but of late onset (started on day 7), up to day 28 that was associated with higher isoniazid concentrations (Table 6.34), lower IL-2 (Th1 response) versus increased IL-10 and IgM (Th2 response; Table 6.33; INH+LMS). Here, the strong Th2 response implies that there was probably a Th1 response that was not captured by the study sampling framework. Treatment with levamisole alone did not cause liver injury. Therefore, immune stimulation led to more severe isoniazid liver injury.

Co-treatment with an enzyme inducer, carbamazepine, led to a mild liver injury (less than for isoniazid alone) up to day 28 (Table 6.34; Figure 6.21). The mild liver injury was associated with lower isoniazid concentrations, mild increase in IL-2 up to day 14 (Th1 response) versus moderate increase in IL-10 from day 2 to day 28 (Th2 response; Table 6.33; INH+CBZ). Interestingly by day 42 the liver injury had worsened, and this was associated with slightly higher isoniazid concentrations and a weak Th2 response, most probably because the enzyme inducing effects were off (Table 6.33; INH+CBZ). Treatment with carbamazepine alone caused mild liver injury up to day 14, and carbamazepine did not induce liver enzyme activity.

Table 6.33: Description of the changes or responses in the test group relative to those in the control or INH group

INH alone

	Period 1 0 – 14 days of treatment	**Period 2 14 – 42 days of treatment**
	INH vs. S	**INH vs. S**
Liver injury (score)	Increased, 8+ (d14)	Decreased, 4+ (d42)
INH conc. (µg/ml)	Peak at d14 (8.6 µg/ml)	Lower than phase 1
IL-2 conc. (pg/ml)	Increased (higher), peaked d7	Decreased, to control (S) by d42
IL-10 conc. (pg/ml)	Moderate increase from d7	Moderate increase from d28 to d42
CYP2E1(nmol/min*mg)	Increased CYP2E1 activity by d2 and continued so	

INH+LMS

	Period 1 0 – 14 days of treatment	**Period 2 14 – 42 days of treatment**
	INH+LMS vs. INH alone	**INH+LMS vs. INH alone**
Liver injury (score)	Increased, 8.5+ (d14)	Worsened, 10.5+ (d28)
INH conc. (µg/ml)	Lower, up to d14	Peak at d28 (9.6 µg/ml)
IL-2 conc. (pg/ml)	Similar up to day d2	Lower, from d7 onwards
IL-10 conc. (pg/ml)	Increase from d2	Higher up to d42

INH+CBZ

	Period 1 0 – 14 days of treatment	**Period 2 14 – 42 days of treatment**
	INH+CBZ vs. INH alone	**INH+CBZ vs. INH alone**
Liver injury (score)	Mild, 5+ (d14)	Improved, 4.5+ (d28)
INH conc. (µg/ml)	Peak at d14 (4.2 µg/ml)	Lower
IL-2 conc. (pg/ml)	Moderate increase, peak at d14	Fell back to INH alone group by d42
IL-10 conc. (pg/ml)	Moderate increase from d2	Increased, up to d28

INH = isoniazid; LMS = levamisole; CBZ = carbamazepine; d = day

Table 6.34: The relation between isoniazid concentrations and histopathological lesions in the INH, INH+LMS and INH+CBZ groups

Group	Lesions score (score)	INH concentration (µg/ml)
Untreated		
0 Days	0+	0.000
INH		
2 Days	8+	1.891
7 Days	11+	4.287
14 Days	8+	8.628
28 Days	7+	2.642
42 Days	4+	1.607
INH+LMS		
2 Days	2.5+	2.299
7 Days	7+	2.360
14 Days	8.5+	3.694
28 Days	10.5+	9.618
42 Days	6+	3.316
INH+CBZ		
2 Days	6+	1.701
7 Days	5.5+	3.596
14 Days	5+	4.347
28 Days	4.5+	0.169
42 Days	8+	2.678

INH = isoniazid; LMS = levamisole; CBZ = carbamazepine

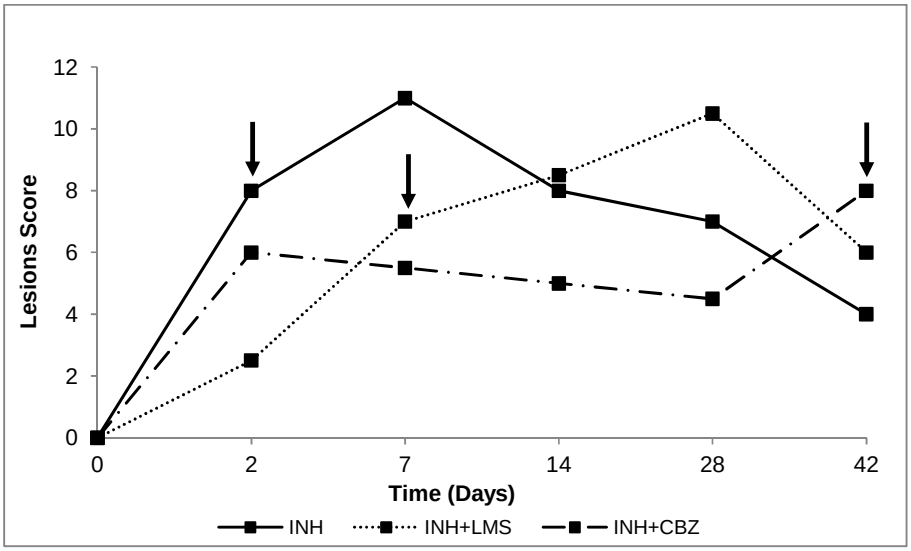

Figure 6.21: Lesions score chart of the INH, INH+LMS and INH+CBZ groups over 42 days

Table 6.34 and Figure 6.21 show that the histopathological lesions correlated with isoniazid concentrations, *i.e.*, the higher the isoniazid concentration the more severe the lesions score. In the first scenario, there was immune activation of the Th1 and Th2 responses followed by liver healing, indicating a counter adaptive immune mechanism against isoniazid to prevent progression to overt hepatotoxicity. Interestingly, in the second scenario, levamisole must have delayed the Th1 response as the lesions score only increased at a later stage of treatment (Table 6.34; Figure 6.21). Nonetheless, the strong Th2 response also suggests adaptation to the drug insult, which resulted in liver healing. However, in the third scenario the lesions score was constant and then increased as soon as the Th2 response returned to normal (Table 6.34; Figure 6.21). This suggests that the immune system is involved in isoniazid-induced liver injury, and that further studies are required to evaluate the role of CYP450.

In conclusion, the data suggest that the immune system is involved in the initial isoniazid-induced liver injury, and that it might also aid in the prevention of progression of the injury.

CHAPTER 7

CYTOCHROME P450 AND THE IMMUNE RESPONSE TO PROLONGED ADMINISTRATION OF NEVIRAPINE

7.0 SUMMARY

Introduction: Nevirapine is associated with hypersensitivity reactions and liver injury/toxicity that have hampered its use, particularly for HIV prophylaxis. Since the immune system and CYP450 enzymes have been implicated in the liver injury, these parameters were investigated during prolonged nevirapine administration.

Methods: The animal experiment was divided into three phases. During phase I, two groups of 25 rats each were administered daily S or NVP (200 mg/kg), while for phase II, two groups of 25 rats each received daily S or NVP in combination with an immune stimulant, LMS (2.5 mg/kg), and lastly, during phase III, two groups of 25 rats each received daily S or NVP in combination with a CYP450 inducer, CBZ (60 mg/kg). In each group, five rats were sacrificed after 2, 7, 14, 28 and 42 days. Blood was analysed for full blood count, CD4 and CD8 counts, liver function, renal function, IL-2, IL-10, IgG, IgM and nevirapine concentrations. A piece of liver was sent for histopathology testing. In addition, rat liver microsomes were analysed for CYP1A2, CYP2E1 and CYP3A2 activity.

Results: Nevirapine alone caused liver injury up to 14 days and this was associated with CYP3A2 induction, low nevirapine concentrations, and a moderate increase in CD4 count and IL-2 concentrations. By day 28 the injury had improved and was associated with increased IL-10 and IgG concentrations. Co-administration with levamisole exacerbated the injury and was associated with low nevirapine concentrations as well as lower IL-10 concentrations, while carbamazepine co-treatment caused less liver injury and was associated with increased IL-2 concentrations, which were counteracted by increased IL-10 concentrations.

Conclusion: This implies that the immune system is involved in initiating nevirapine-induced liver injury, and might also be a source of prevention to overt hepatotoxicity.

7.1 INTRODUCTION

Nevirapine is liable to hypersensitivity reactions such as skin rash and hepatotoxicity. Although involvement of the immune system in nevirapine-induced skin rash has already been proven (Popovic *et al.*, 2006; Shenton *et al.*, 2003), this is still unclear for nevirapine-induced liver injury, as other factors such as CYP450 enzymes may be involved (Walubo *et al*., 2006; Wen *et al*., 2009). This chapter describes the role of the CYP450 and immune response in nevirapine-induced liver injury. The results are reported under the following parameters: physiological observations (function tests), *i.e.*, full blood count, renal function tests, liver function tests and liver histopathology; nevirapine concentrations; specific immune tests, *i.e.*, direct observations, cytokines, CD4 and CD8 counts and immunoglobulins; and activity of rat CYP1A2, CYP2E1 and CYP3A2.

7.2 METHODS

A. Materials

7.2.1 Apparatus

All apparatus used are the same as described in Chapter 6, Section 6.2.1.

7.2.2 Chemicals

Viramune® (nevirapine) oral suspension (50 mg/5 ml) and tablets (200 mg; Boehringer Ingelheim Pharmaceuticals, Inc., Ridgefield, CT, U.S.A) were purchased from a local pharmacy, while the nevirapine standard was obtained from Sigma-Aldrich™ (St. Louis, MO, U.S.A). All other chemicals were of analytical grade, and the same as discussed in Chapter 6, Section 6.2.2.

7.2.3 Preparation of drugs for oral administration

200 mg/kg nevirapine was prepared by mixing Viramune® oral suspension and tablets, since the tablets are poorly soluble in water, while levamisole and carbamazepine were prepared as described in Chapter 6, Section 6.2.3.

7.2.4 Buffers and reagents

All buffers and reagents used were the same as discussed in Chapter 6, Section 6.2.4.

B. Methods

7.2.5 Experimental design

Here, a total of 155 rats were used. Five rats were not treated with any drug, and used for baseline data. Furthermore, the study was divided into three phases.

7.2.5.1 Phase I – Treatment with nevirapine alone

Rats were weighed and divided into two groups of 25 animals each, namely the S group (control) and the NVP group (test). Rats received saline solution or nevirapine once daily for 2, 7, 14, 28, and 42 days, respectively. At each time frame, five rats were sacrificed on the day following the last day of dosing. Saline solution and nevirapine were administered orally on a daily basis as follows (Figure 7.1):

- S group: saline solution (1 ml, orally)
- NVP group: 200 mg/kg nevirapine (1 ml, orally)

The dose of nevirapine was from a previous departmental study (Bekker *et al.*, 2012).

7.2.5.2 Phase II – Co-treatment with an immune stimulant

Rats were weighed and divided into two groups of 25 animals each, namely the S+LMS group (control) and the NVP+LMS group (test). Rats received saline solution plus levamisole or nevirapine plus levamisole for 2, 7, 14, 28, and 42 days, respectively. Levamisole administration commenced on the day before the start of saline or nevirapine dosing, thereafter saline and nevirapine were administered daily in the morning, and levamisole daily in the afternoon to avoid a potential drug interaction. At each time frame, five rats were sacrificed on the day following the last day of dosing. Saline solution, nevirapine and levamisole were administered orally as follows (Figure 7.1):

- S+LMS group: saline solution (1 ml, orally) and 2.5 mg/kg levamisole (500 µl, orally)
- NVP+LMS group: 200 mg/kg nevirapine (1 ml, orally) and 2.5 mg/kg levamisole (500 µl, orally)

The dose of levamisole was from a report by Gautam *et al*. (2009).

7.2.5.3 Phase III – Co-treatment with a CYP450 inducer

Rats were weighed and divided into two groups of 25 animals each, namely the S+CBZ group (control) and the NVP+CBZ group (test). Rats received saline solution plus carbamazepine or nevirapine plus carbamazepine for 2, 7, 14, 28, and 42 days, respectively. Carbamazepine administration was begun on the day before the start of saline or nevirapine dosing, thereafter saline and nevirapine were administered daily in the morning, and carbamazepine daily in the afternoon to avoid a potential drug interaction. At each time frame, five rats were sacrificed on the day following the last day of dosing. Saline solution, nevirapine and carbamazepine were administered orally as follows (Figure 7.1):

- S+CBZ group: saline solution (1 ml, orally) and 60 mg/kg carbamazepine (500 µl, orally)
- NVP+CBZ group: 200 mg/kg nevirapine (1 ml, orally) and 60 mg/kg carbamazepine (500 µl, orally)

The dose of carbamazepine was from a report by Tateishi and co-workers (1999).

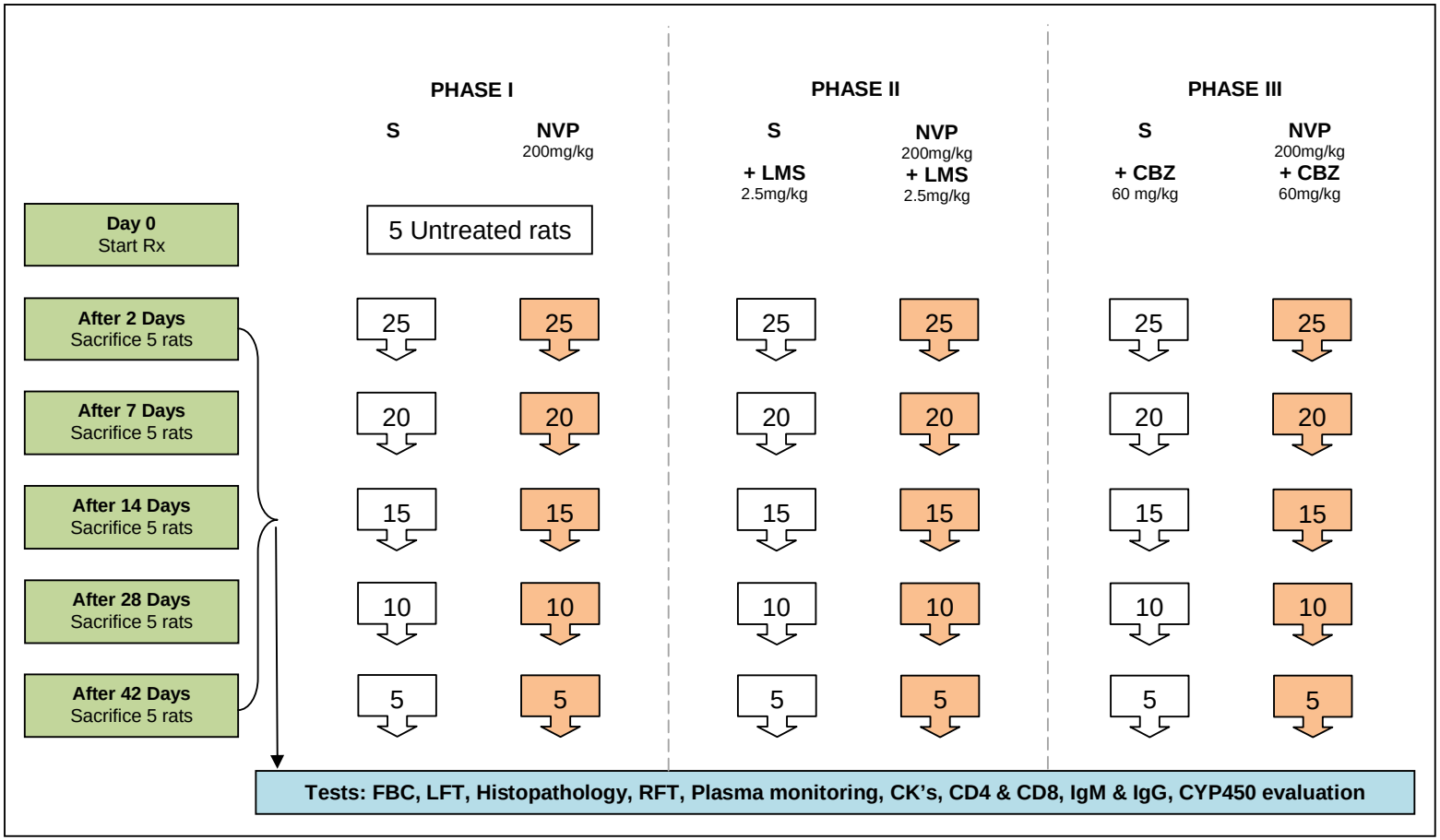

Figure 7.1: A schematic illustration of the experimental design of Phase I, II and III of prolonged nevirapine administration

7.2.6 Animal care

Ethical approval and animal care are the same is discussed in Chapter 6, Section 6.2.6.

7.2.7 Animal weighing, blood collection and liver removal

The process of animal weighing, blood collection and liver removal, is the same as discussed in Chapter 6, Section 6.2.7.

7.2.8 Analysis of function tests

The analysis of full blood count, CD4 and CD8 counts, liver and renal function tests, and histopathology of the rat livers, are as described in Chapter 6, Section 6.2.8.

7.2.9 Analysis of cytokines by enzyme-linked immunosorbent assay

The analysis of IL-2 and IL-10 by ELISA, is as described in Chapter 6, Section 6.2.9.

7.2.10 Analysis of immunoglobulins by enzyme-linked immunosorbent assay

The analysis of IgG and IgM by ELISA, is as discussed in Chapter 6, Section 6.2.10.

7.2.11 Analysis of nevirapine concentrations in rat plasma

Plasma concentrations of nevirapine in rats were monitored using the HPLC assay as developed and described in Chapter 5. For nevirapine, a standard curve was generated from five known calibration standards, from which nevirapine concentrations in rat plasma were derived.

7.2.12 Preparation of rat liver microsomes

Microsomes were prepared as discussed in Chapter 6, Section 6.2.12.

7.2.13 Protein assay

The protein assay was conducted as described in Chapter 6, Section 6.2.13.

7.2.14 Determination of rat CYP1A2, CYP2E1 and CYP3A2 activity *in vivo*

The assays which were used to determine rat CYP1A2, CYP2E1 and CYP3A2 activity *in vivo*, are the same as described in Chapter 6, Section 6.2.14.

7.2.15 Statistical analysis of results

Data were analysed by non-parametric methods using the GraphPad Instat statistical program. Accordingly, parameters were reported as mean and standard deviation (SD), and the Mann-Whitney Test was used for data comparison with the level of significance set at $p < 0.05$.

7.3 RESULTS

The results are divided into three phases: Phase I – treatment with nevirapine alone, Phase II – co-treatment with an immune stimulant, and Phase III – co-treatment with a CYP450 inducer, and all parameters are reported as such.

A. Phase I: Treatment with nevirapine alone

7.3.1 Physiological observations (function tests)

7.3.1.1 Full blood count

Table 7.1 shows results of the full blood count of the S and NVP groups, while Figures 7.1 a – e are graphical illustrations of red blood count parameters. By day 42 in the NVP group red cell count ($p = 0.0500$), haemoglobin ($p = 0.0620$) and MCHC ($p = 0.0500$) increased, while MCV and MCH values decreased ($p = 0.0500$). Although a similar pattern of change was observed for the same parameters in the S group, these changes were not statistically significant. The elevated red cell count and haemoglobin signify higher oxygen requirements, while ageing of red blood cells was associated with a small MCV, and subsequent increased MCHC. Furthermore, the white cell count, lymphocytes, neutrophils and monocytes were slightly elevated ($p = 0.0500$), and higher than in the S group ($p = 0.0500$).

Table 7.1: Average (mean ± SD) full blood count and platelets results of the S and NVP groups

Group (n = 3)	RCC (x10^{12}/l)	Hb (g/dl)	Hct (l/l)	MCV (fl)	MCH (pg)	MCHC (g/dl)	Plt (x10^{9}/l)	WCC (x10^{9}/l)	Neu (x10^{9}/l)	Ly (x10^{9}/l)	Mo (x10^{9}/l)	Eos (x10^{9}/l)	Bas (x10^{9}/l)
Untreated													
0 Days	6.28±0.2	12.9±0.3	0.398±0.01	63.5±2.5	20.5±0.4	32.3±0.8	860±221.1	6.95±2.7	0.77±0.2	4.67±1.8	0.19±0.1	0.02±0.0	0.00±0.0
S													
2 Days	6.67±0.2	13.7±0.1	0.422±0.01	63.3±2.3	20.6±0.6	32.4±0.6	849±81.6	6.50±0.9	0.60±0.2	5.18±0.7	0.21±0.0	0.50±0.2	0.01±0.0
7 Days	7.53±0.9	15.3±1.7	0.451±0.04	60.1±2.5	20.3±0.2	33.9±1.2	1033±79.8	5.44±2.4	1.03±0.8	4.07±2.0	0.30±0.3	0.04±0.0	0.01±0.0
14 Days	6.77±0.6	13.9±1.1	0.417±0.03	61.8±2.8	20.5±0.6	33.2±0.6	721±196.4	5.22±1.2	0.63±0.5	4.21±0.7	0.18±0.1	0.18±0.1	0.05±0.0
28 Days	7.07±0.7	13.9±1.3	0.390±0.04	55.1±1.0	19.7±0.1	35.8±0.6	961±172.5	7.38±1.0	0.91±0.2	6.15±0.8	0.24±0.1	0.07±0.0	0.01±0.0
42 Days	6.93±0.8	13.4±1.8	0.374±0.05	53.9±1.0	19.3±0.4	35.8±0.2	839±166.0	3.93±0.3	0.54±0.1	3.23±0.3	0.11±0.0	0.04±0.0	0.01±0.0
NVP													
2 Days	6.51±0.2	13.6±0.4	0.408±0.01	62.7±0.8	20.9±0.1	33.2±0.3	854±209.3	3.48±0.8	1.02±0.1	1.88±0.3	0.10±0.0	0.48±0.4	0.00±0.0
7 Days	7.14±0.3	14.2±0.5	0.424±0.02	59.5±1.8	19.9±0.6	33.5±0.5	779±141.3	5.54±1.3	0.76±0.3	3.81±0.9	0.49±0.2	0.48±0.2	0.01±0.0
14 Days	6.97±0.3	13.9±0.4	0.415±0.01	59.5±2.2	19.9±0.6	33.5±0.3	1013±73.0	4.86±0.7	1.08±0.2	3.38±0.4	0.37±0.1	0.02±0.0	0.01±0.0
28 Days	7.36±0.6	14.2±1.1	0.404±0.03	55.0±0.7	19.3±0.3	35.0±0.6	876±17.2	6.07±0.6	1.09±0.2	4.62±0.7	0.28±0.2	0.08±0.1	0.01±0.0
42 Days	7.73±0.3	14.5±0.3	0.405±0.00	52.5±2.3	18.8±0.5	35.8±0.8	1052±115.6	5.42±0.1	1.52±0.6	3 .52±0.6	0.33±0.0	0.04±0.0	0.01±0.0

RCC = red cell count; Hb = haemoglobin; Hct = haematocrit; MCV = mean corpuscular volume; MCH = mean corpuscular haemoglobin; MCHC = mean corpuscular haemoglobin concentration; Plt = platelets; WCC = white cell count; Neu = neutrophils; Ly = lymphocytes; Mo = monocytes; Eos = eosinophils; Bas = basophils; S = saline; NVP = nevirapine

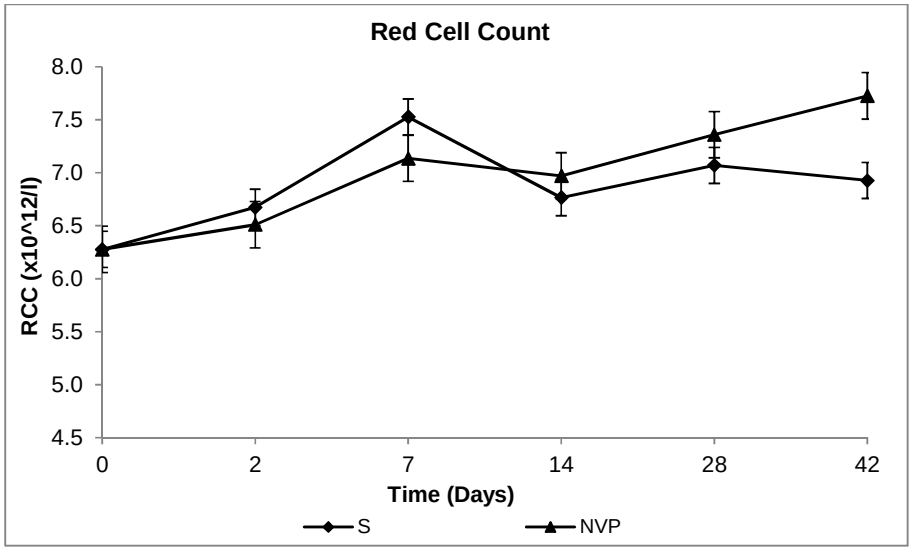

Figure 7.2 a): Red cell counts of the S and NVP groups over 42 days

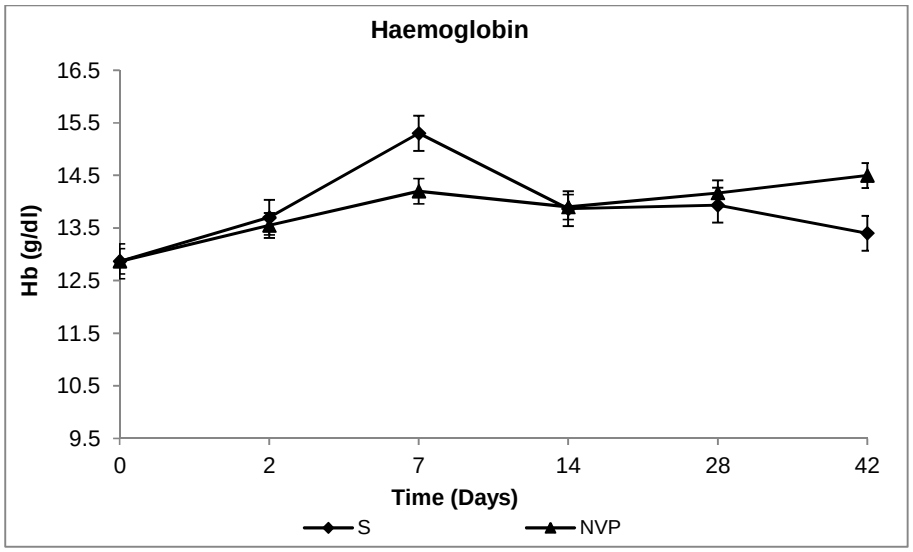

Figure 7.2 b): Haemoglobin concentrations of the S and NVP groups over 42 days

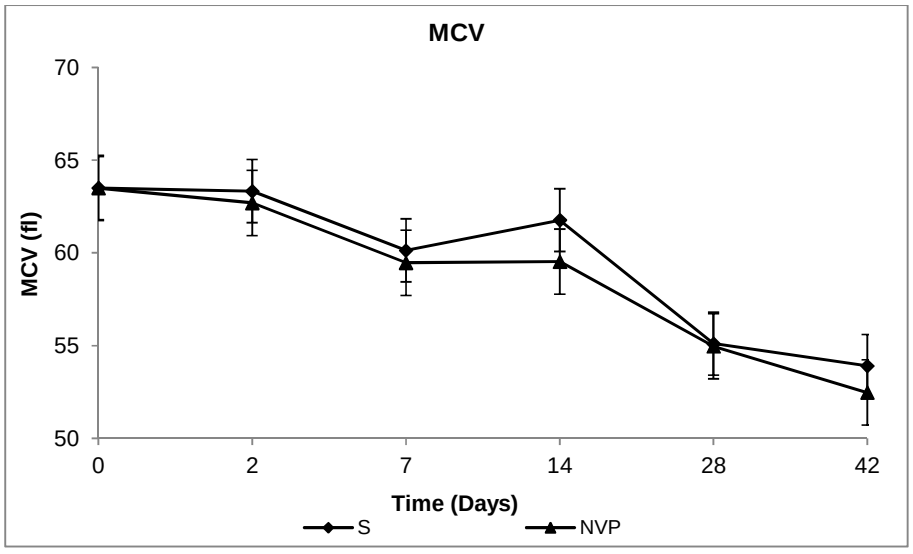

Figure 7.2 c): MCV of the S and NVP groups over 42 days

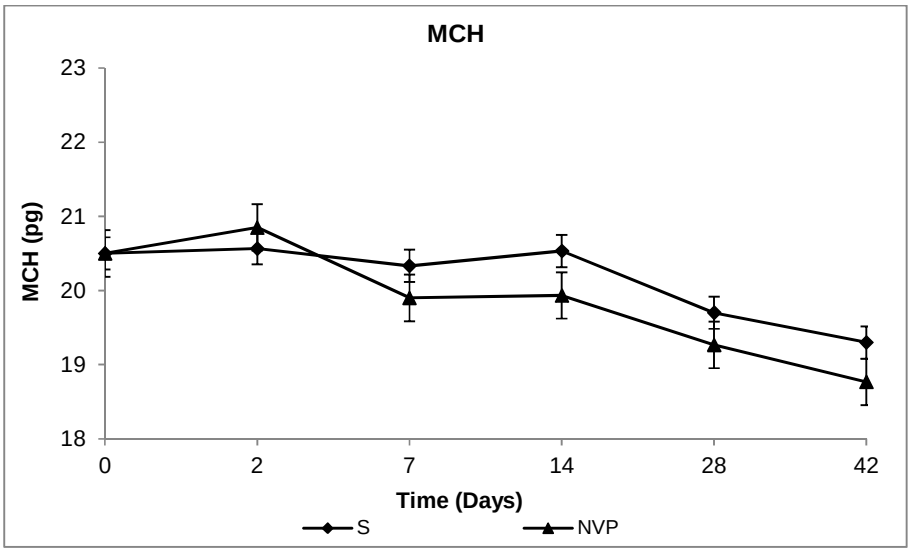

Figure 7.2 d): MCH of the S and NVP groups over 42 days

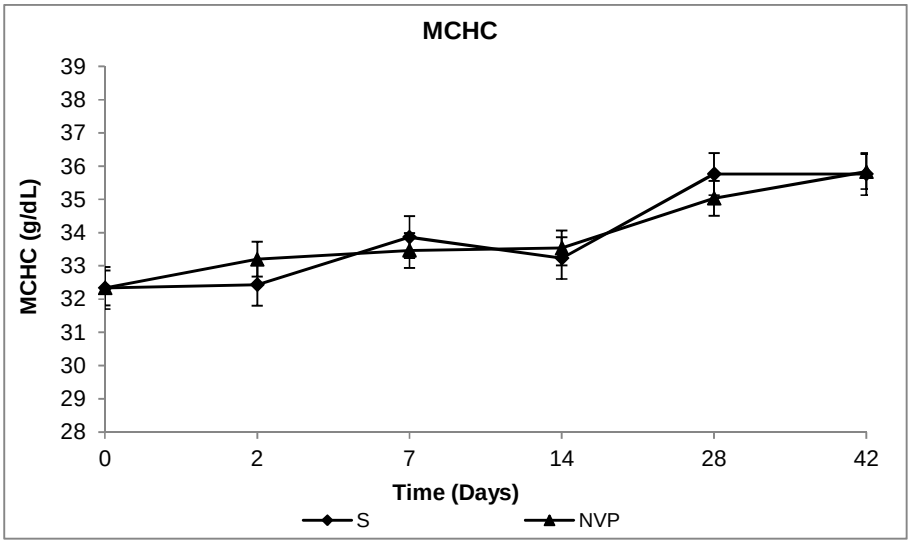

Figure 7.2 e): MCHC of the S and NVP groups over 42 days

7.3.1.2 Renal function tests

Table 7.2 shows the changes of BUN and Cr of the S and NVP groups. In both groups BUN and Cr levels were normal, in spite of the spike on day 28 ($p = 0.0500$), as this was still within the normal range.

Table 7.2: Average (mean ± SD) renal function test results of the S and NVP groups

Group	**RFT**	
	BUN	**Cr**
(n = 3)	**(mmol/l)**	**(µmol/l)**
Untreated		
0 Days	7.2 ± 1	37 ± 8
S		
2 Days	7.3 ± 1	39 ± 2
7 Days	8.1 ± 0	46 ± 7
14 Days	7.5 ± 1	39 ± 3
28 Days	10.6 ± 2	73 ± 17
42 Days	5.8 ± 1	38 ± 9
NVP		
2 Days	7.9 ± 1	36 ± 1
7 Days	8.7 ± 0	46 ± 2
14 Days	9.1 ± 1	41 ± 9
28 Days	8.5 ± 1	63 ± 7
42 Days	7.0 ± 0	27 ± 3

RFT = renal function test; BUN = blood urea nitrogen; Cr = creatinine; S = saline; NVP = nevirapine

7.3.1.3 Liver function tests

Table 7.3 shows the changes of ALT, AST and ALP of the S and NVP groups. Liver function was normal as there were no differences in ALT, AST and ALP between the two groups.

Table 7.3: Mean ± SD values of liver function test results of the S and NVP groups

Group	LFT		
	ALT	AST	ALP
(n = 3)	(U/l)	(U/l)	(U/l)
Untreated			
0 Days	50 ± 5	88 ± 14	352 ± 76
S			
2 Days	46 ± 2	90 ± 7	400 ± 7
7 Days	49 ± 10	103 ± 25	304 ± 13
14 Days	58 ± 4	127 ± 37	508 ± 37
28 Days	47 ± 2	115 ± 44	216 ± 19
42 Days	46 ± 6	76 ± 28	109 ± 76
NVP			
2 Days	63 ± 7	107 ± 10	359 ± 43
7 Days	87 ± 36	169 ± 115	447 ± 78
14 Days	72 ± 3	109 ± 33	443 ± 43
28 Days	53 ± 4	128 ± 44	166 ± 37
42 Days	54 ± 2	70 ± 4	14 ± 9

LFT = liver function test; ALT = alanine aminotransferase; AST = aspartate aminotransferase; ALP = alkaline phosphatase; S = saline; NVP = nevirapine

7.3.1.4 Liver histopathology

(a) Liver histopathology reports

Liver sections for histopathology (Figures 7.3 a – k) were randomly selected, and the main histopathology lesions are summarised in the tally table (Table 7.4). The following report is a summary of the features of the lesion:

i. Figure 7.3 a: Liver section from an untreated rat at day 0

A representative photograph of a rat liver from an untreated rat. The report: “No pathology appears to be present in the two control (untreated) animals.”

ii. Figures 7.3 b and c: Liver sections A and B from the NVP group after 2 days of nevirapine alone treatment

Representative photographs of rat livers, after 2 days of daily nevirapine alone treatment. The report: “Mild granular vacuolar degeneration and cell swelling were the earlier cell changes observed in both liver sections A and B. Scattered cytonecrosis was mild in liver section B, as presented by dark eosinophilic staining and nuclear pyknosis. Zonal necrosis was only minimally present in both liver sections. Mitotic figures suggestive of cell regeneration which usually follows on the

cytonecrosis could be confirmed in both of the liver sections, and was very prominent in liver section A."

iii. Figures 7.3 d and e: Liver sections A and B from the NVP group after 7 days of nevirapine alone treatment

Representative photographs of rat livers, after 7 days of daily nevirapine alone treatment. The report: "Granular vacuolar degeneration and cell swelling were moderate in liver sections A and B. Single cell necrosis (cytonecrosis) appeared to be mild in both sections. Only minimal centrilobular necrosis was present in section A, while one mitotic figure could be observed in section B."

iv. Figures 7.3 f and g): Liver sections A and B from the NVP group after 14 days of nevirapine alone treatment

Representative photographs of rat livers, after 14 days of daily nevirapine alone treatment. The report: "Mild to moderate cellular swelling, vacuolar hepatopathy (degeneration) and granular cytoplasm were present in both liver sections A and B. Hepatic parenchymal cell necrosis (cytonecrosis), and centrilobular zonal necrosis were graded moderate to severe in liver section A as represented by loss of nuclei, disarrangement of the hepatocytic cords and sinusoidal dilatation."

v. Figures 7.3 h and i: Liver sections A and B from the NVP group after 28 days of nevirapine alone treatment

Representative photographs of rat livers, after 28 days of daily nevirapine alone treatment. The report: "Granular vacuolar degeneration and cell swelling were minimal to mild in both the liver sections A and B, as was cytonecrosis. No centrilobular zonal necrosis was observed, while hepatocyte mitosis was only minimally present in section A."

vi. Figures 7.3 j and k: Liver sections A and B from the NVP group after 42 days of nevirapine alone treatment

Representative photographs of rat livers, after 42 days of daily nevirapine alone treatment. The report: "Minimal to mild granular vacuolar degeneration, cell swelling and cytonecrosis were present in both the liver sections A and B. Centrilobular zonal necrosis was minimal in section A, while no hepatocyte mitosis was recorded."

In view of the histopathology photographs (Figures 7.3 a – k), reports and tally table (Table 7.4), it was concluded that treatment with nevirapine alone caused liver injury up to 14 days, and by day 28 this had improved.

(b) Liver histopathology photographs

Figures 7.3 a – k are representative of randomly selected liver sections of untreated rats, as well as the NVP group after nevirapine alone treatment.

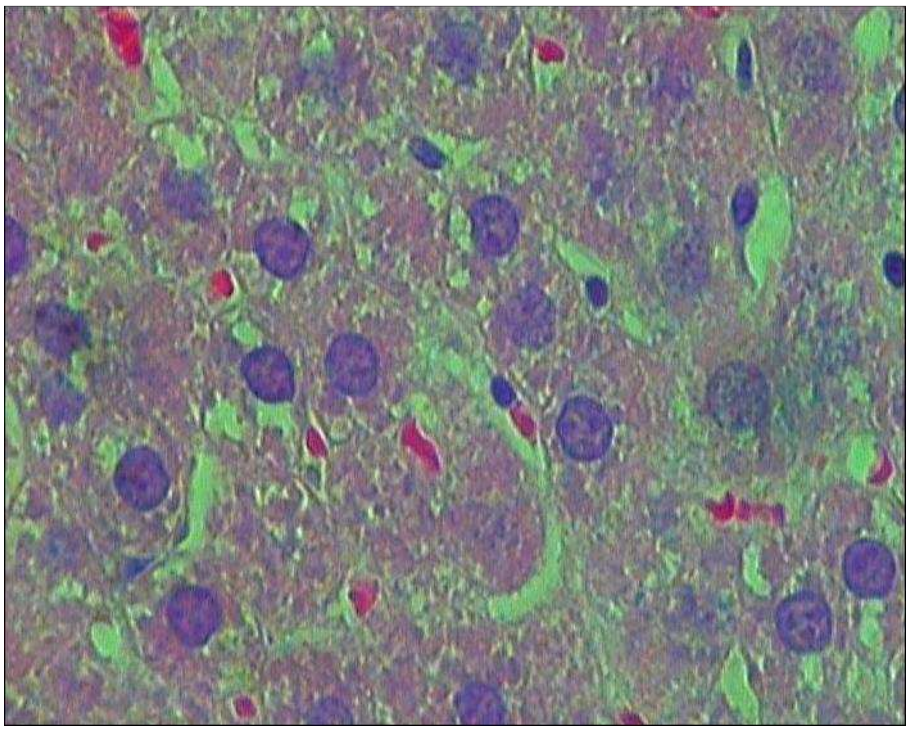

Figure 7.3 a): Liver section from an untreated rat at day 0, showing a normal liver with no inflammation

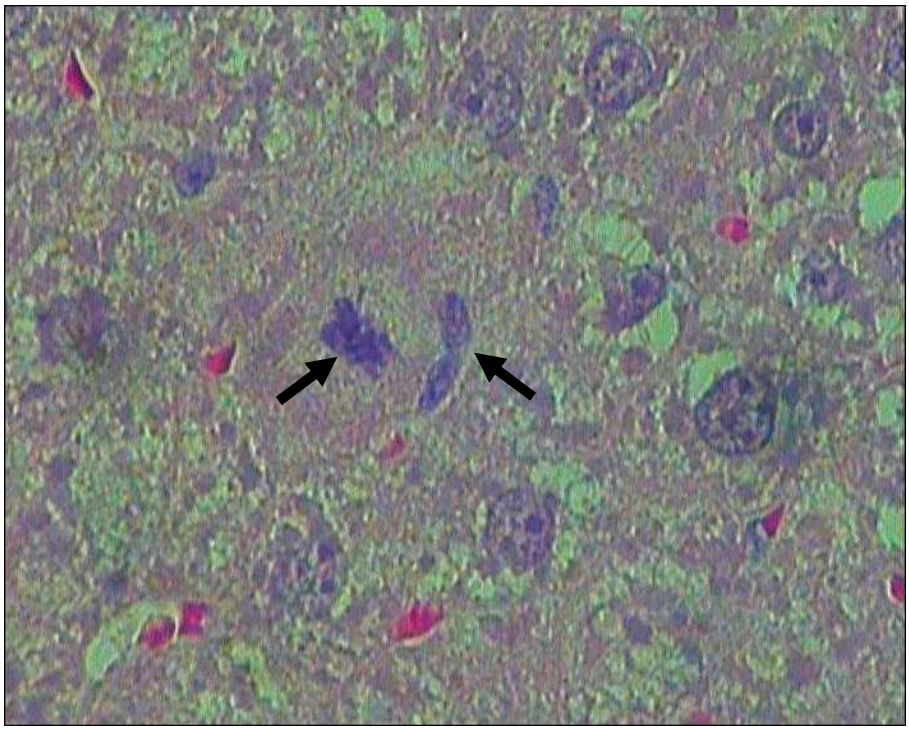

Figure 7.3 b): Liver section A from the NVP group after 2 days of treatment, showing scattered cytonecrosis and prominent mitotic figures

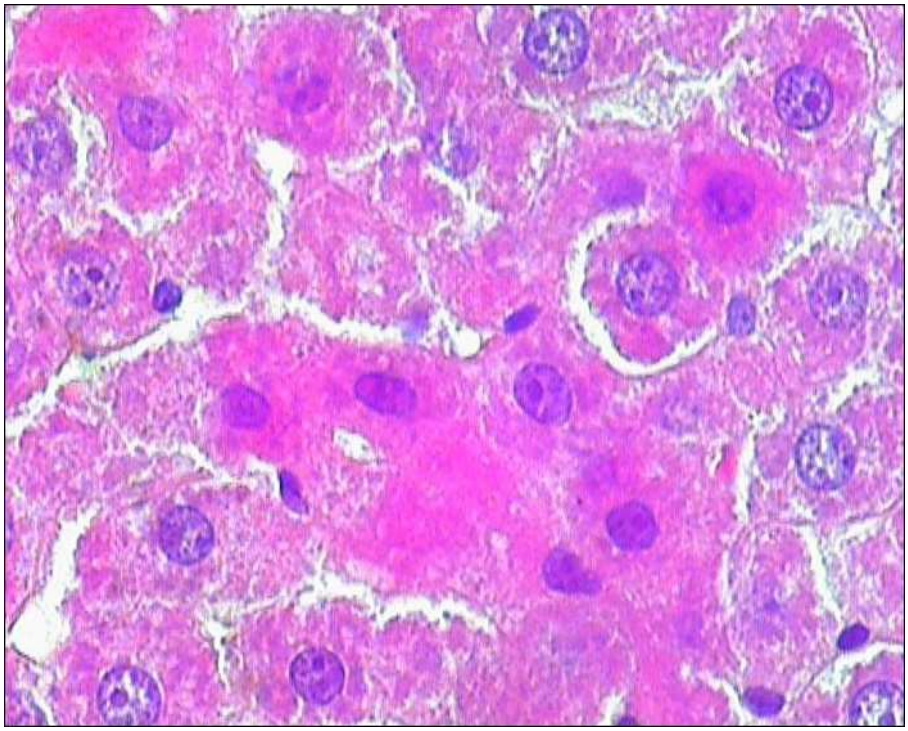

Figure 7.3 c): Liver section B from the NVP group after 2 days of treatment, showing mild cytonecrosis with dark eosinophilic staining and nuclear pyknosis

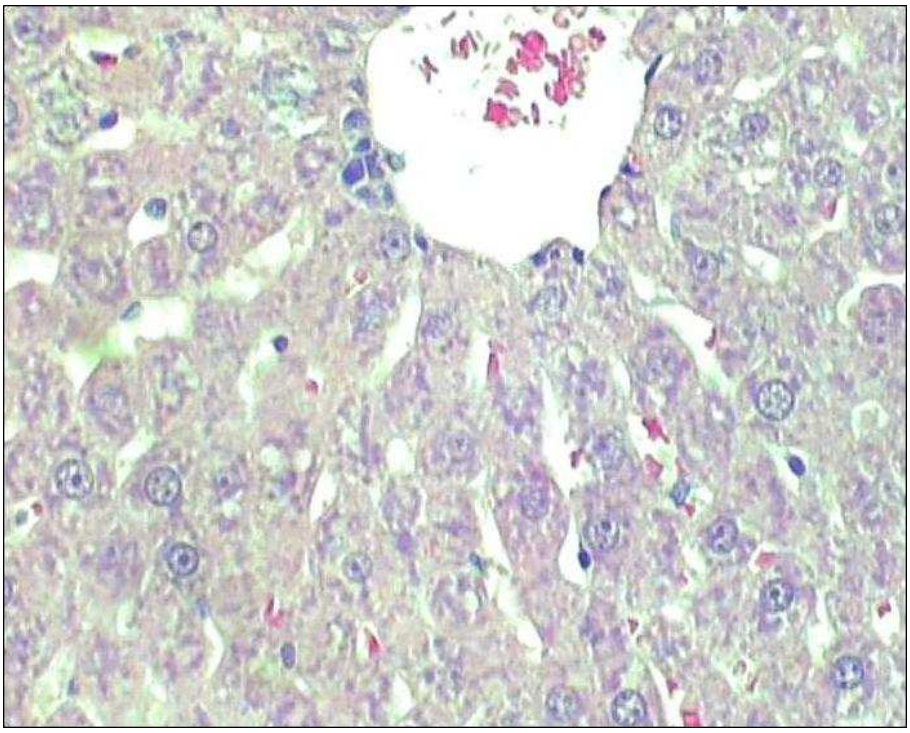

Figure 7.3 d): Liver section A from the NVP group after 7 days of treatment, showing minimal centrilobular necrosis in the liver parenchyma

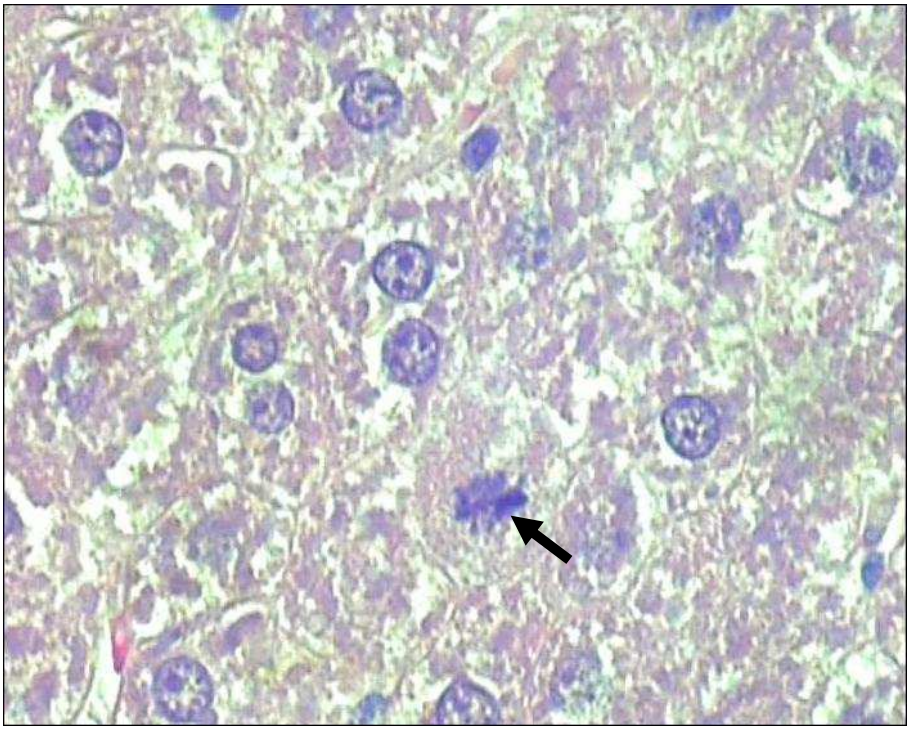

Figure 7.3 e): Liver section B from the NVP group after 7 days of treatment, showing a mitotic figure in the area of necrosis

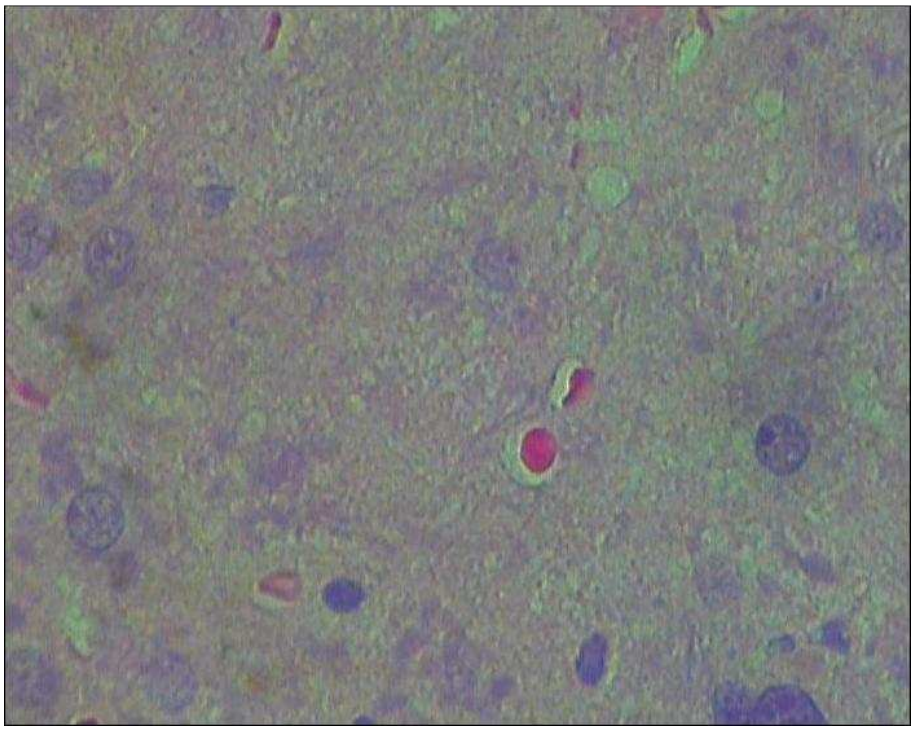

Figure 7.3 f): Liver section A from the NVP group after 14 days of treatment, showing cytonecrosis, and centrilobular zonal necrosis with loss of nuclei and disarrangement of the hepatocytic cords

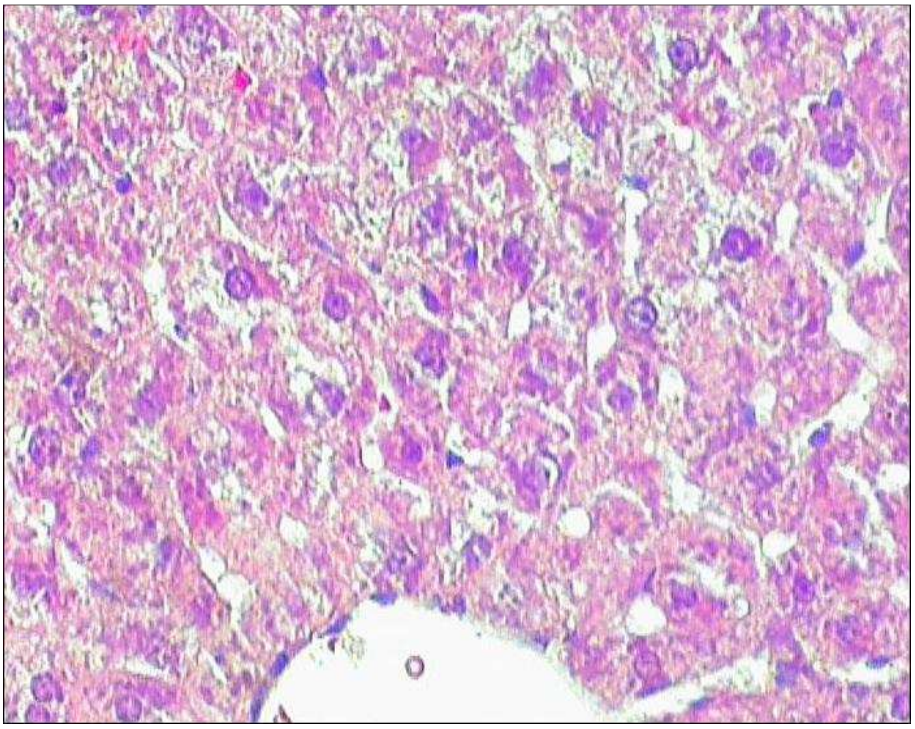

Figure 7.3 g): Liver section B from the NVP group after 14 days of treatment, showing moderate cellular swelling, vacuolar hepatopathy and granular cytoplasm

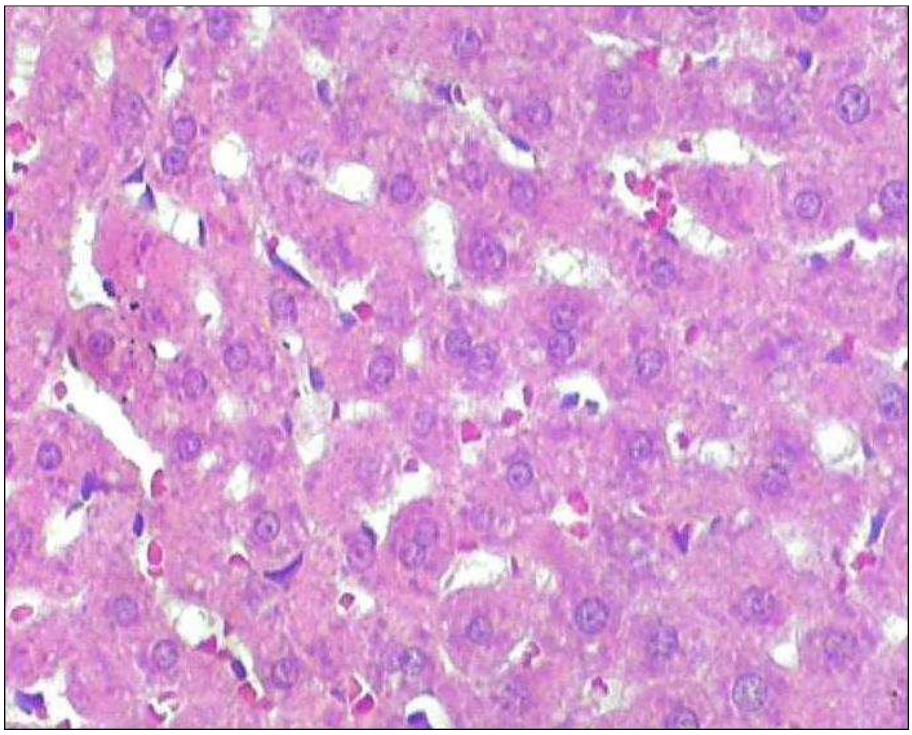

Figure 7.3 h): Liver section A from the NVP group after 28 days of treatment, showing hepatocytic cords and mild degenerative changes

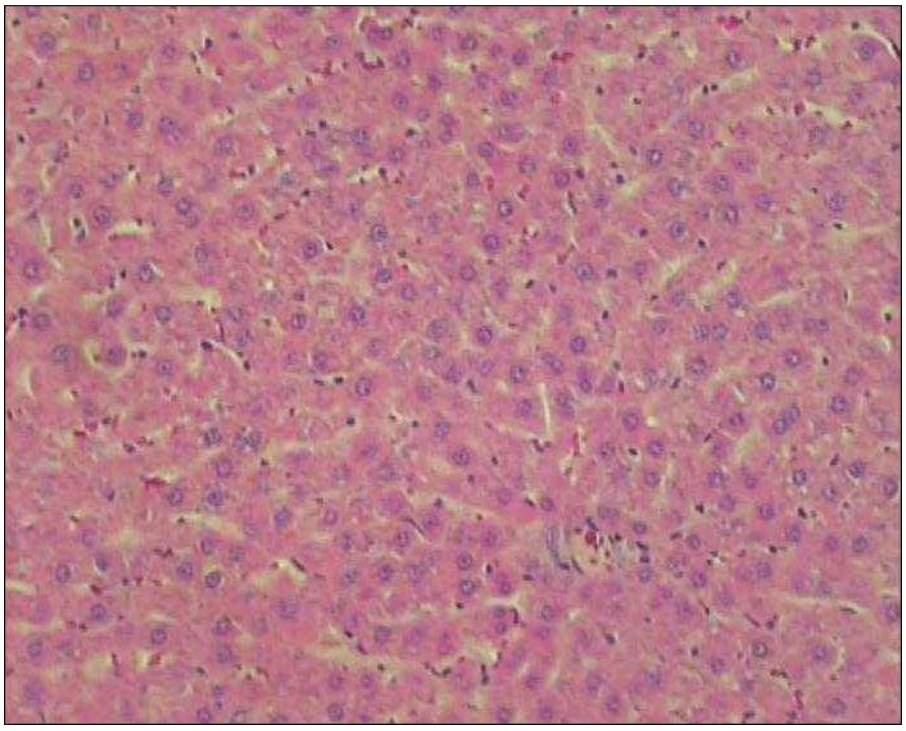

Figure 7.3 i): Liver section B from the NVP group after 28 days of treatment, showing minimal granular vacuolar degeneration and cellular swelling, as well as cytonecrosis

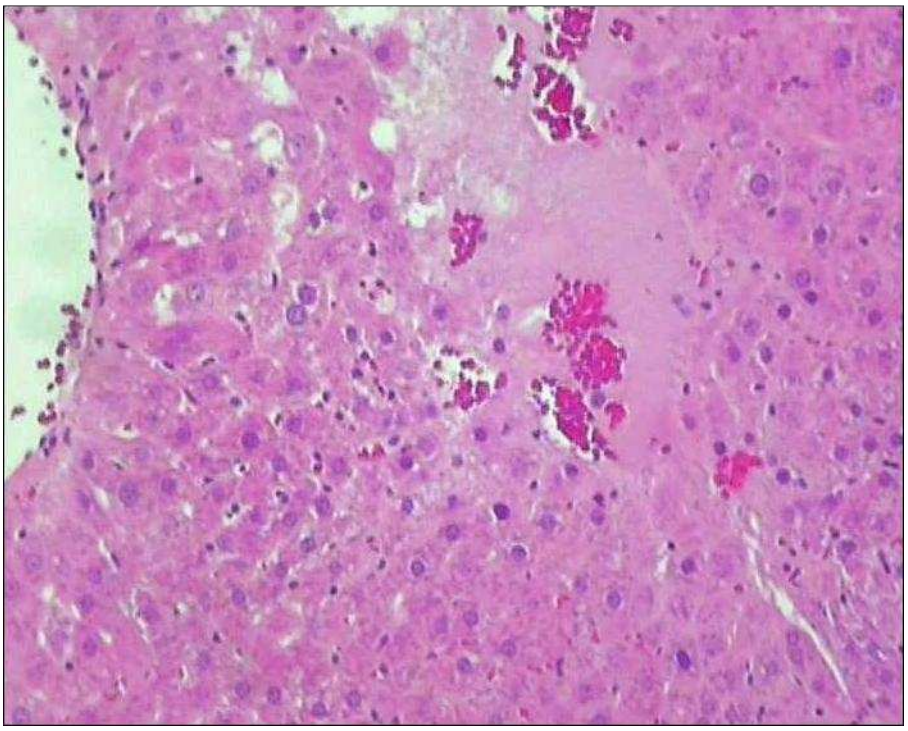

Figure 7.3 j): Liver section A from the NVP group after 42 days of treatment, showing cytonecrosis and minimal centrilobular necrosis

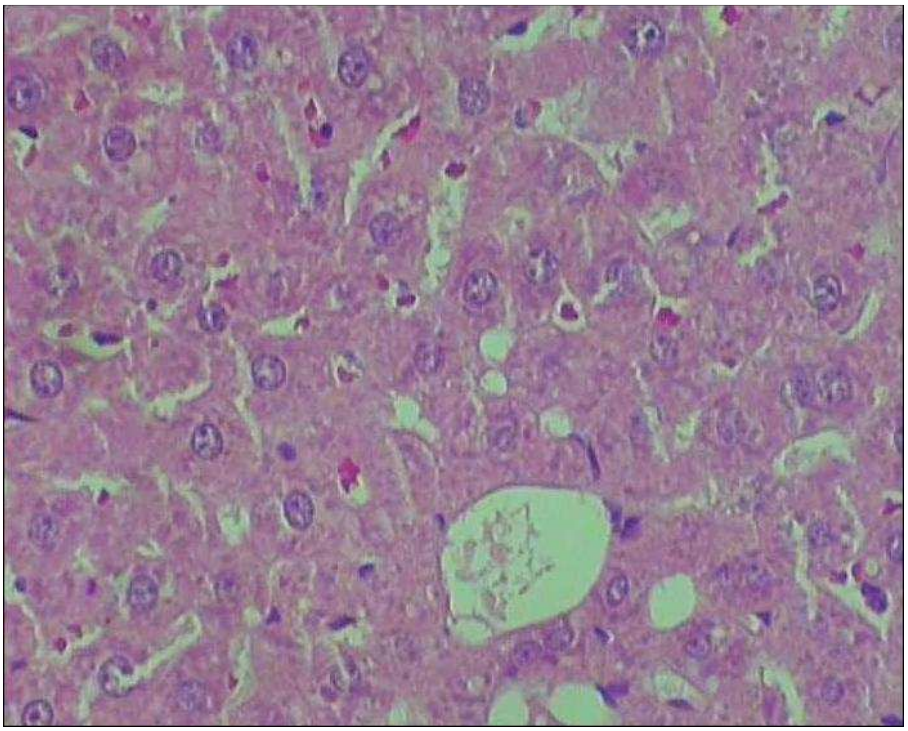

Figure 7.3 k): Liver section B from the NVP group after 42 days of treatment, showing centrilobular hepatocytes with minimal degeneration

Table 7.4: Tally of main pathology lesions (lesions score) in livers of untreated rats and the NVP group

Group	UnRx	NVP									
		2 Days		7 Days		14 Days		28 Days		42 Days	
(n = 2)	Fig.7.3a	Fig.7.3b	Fig.7.3c	Fig.7.3d	Fig.7.3e	Fig.7.3f	Fig.7.3g	Fig.7.3h	Fig.7.3i	Fig.7.3j	Fig.7.3k
Histopathological lesions											
Vacuolar degeneration	0	2+	2+	3+	3+	2+	3+	2+	1+	2+	1+
Cell swelling	0	2+	2+	3+	3+	2+	3+	2+	1+	2+	1+
Cytonecrosis	0	2+	1+	2+	2+	3+	1+	2+	1+	2+	1+
Centrilobular necrosis	0	1+	1+	1+	0	3+	1+	0	0	1+	0
Hepatocyte mitosis	0	3+	1+	0	1+	0	0	1+	0	0	0
Average lesions score											
Vacuolar degeneration	0	2+		3+		2.5+		1.5+		1.5+	
Cell swelling	0	2+		3+		2.5+		1.5+		1.5+	
Cytonecrosis	0	1.5+		2+		2+		1.5+		1.5+	
Centrilobular necrosis	0	1+		0.5+		2+		0		0.5+	
Hepatocyte mitosis	0	2+		0.5+		0		0.5+		0	
Total lesion score	**0**	**8.5+**		**9+**		**9+**		**5+**		**5+**	

UnRx = untreated; NVP = nevirapine

7.3.2 Nevirapine concentrations

Table 7.5 shows nevirapine concentrations of the NVP group, while Figure 7.4 is a graphical illustration of the same. By day 42, nevirapine plasma concentrations in the NVP group had declined (p = 0.0159), and this shows that nevirapine was extensively metabolised due to enzyme induction (Section 7.3.4.2).

Table 7.5: Average (mean ± SD) nevirapine concentrations of the NVP group

Group	**NVP**
	NVP concentration
(n = 5)	**(µg/ml)**
2 Days	4.292 ± 2.69
7 Days	2.008 ± 0.67
14 Days	1.983 ± 0.58
28 Days	1.721 ± 3.04
42 Days	0.497 ± 1.11

NVP = nevirapine

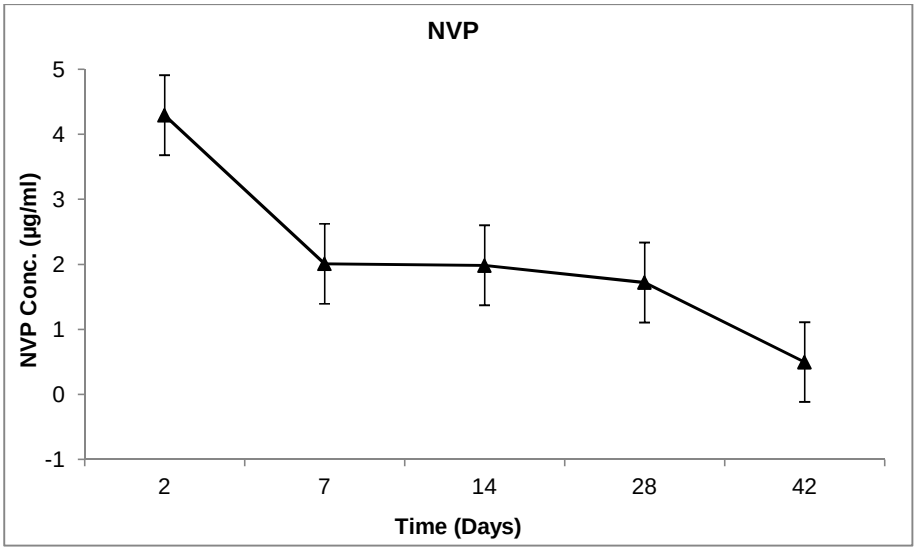

Figure 7.4: Nevirapine concentrations of the NVP group over 42 days

7.3.3 Specific immunology tests

7.3.3.1 Direct observations

Table 7.6 shows changes in body weight of the S and NVP groups over the treatment period. Both groups showed weight gain at all times, but the weight gain was greater in the S group from days 14 to 42 (Refer to Appendix H-1 and H-3 for baseline weights).

Table 7.6: Average (mean ± SD) change in rat weights of the S and NVP groups

Group (n = 5)	S change in weight (g)	NVP change in weight (g)
2 Days	9.2±4	10.6±8
7 Days	35.7±8	28.5±1
14 Days	84.6±5	82.8±2
28 Days	107.8±10	99.7±16
42 Days	171.4±27	105.6±16

S = saline; NVP = nevirapine

7.3.3.2 Cytokines

Table 7.7 shows IL-2 and IL-10 concentrations of the S and NVP groups, while Figures 7.5 a – b are graphical illustrations of the same. There was an increase in IL-2 up to day 7 ($p = 0.0500$). Thereafter, it gradually declined until day 42 ($p = 0.0500$), and was lower than in the S group on the same day ($p = 0.0500$). By day 42 IL-10 concentrations in the NVP group were elevated, but this was not statistically significant. However, it was higher than in the S group ($p = 0.0500$).

Table 7.7: Average (mean ± SD) cytokine concentrations of the S and NVP groups

Group	Cytokine	
(n = 3)	IL-2 (pg/ml)	IL-10 (pg/ml)
Untreated		
0 Days	65.46 ± 2.0	31.08 ± 1.2
S		
2 Days	74.87 ± 6.5	29.96 ± 2.8
7 Days	77.26 ± 5.8	34.57 ± 0.7
14 Days	77.58 ± 6.6	35.69 ± 5.4
28 Days	78.81 ± 4.6	32.46 ± 4.2
42 Days	74.39 ± 5.7	32.03 ± 2.5
NVP		
2 Days	76.55 ± 1.2	34.77 ± 2.5
7 Days	82.21 ± 4.6	36.63 ± 3.6
14 Days	73.07 ± 7.9	34.87 ± 6.8
28 Days	71.82 ± 10.6	39.64 ± 3.5
42 Days	61.92 ± 4.5	41.89 ± 6.5

IL = interleukin; S = saline; NVP = nevirapine

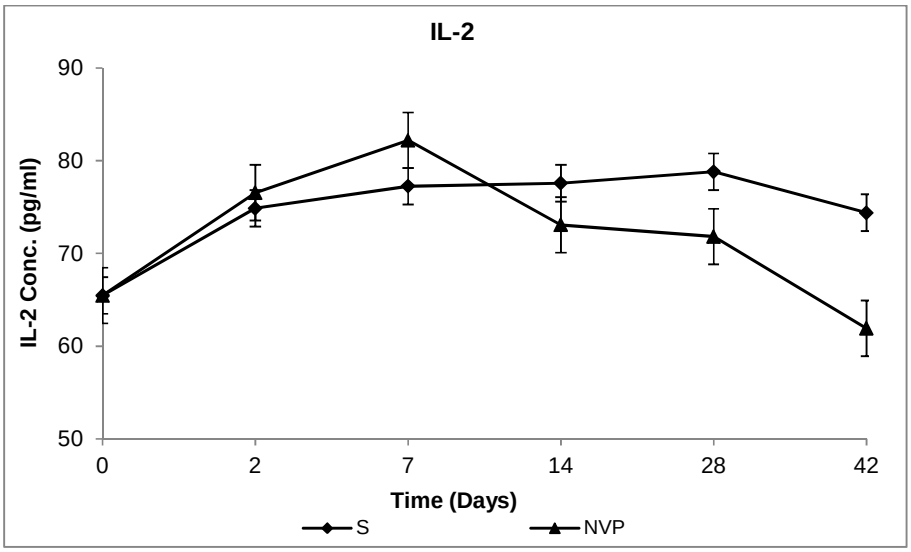

Figure 7.5 a): IL-2 concentrations of the S and NVP groups over 42 days

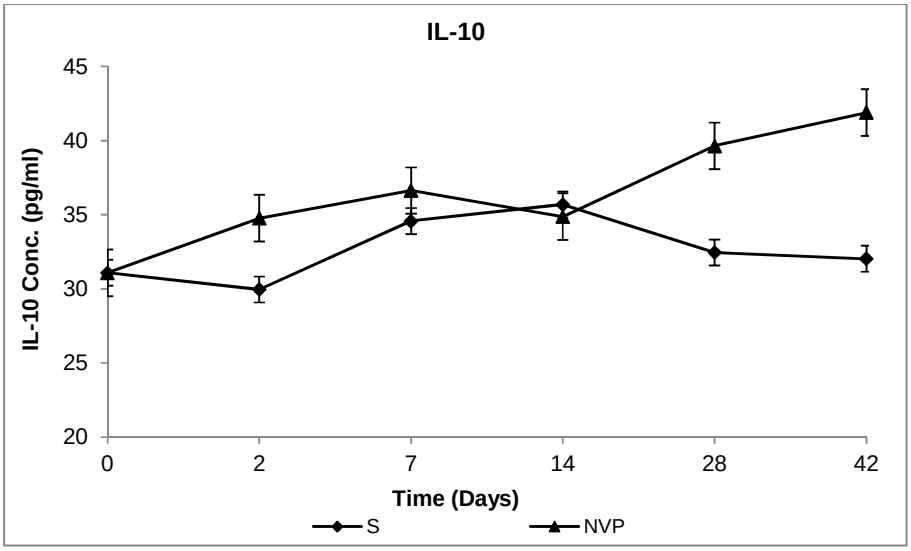

Figure 7.5 b): IL-10 concentrations of the S and NVP groups over 42 days

7.3.3.3 CD4 and CD8 counts

Table 7.8 shows CD4 and CD8 counts of the S and NVP groups, while Figures 7.6 a – b are graphical illustrations of the same. Lymphocyte, CD4 and CD8 counts of the NVP group increased up to day 28 ($p = 0.0500$), and then declined ($p = 0.0500$). Although the changes of lymphocyte, CD4 and CD8 counts of the S group carried no statistical significance, the lymphocyte and CD4 count was higher than in the NVP group ($p = 0.0500$).

Table 7.8: Average (mean ± SD) CD4 and CD8 counts of the S and NVP groups

Group	**Ly**	**T-Ly**	
		CD4	**CD8**
(n = 3)	**($x10^9$/l)**	**($x10^9$/l)**	**($x10^9$/l)**
Untreated			
0 Days	4.67±1.8	2.23±1.3	1.42±0.7
S			
2 Days	5.18±0.7	2.27±0.3	1.35±0.2
7 Days	4.07±2.0	1.72±0.8	1.07±0.5
14 Days	4.21±0.7	1.69±0.2	1.17±0.2
28 Days	6.15±0.8	2.45±0.2	1.58±0.3
42 Days	3.23±0.3	1.47±0.1	0.79±0.2
NVP			
2 Days	1.88±0.3	1.07±0.2	0.59±0.1
7 Days	3.81±0.9	1.58±0.3	1.07±0.3
14 Days	3.38±0.4	1.43±0.1	0.85±0.2
28 Days	4.62±0.7	1.78±0.4	1.26±0.2
42 Days	3.52±0.6	1.23±0.2	0.81±0.2

Ly = lymphocytes; CD = cluster of differentiation; S = saline; NVP = nevirapine

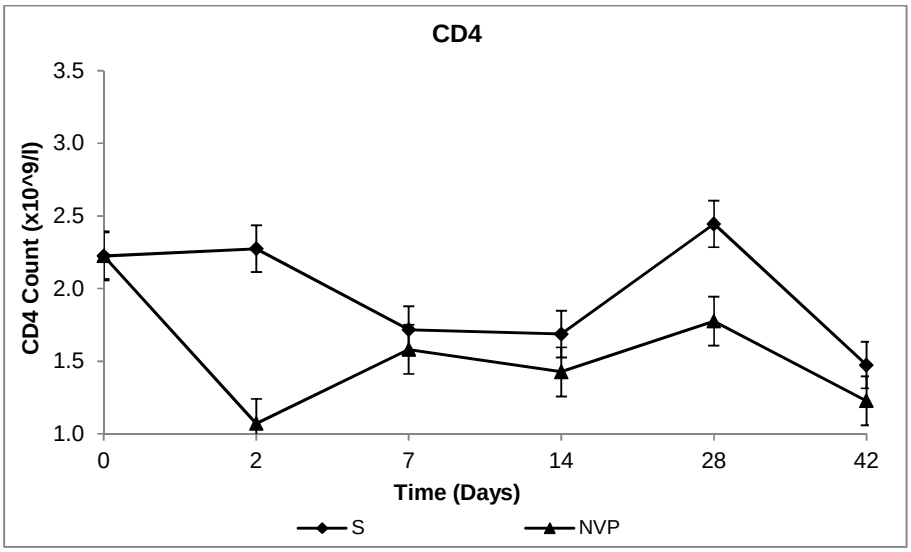

Figure 7.6 a): CD4 counts of the S and NVP groups over 42 days

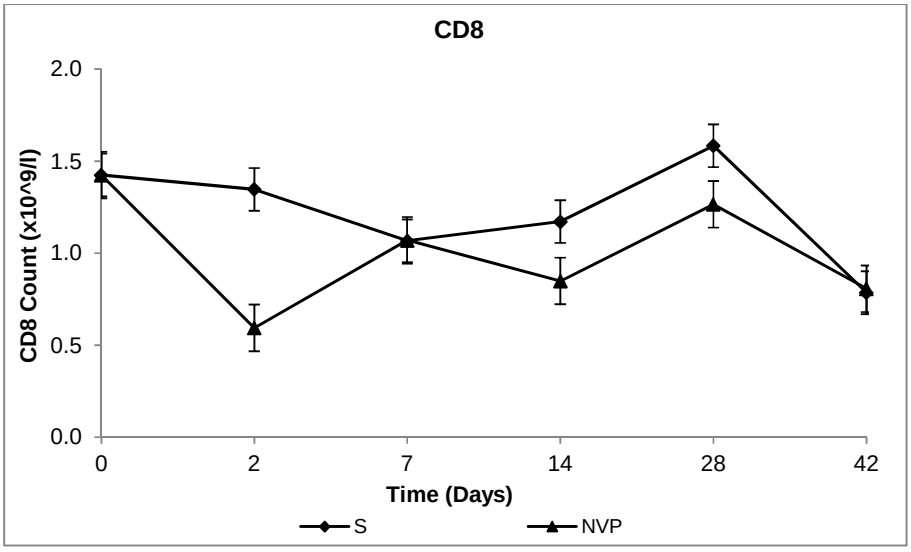

Figure 7.6 b): CD8 counts of the S and NVP groups over 42 days

7.3.3.4 Immunoglobulins

Table 7.9 shows concentrations of IgM and IgG of the S and NVP groups, while Figures 7.7 a – b are graphical illustrations of the same. By day 42 IgM concentrations in the NVP group were elevated (p = 0.0500), while in the S group they declined (p = 0.0500). For IgG, both groups showed elevated levels by day 42 (p = 0.0500).

Table 7.9: Average (mean ± SD) immunoglobulin concentrations of the S and NVP groups

Group	**Immunoglobulin**	
	IgM	**IgG**
(n = 3)	**(mg/ml)**	**(mg/ml)**
Untreated		
0 Days	0.109 ± 0.02	14.434 ± 1.10
S		
2 Days	0.104 ± 0.04	14.137 ± 0.91
7 Days	0.110 ± 0.04	14.302 ± 0.70
14 Days	0.110 ± 0.03	12.617 ± 0.29
28 Days	0.075 ± 0.03	16.350 ± 1.00
42 Days	0.046 ± 0.01	17.109 ± 0.26
NVP		
2 Days	0.011 ± 0.01	13.971 ± 1.63
7 Days	0.012 ± 0.00	13.311 ± 0.57
14 Days	0.116 ± 0.03	12.517 ± 1.05
28 Days	0.087 ± 0.02	12.022 ± 0.51
42 Days	0.089 ± 0.04	16.724 ± 2.23

IgM = immunoglobulin M; IgG = immunoglobulin G; S = saline; NVP = nevirapine

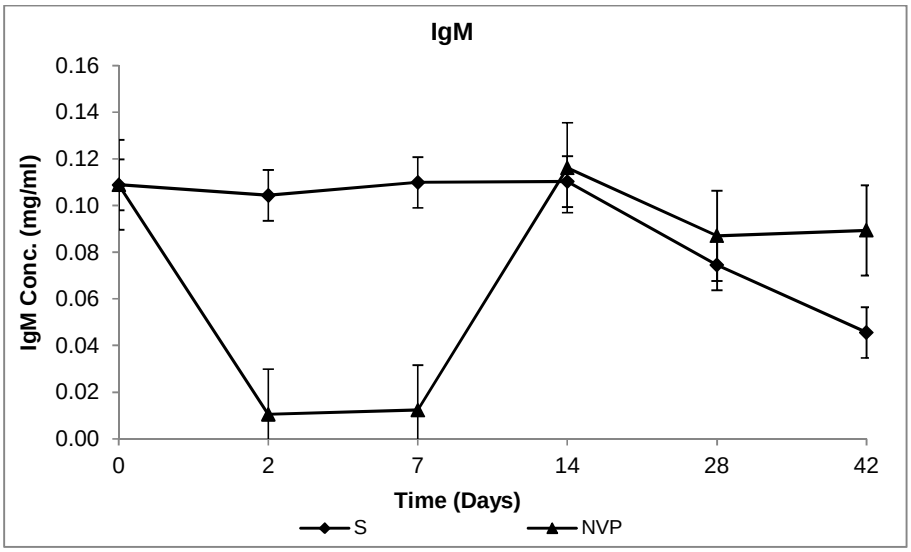

Figure 7.7 a): IgM concentrations of the S and NVP groups over 42 days

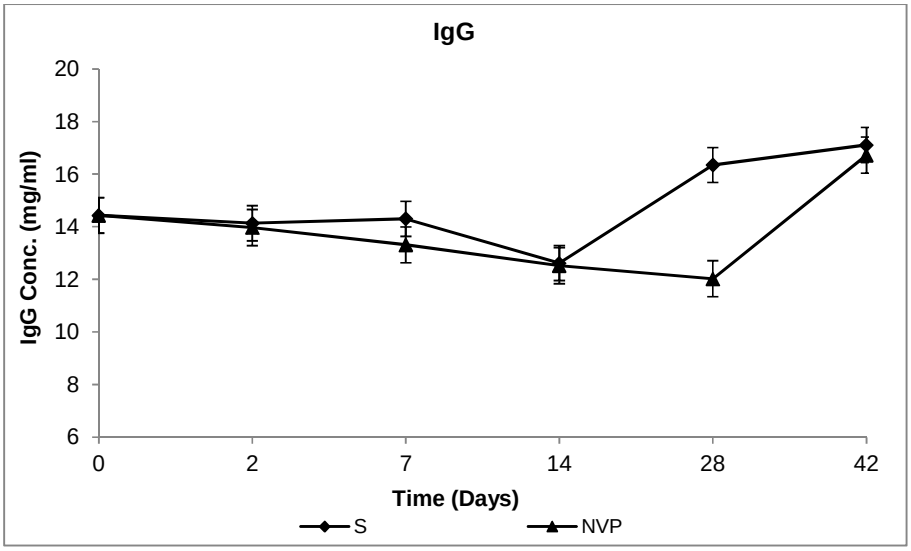

Figure 7.7 b): IgG concentrations of the S and NVP groups over 42 days

7.3.4 Activity of rat CYP1A2, CYP2E1 and CYP3A2 *in vivo*

7.3.4.1 Protein concentrations

Table 7.10 and Figure 7.8 show the results of BSA calibration samples, used for the protein assay, while Table 7.11 is that of microsomal protein concentrations of untreated rats and the NVP group. The absorption plot (Figure 7.8) is linear with a correlation coefficient (r^2) of 0.9970 and regression equation of y = 0.02x – 0.002. Final protein concentrations of microsomal liver samples from selected rats from the untreated and NVP groups were calculated as indicated in Table 7.11.

Table 7.10: Protein assay calibration data

BSA concentration (mg/ml)	Absorption (nm)
0.5	0.01
1	0.02
2.5	0.05
5	0.09
10	0.21

BSA = bovine serum albumin

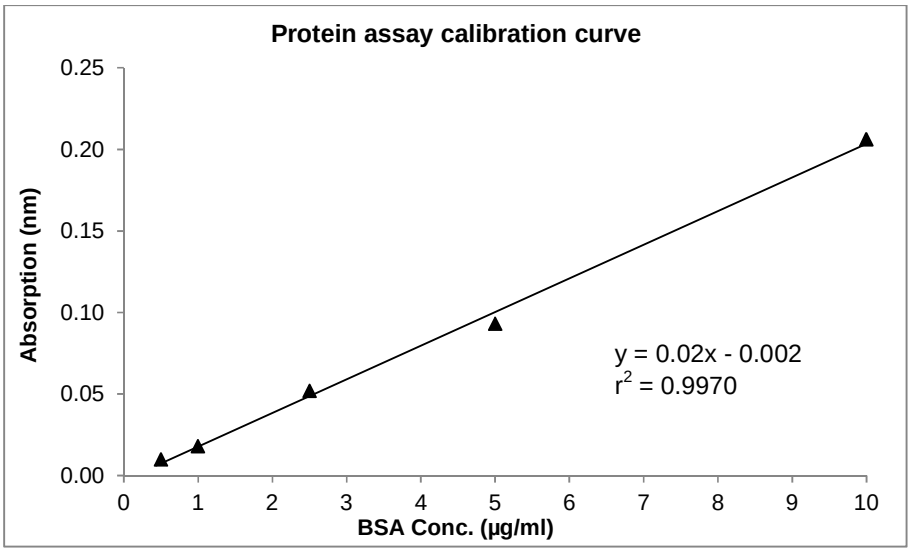

Figure 7.8: Calibration curve of BSA standards

Table 7.11: Average (mean ± SD) microsomal protein concentrations of the untreated and NVP groups

Selected liver (n = 3)	Protein concentration (mg/ml)	Absorption (nm)
Untreated		
Rat 1	49.40 ± 6.8	0.132 ± 0.02
Rat 2	55.02 ± 1.1	0.146 ± 0.00
Rat 4	51.00 ± 5.1	0.136 ± 0.01
NVP-2D		
Rat 2	44.01 ± 3.5	0.087 ± 0.01
Rat 3	55.32 ± 10.7	0.124 ± 0.03
Rat 4	62.63 ± 8.3	0.139 ± 0.02
NVP-7D		
Rat 2	43.95 ± 5.5	0.078 ± 0.01
Rat 3	54.70 ± 4.3	0.098 ± 0.01
Rat 5	50.02 ± 5.5	0.089 ± 0.01
NVP-14D		
Rat 2	56.85 ± 3.8	0.115 ± 0.01
Rat 3	54.48 ± 5.2	0.112 ± 0.01
Rat 5	50.52 ± 3.1	0.102 ± 0.01

NVP = nevirapine; D = days

7.3.4.2 CYP1A2, CYP2E1 and CYP3A2 activity in vivo

Table 7.12 shows CYP1A2, CYP2E1 and CYP3A2 activity after 2, 7 and 14 days of nevirapine alone treatment, while Figures 7.9 a – c are graphical illustrations of the same. Treatment with nevirapine alone increased CYP1A2 reaction rate on each occasion, and was most different from the normal on days 2 and 7 ($p = 0.0286$). CYP2E1 reaction rate was slightly elevated on all occasions, but not statistically different from the normal. CYP3A2 activity was induced on all occasions and was significantly different from the normal ($p = 0.0083$).

Table 7.12: Average (mean ± SD) CYP1A2, CYP2E1 and CYP3A2 activity

Group (n = 3)	CYP1A2 (pmol/min*mg)	CYP2E1 (nmol/min*mg)	CYP3A2 (pmol/min*mg)
Untreated			
0 Days	4.40 ± 0.8	0.77 ± 0.1	84.63 ± 6.9
NVP			
2 Days	13.63 ± 1.5	0.87 ± 0.0	107.17 ± 8.7
7 Days	7.35 ± 1.1	0.91 ± 0.0	94.28 ± 5.2
14 Days	5.31 ± 1.6	1.15 ± 0.6	105.79 ± 11.0

NVP = nevirapine

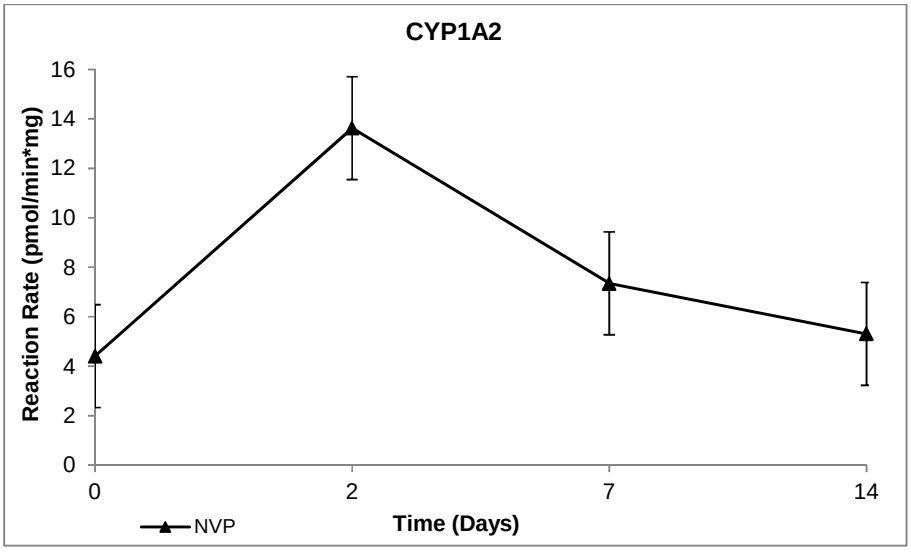

Figure 7.9 a): CYP1A2 activity after nevirapine alone treatment

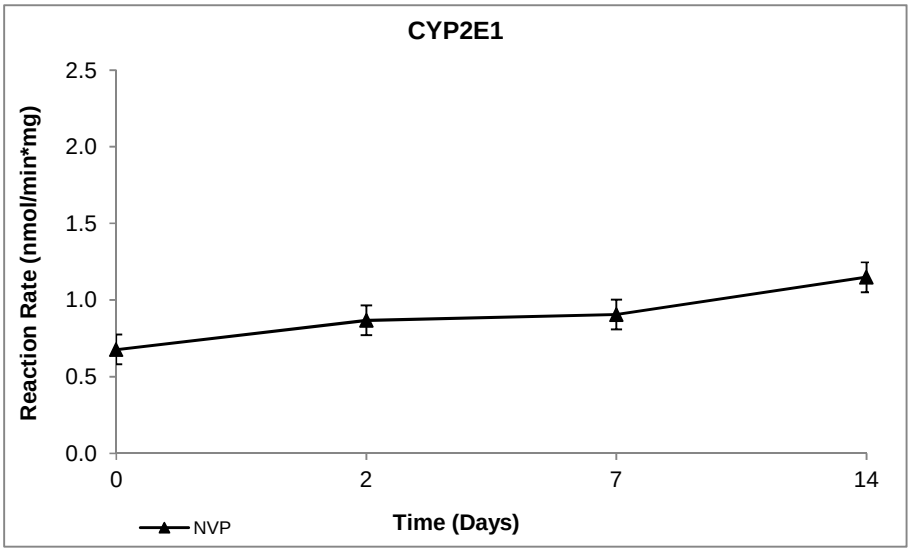

Figure 7.9 b): CYP2E1 activity after nevirapine alone treatment

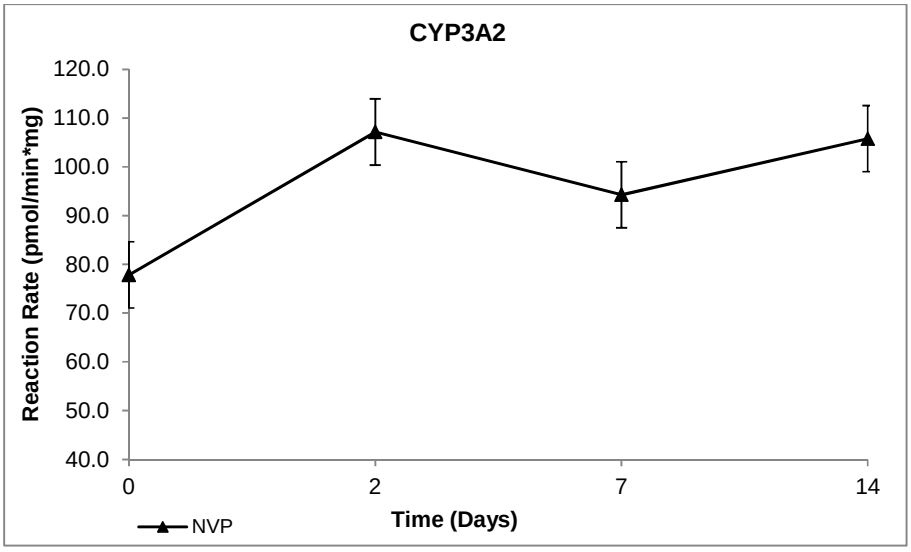

Figure 7.9 c): CYP3A2 activity after nevirapine alone treatment

7.3.5 Main observations

- ALT observations did not correlate with the histopathology changes and this implies that the liver injury was subclinical.
- The histopathology showed that nevirapine caused liver injury up to 14 days, and this had improved by day 28.
- By day 42, nevirapine concentrations were very low.
- A Th1 response (increased IL-2) was observed early (day 7) with nevirapine alone treatment, and this shifted to a later (day 42) Th2 response (increased IL-10).
- The CD4 count was moderately elevated, but lower than in the control.
- The increased IgG on day 42 was not different from the control.
- Nevirapine increased CYP2A1 activity up to day 2, while its effect on CYP2E1 activity was minimal. CYP3A4 activity continued to increase and correlated with the low nevirapine concentrations.

B. Phase II: Co-treatment with an immune stimulant

7.3.6 Physiological observations (function tests)

7.3.6.1 Full blood count

Table 7.13 shows results of the full blood count of the S, S+LMS, NVP and NVP+LMS groups. The changes of red blood count parameters as observed for nevirapine and levamisole co-treatment, share a common pattern with that of nevirapine alone (Section 7.3.1.1). Between the two groups the only significant difference was observed on day 2, when the red cell count, haemoglobin and haematocrit were lower in the NVP+LMS group than in the NVP group ($p = 0.0500$). Furthermore, the white cell count, neutrophils, lymphocytes and monocytes of the NVP+LMS group increased up to day 14 ($p = 0.0500$) and were always higher than in the NVP group ($p = 0.0500$).

Table 7.13: Average (mean ± SD) full blood count and platelets results of the S, S+LMS, NVP and NVP+LMS groups

Group (n = 3)	RCC ($x10^{12}$/l)	Hb (g/dl)	Hct (l/l)	MCV (fl)	MCH (pg)	MCHC (g/dl)	Plt ($x10^9$/l)	WCC ($x10^9$/l)	Neu ($x10^9$/l)	Ly ($x10^9$/l)	Mo ($x10^9$/l)	Eos ($x10^9$/l)	Bas ($x10^9$/l)
Untreated													
0 Days	6.28±0.2	12.9±0.3	0.398±0.01	63.5±2.5	20.5±0.4	32.3±0.8	860±221.1	6.95±2.7	0.77±0.2	4.67±1.8	0.19±0.1	0.02±0.0	0.00±0.0
S													
2 Days	6.67±0.2	13.7±0.1	0.422±0.01	63.3±2.3	20.6±0.6	32.4±0.6	849±81.6	6.50±0.9	0.60±0.2	5.18±0.7	0.21±0.0	0.50±0.2	0.01±0.0
7 Days	7.53±0.9	15.3±1.7	0.451±0.04	60.1±2.5	20.3±0.2	33.9±1.2	1033±79.8	5.44±2.4	1.03±0.8	4.07±2.0	0.30±0.3	0.04±0.0	0.01±0.0
14 Days	6.77±0.6	13.9±1.1	0.417±0.03	61.8±2.8	20.5±0.6	33.2±0.6	721±196.4	5.22±1.2	0.63±0.5	4.21±0.7	0.18±0.1	0.18±0.1	0.05±0.0
28 Days	7.07±0.7	13.9±1.3	0.390±0.04	55.1±1.0	19.7±0.1	35.8±0.6	961±172.5	7.38±1.0	0.91±0.2	6.15±0.8	0.24±0.1	0.07±0.0	0.01±0.0
42 Days	6.93±0.8	13.4±1.8	0.374±0.05	53.9±1.0	19.3±0.4	35.8±0.2	839±166.0	3.93±0.3	0.54±0.1	3.23±0.3	0.11±0.0	0.04±0.0	0.01±0.0
S+LMS													
2 Days	6.53±0.4	13.2±0.6	0.408±0.03	62.5±0.5	20.3±0.5	32.4±0.7	755±239.0	7.33±0.2	0.66±0.0	5.99±0.4	0.35±0.0	0.34±0.1	0.01±0.0
7 Days	6.91±0.0	13.4±0.0	0.408±0.00	59.0±0.0	19.4±0.0	32.8±0.0	850±00.0	6.75±0.0	0.93±0.0	5.10±0.0	0.38±0.0	0.33±0.0	0.01±0.0
14 Days	6.99±0.4	13.8±0.8	0.427±0.02	61.1±1.6	19.8±0.1	32.4±0.8	718±204.1	4.65±1.7	0.53±0.3	3.82±1.8	0.12±0.1	0.18±0.3	0.00±0.0
28 Days	7.41±0.2	14.9±0.4	0.436±0.01	58.8±0.2	20.1±0.4	34.1±0.8	578±62.1	5.13±1.8	0.71±0.1	4.27±1.6	0.10±0.1	0.04±0.0	0.01±0.0
42 Days	7.91±0.2	15.6±0.4	0.455±0.02	57.5±0.7	19.7±0.2	34.2±0.5	701±116.7	6.76±0.8	0.85±0.1	5.69±0.9	0.16±0.1	0.04±0.0	0.00±0.0
NVP													
2 Days	6.51±0.2	13.6±0.4	0.408±0.01	62.7±0.8	20.9±0.1	33.2±0.3	854±209.3	3.48±0.8	1.02±0.1	1.88±0.3	0.10±0.0	0.48±0.4	0.00±0.0
7 Days	7.14±0.3	14.2±0.5	0.424±0.02	59.5±1.8	19.9±0.6	33.5±0.5	779±141.3	5.54±1.3	0.76±0.3	3.81±0.9	0.49±0.2	0.48±0.2	0.01±0.0
14 Days	6.97±0.3	13.9±0.4	0.415±0.01	59.5±2.2	19.9±0.6	33.5±0.3	1013±73.0	4.86±0.7	1.08±0.2	3.38±0.4	0.37±0.1	0.02±0.0	0.01±0.0
28 Days	7.36±0.6	14.2±1.1	0.404±0.03	55.0±0.7	19.3±0.3	35.0±0.6	876±17.2	6.07±0.6	1.09±0.2	4.62±0.7	0.28±0.2	0.08±0.1	0.01±0.0
42 Days	7.73±0.3	14.5±0.3	0.405±0.00	52.5±2.3	18.8±0.5	35.8±0.8	1052±115.6	5.42±0.1	1.52±0.6	3 .52±0.6	0.33±0.0	0.04±0.0	0.01±0.0
NVP+LMS													
2 Days	4.93±0.5	10.1±0.8	0.314±0.02	63.6±1.0	20.5±0.4	32.1±0.1	682±174.7	4.92±0.3	1.22±0.0	3.27±0.0	0.39±0.4	0.03±0.0	0.02±0.0
7 Days	7.26±0.2	14.2±0.2	0.418±0.01	57.6±2.5	19.5±0.9	33.9±0.4	861±136.2	5.03±1.2	1.31±1.1	3.12±0.3	0.45±0.2	0.14±0.1	0.01±0.0
14 Days	7.31±0.3	14.3±0.7	0.435±0.02	59.4±1.1	19.6±0.1	32.9±0.4	1000±51.7	7.96±2.8	1.30±0.2	6.11±2.5	0.52±0.1	0.02±0.0	0.01±0.0
28 Days	7.60±0.2	14.8±0.1	0.439±0.01	57.8±0.6	19.5±0.5	33.8±0.5	707±88.1	5.27±1.3	0.97±0.2	4.05±1.0	0.22±0.1	0.03±0.0	0.00±0.0
42 Days	7.85±0.6	15.0±0.9	0.435±0.02	55.6±2.3	19.1±0.7	34.4±0.5	665±83.2	4.39±1.1	0.80±0.1	3.32±0.8	0.23±0.1	0.04±0.0	0.00±0.0

RCC = red cell count; Hb = haemoglobin; Hct = haematocrit; MCV = mean corpuscular volume; MCH = mean corpuscular haemoglobin; MCHC = mean corpuscular haemoglobin concentration; Plt = platelets; WCC = white cell count; Neu = neutrophils; Ly = lymphocytes; Mo = monocytes; Eos = eosinophils; Bas = basophils; S = saline; LMS = levamisole; NVP = nevirapine

7.3.6.2 Renal function tests

Table 7.14 shows the changes of BUN and Cr of the S, S+LMS, NVP and NVP+LMS groups. In all groups BUN and Cr levels were normal. Here, Cr levels also spiked on day 28, but were still within the normal range (p = 0.0500).

Table 7.14: Average (mean ± SD) renal function test results of the S, S+LMS, NVP and NVP+LMS groups

Group	**RFT**		**Group**	**RFT**	
	BUN	**Cr**		**BUN**	**Cr**
(n = 3)	**(mmol/l)**	**(µmol/l)**	**(n = 3)**	**(mmol/l)**	**(µmol/l)**
Untreated					
0 Days	7.2 ± 1	37 ± 8			
S			**NVP**		
2 Days	7.3 ± 1	39 ± 2	2 Days	7.9 ± 1	36 ± 1
7 Days	8.1 ± 0	46 ± 7	7 Days	8.7 ± 0	46 ± 2
14 Days	7.5 ± 1	39 ± 3	14 Days	9.1 ± 1	41 ± 9
28 Days	10.6 ± 2	73 ± 17	28 Days	8.5 ± 1	63 ± 7
42 Days	5.8 ± 1	38 ± 9	42 Days	7.0 ± 0	27 ± 3
S+LMS			**NVP+LMS**		
2 Days	8.3 ±1	38 ± 5	2 Days	8.4 ± 1	33 ± 8
7 Days	7.8 ± 1	40 ± 18	7 Days	7.5 ± 1	33 ± 8
14 Days	6.5 ± 1	35 ± 5	14 Days	6.6 ± 1	31 ± 12
28 Days	6.1 ± 0	60 ± 3	28 Days	5.8 ± 0	56 ± 6
42 Days	6.5 ± 1	21 ± 1	42 Days	7.4 ± 1	10 ± 1

RFT = renal function test; BUN = blood urea nitrogen; Cr = creatinine; S = saline; LMS = levamisole; NVP = nevirapine

7.3.6.3 Liver function tests

Table 7.15 shows the changes of ALT, AST and ALP of the S, S+LMS, NVP and NVP+LMS groups. Over the 42 days, the results were similar in all groups.

Table 7.15: Average (mean ± SD) liver function test results of the S, S+LMS, NVP and NVP+LMS groups

Group	LFT			Group	LFT		
	ALT	AST	ALP		ALT	AST	ALP
(n = 3)	(U/l)	(U/l)	(U/l)	(n = 3)	(U/l)	(U/l)	(U/l)
Untreated							
0 Days	50±5	88±14	352±76				
S				**NVP**			
2 Days	46±2	90±7	400±7	2 Days	63±7	107±10	359±43
7 Days	49±10	103±25	304±13	7 Days	87±36	169±115	447±78
14 Days	58±4	127±37	508±37	14 Days	72±3	109±33	443±43
28 Days	47±2	115±44	216±19	28 Days	53±4	128±44	166±37
42 Days	46±6	76±28	109±76	42 Days	54±2	70±4	14±9
S+LMS				**NVP+LMS**			
2 Days	40±3	113±53	541±9	2 Days	75±28	182±174	524±80
7 Days	52±15	90±27	483±130	7 Days	57±10	70±10	423±58
14 Days	48±12	73±16	478±105	14 Days	52±9	92±29	378±72
28 Days	50±7	75±19	127±63	28 Days	51±3	67±13	67±64
42 Days	46±2	75±6	24±11	42 Days	49±6	70±17	12±3

LFT = liver function test; ALT = alanine aminotransferase; AST = aspartate aminotransferase; ALP = alkaline phosphatase; S = saline; NVP = nevirapine; LMS = levamisole

7.3.6.4 Liver histopathology

(a) Liver histopathology reports

Liver sections for histopathology (Figures 6.10 a – j and 7.10 a – j) were randomly selected, and the main histopathology lesions are summarised in the tally tables (Tables 7.16 a and b). The following report is a summary of the features of the lesion:

i. Figures 6.10 a and b: Liver sections A and B from the S+LMS group after 2 days of saline and levamisole co-treatment

The report is the same as in Chapter 6, Section 6.3.6.4 i.

ii. Figures 6.10 c and d: Liver sections A and B from the S+LMS group after 7 days of saline and levamisole co-treatment

The report is the same as in Chapter 6, Section 6.3.6.4 ii.

iii. Figures 6.10 e and f: Liver sections A and B from the S+LMS group after 14 days of saline and levamisole co-treatment

The report is the same as in Chapter 6, Section 6.3.6.4 iii.

iv. Figures 6.10 g and h: Liver sections A and B from the S+LMS group after 28 days of saline and levamisole co-treatment

The report is the same as in Chapter 6, Section 6.3.6.4 iv.

v. Figures 6.10 i and j: Liver sections A and B from the S+LMS group after 42 days of saline and levamisole co-treatment

The report is the same as in Chapter 6, Section 6.3.6.4 v.

vi. Figures 7.10 a and b: Liver sections A and B from the NVP+LMS group after 2 days of nevirapine and levamisole co-treatment

Representative photographs of rat livers after 2 days of daily nevirapine and levamisole co-treatment. The report: “In liver sections A and B minimal hepatocellular degeneration could be detected in the parenchyma. Section A demonstrated minimal mitosis, while there were multiple mitotic figures present in section B.”

vii. Figures 7.10 c and d: Liver sections A and B from the NVP+LMS group after 7 days of nevirapine and levamisole co-treatment

Representative photographs of rat livers after 7 days of daily nevirapine and levamisole co-treatment. The report: “Although the degenerative changes were graded as minimal in liver sections A and B, mild single cell necrosis could be confirmed within the hepatic parenchyma of section A.”

viii. Figures 7.10 e and f: Liver sections A and B from the NVP+LMS group after 14 days of nevirapine and levamisole co-treatment

Representative photographs of rat livers after 14 days of daily nevirapine and levamisole co-treatment. The report: “Moderate degeneration, cellular swelling and granular cytoplasm was present in liver sections A and B. Cytonecrosis was graded minimal to mild, and accompanied by loss of cell boundaries. Centrilobular zonal necrosis was absent or minimal, and there were no mitotic figures present.”

ix. Figures 7.10 g and h: Liver sections A and B from the NVP+LMS group after 28 days of nevirapine and levamisole co-treatment

Representative photographs of rat livers after 28 days of daily nevirapine and levamisole co-treatment. The report: “Moderate granular vacuolar degeneration and cell swelling were still present in liver sections A and B. Cytonecrosis was graded minimal to mild and caused the cytoplasm to have a granular appearance. No centrilobular zonal necrosis or hepatocyte mitosis was observed.”

x. Figures 7.10 i and j: Liver sections A and B from the NVP+LMS group after 42 days of nevirapine and levamisole co-treatment

Representative photographs of rat livers after 42 days of daily nevirapine and levamisole co-treatment. The report: “The degeneration and cytonecrosis are grade minimal to mild in liver sections A and B, respectively.”

In view of the histopathology photographs (Figures 7.10 a – j), reports and tally tables (Tables 7.16 a and b), it was concluded that co-treatment with levamisole caused liver injury up to 28 days, and this was the same as with nevirapine alone.

(b) Liver histopathology photographs

Histopathology photographs of the S+LMS group (Figures 6.10 a – j) are presented in Chapter 6, Section 6.3.6.4 b. Figures 7.10 a – j are representative of randomly selected liver sections of the NVP+LMS group.

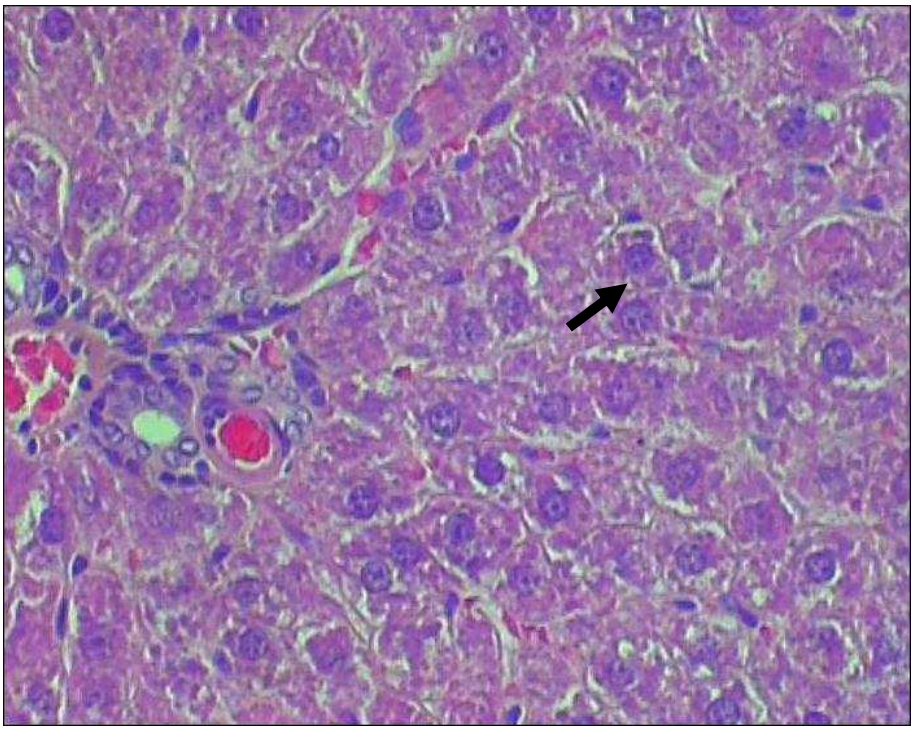

Figure 7.10 a): Liver section A from the NVP+LMS group after 2 days of treatment, showing minimal hepatocellular degeneration and mitosis

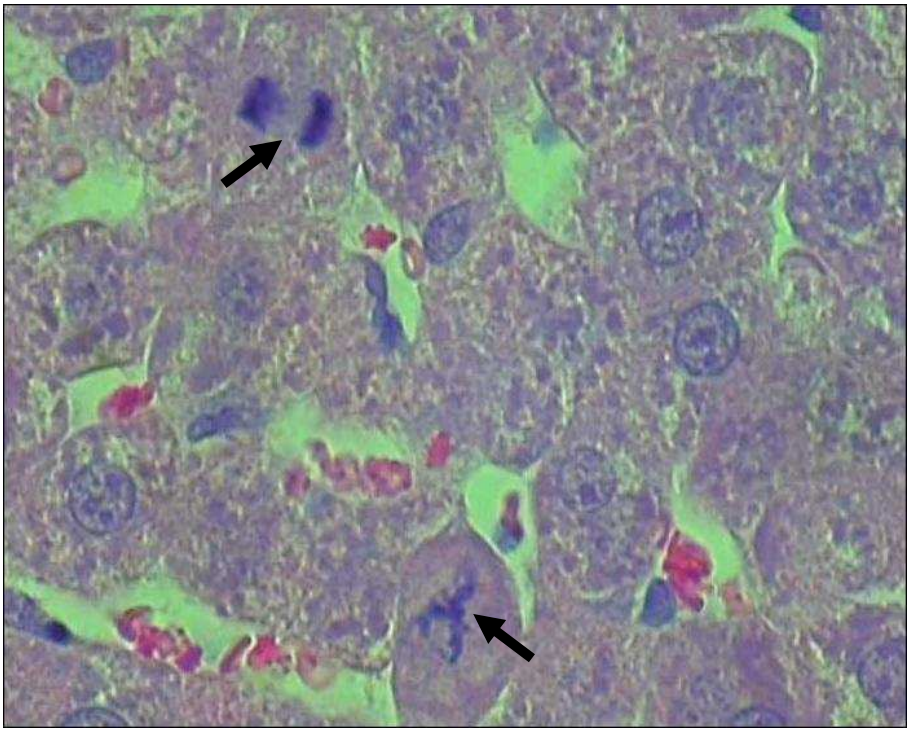

Figure 7.10 b): Liver section B from the NVP+LMS group after 2 days of treatment, showing minimal parenchymal degeneration and multiple mitotic figures

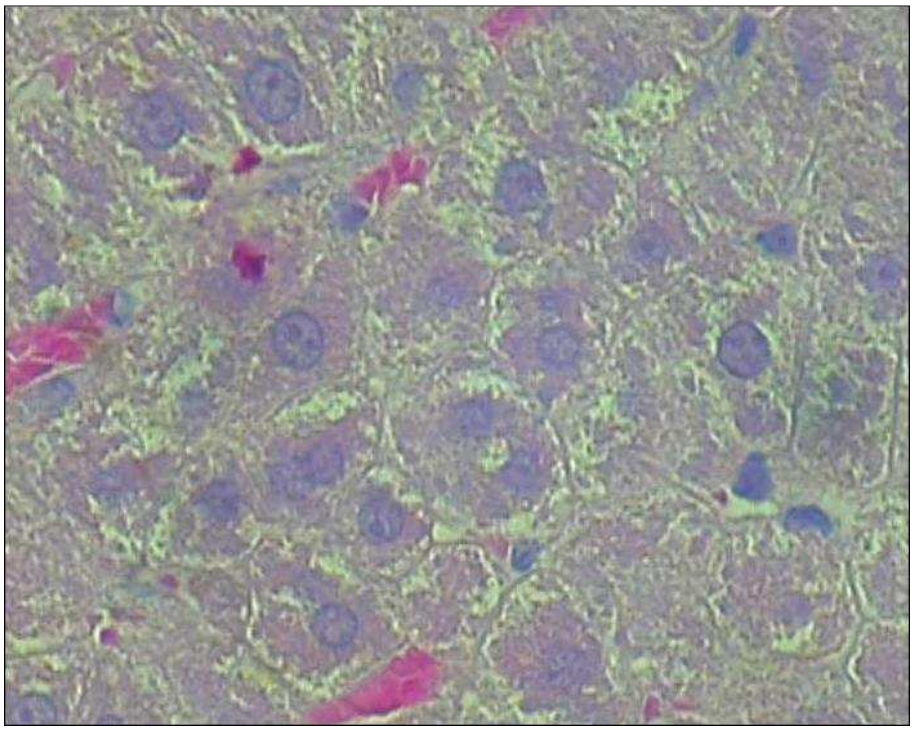

Figure 7.10 c): Liver section A from the NVP+LMS group after 7 days of treatment, showing minimal degenerative changes

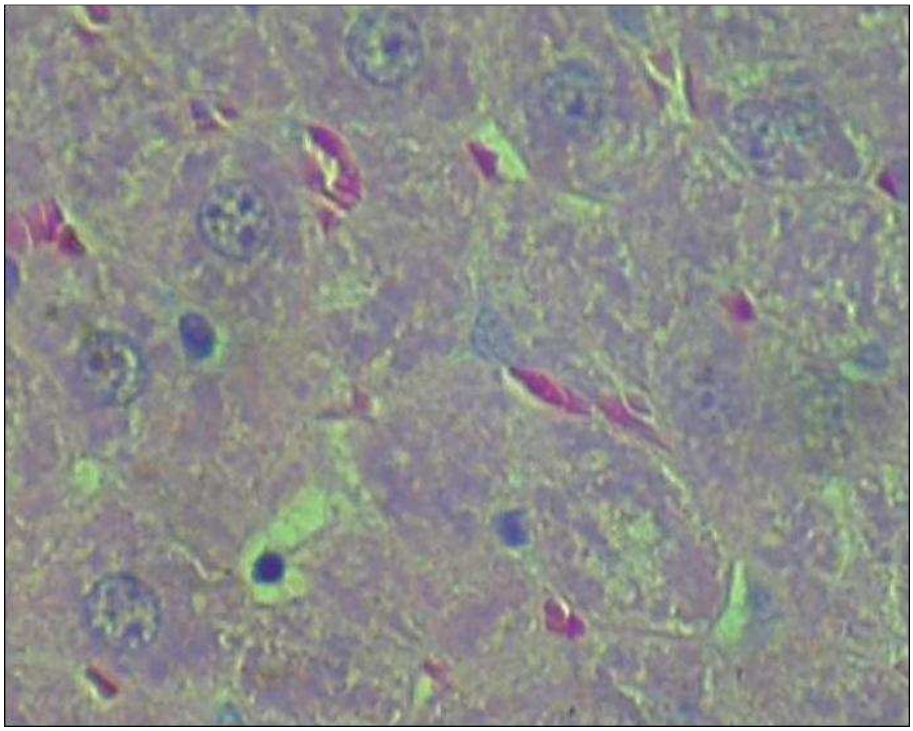

Figure 7.10 d): Liver section B from the NVP+LMS group after 7 days of treatment, showing minimal degeneration and mild single cell necrosis

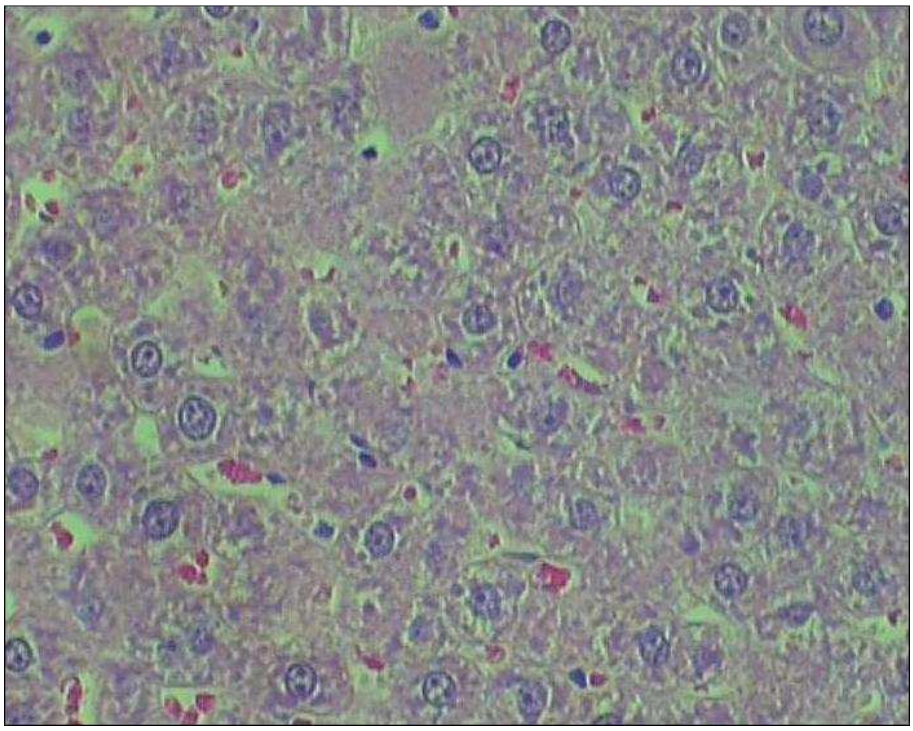

Figure 7.10 e): Liver section A from the NVP+LMS group after 14 days of treatment, showing moderate degeneration and minimal cytonecrosis

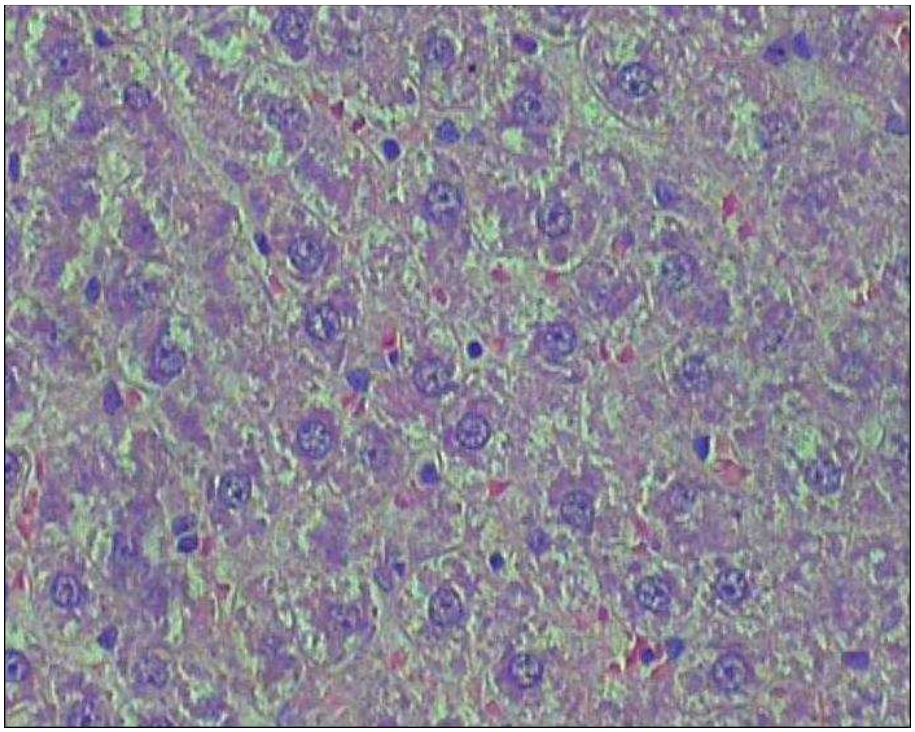

Figure 7.10 f): Liver section B from the NVP+LMS group after 14 days of treatment, showing moderate degeneration, cellular swelling, granular cytoplasm, and mild loss of cell boundaries

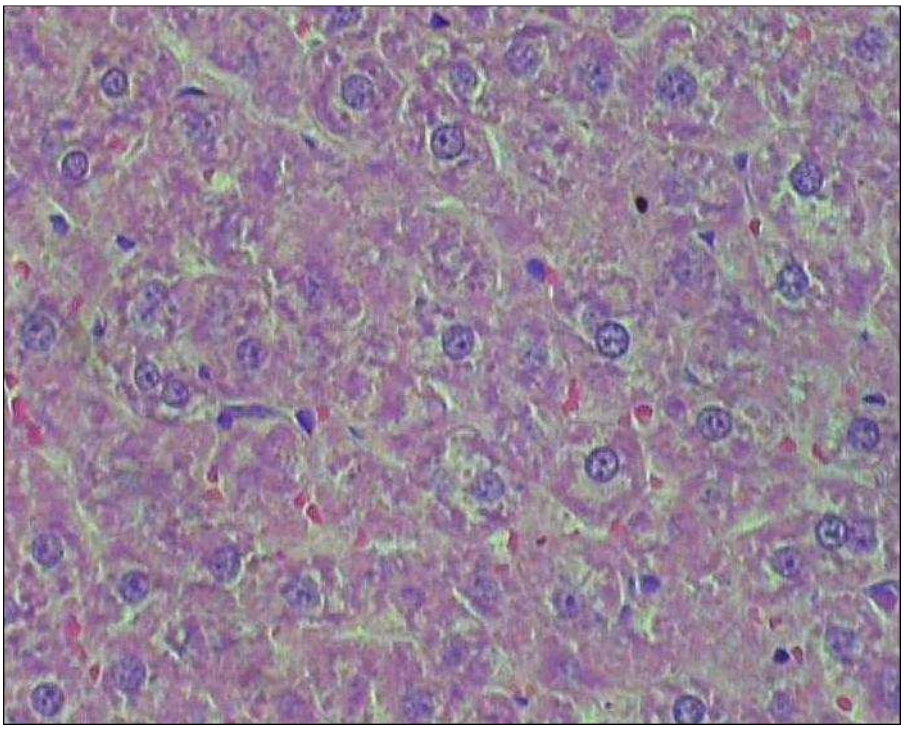

Figure 7.10 g): Liver section A from the NVP+LMS group after 28 days of treatment, showing moderate granular vacuolar degeneration and cell swelling with loss of cell boundaries

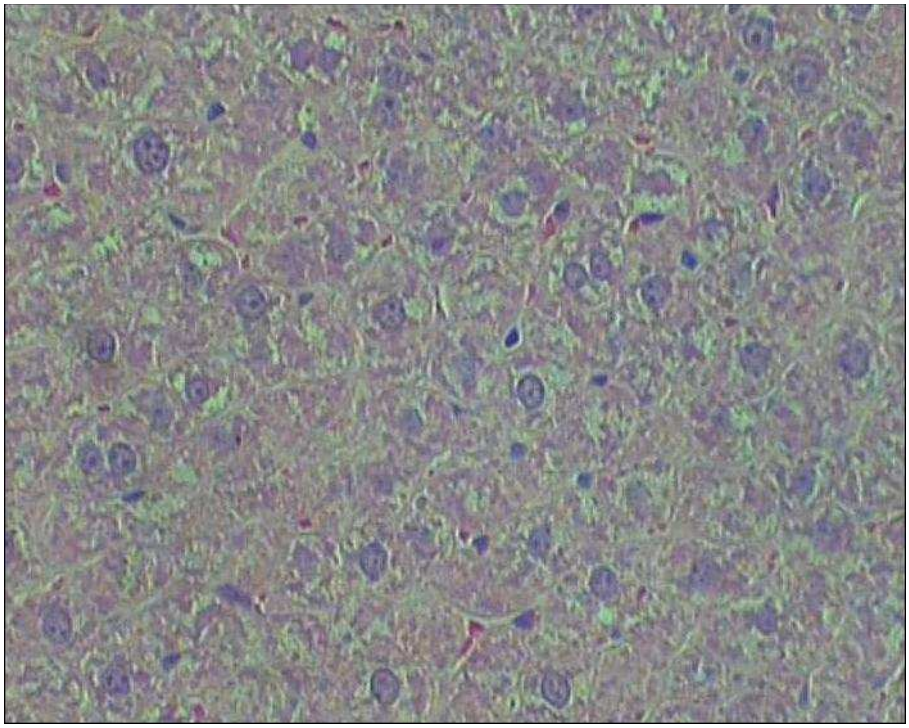

Figure 7.10 h): Liver section B from the NVP+LMS group after 28 days of treatment, showing moderate degeneration, mild cytonecrosis and loss of cell boundaries

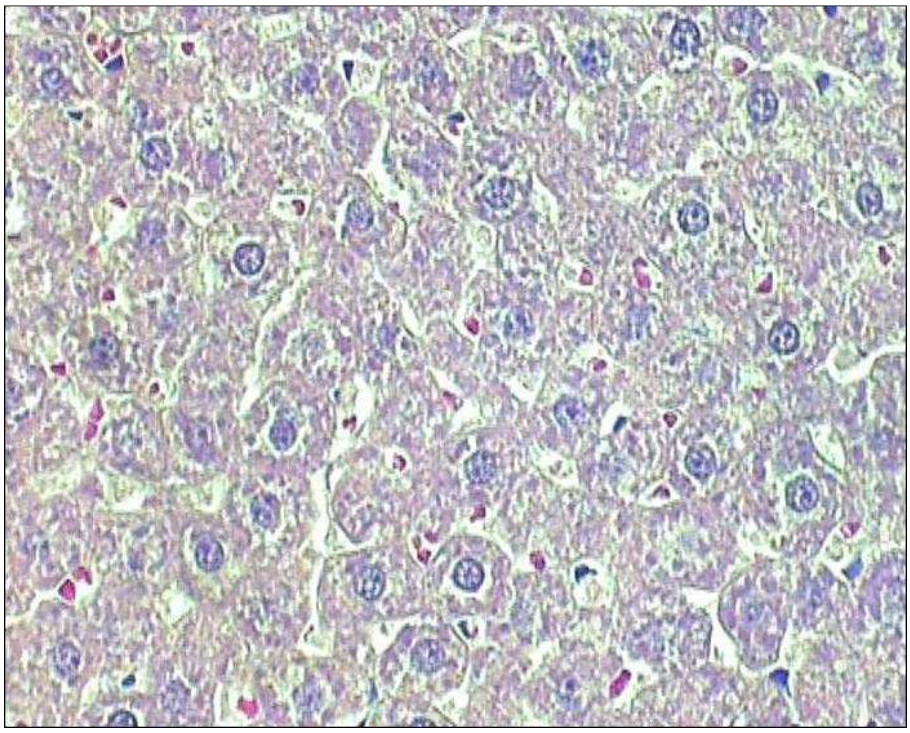

Figure 7.10 i): Liver section A from the NVP+LMS group after 42 days of treatment, showing minimal degeneration and cytonecrosis

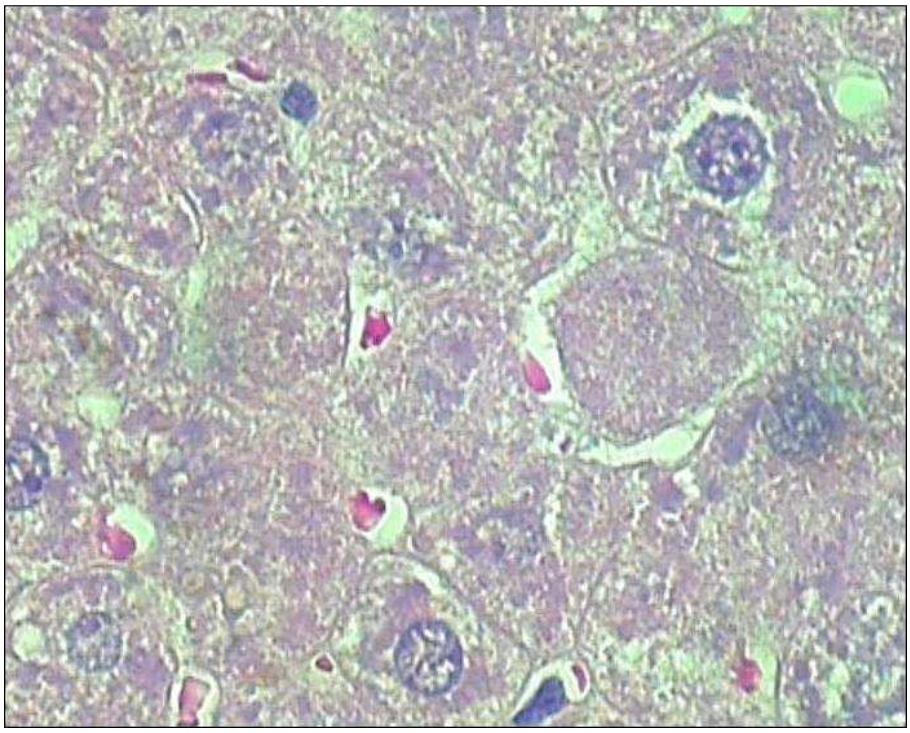

Figure 7.10 j): Liver section B from the NVP+LMS group after 42 days of treatment, showing mild degeneration and cytonecrosis

Table 7.16 a): Tally of main pathology lesions (lesions score) in livers of untreated rats and the S+LMS group

Group	UnRx	S+LMS									
		2 Days		7 Days		14 Days		28 Days		42 Days	
(n = 2)	Fig.6.3a	Fig.6.10a	Fig.6.10b	Fig.6.10c	Fig.6.10d	Fig.6.10e	Fig.6.10f	Fig.6.10g	Fig.6.10h	Fig.6.10i	Fig.6.10j
Histopathological lesions											
Vacuolar degeneration	0	0	0	0	0	0	1+	1+	1+	1+	1+
Cell swelling	0	0	0	0	0	0	1+	1+	1+	1+	1+
Cytonecrosis	0	0	0	0	0	0	0	0	0	0	0
Centrilobular necrosis	0	0	0	0	0	0	0	0	0	0	0
Hepatocyte mitosis	0	0	0	1+	2+	0	0	0	1+	0	0
Average lesions score											
Vacuolar degeneration	0	0		0		0.5+		1+		1+	
Cell swelling	0	0		0		0.5+		1+		1+	
Cytonecrosis	0	0		0		0		0		0	
Centrilobular necrosis	0	0		0		0		0		0	
Hepatocyte mitosis	0	0		1.5+		0		0.5+		0	
Total lesion score	**0**	**0**		**1.5+**		**1+**		**2.5+**		**2+**	

UnRx = untreated; S = saline; LMS = levamisole

Table 7.16 b): Tally of main pathology lesions (lesions score) in livers of untreated rats and the NVP+LMS group

Group	UnRx	NVP+LMS									
		2 Days		7 Days		14 Days		28 Days		42 Days	
(n = 2)	Fig.7.3a	Fig.7.10a	Fig.7.10b	Fig.7.10c	Fig.7.10d	Fig.7.10e	Fig.7.10f	Fig.7.10g	Fig.7.10h	Fig.7.10i	Fig.7.10j
Histopathological lesions											
Vacuolar degeneration	0	1+	1+	1+	1+	3+	3+	3+	3+	1+	2+
Cell swelling	0	1+	1+	1+	1+	3+	3+	3+	3+	1+	2+
Cytonecrosis	0	0	0	0	2+	1+	2+	1+	2+	1+	2+
Centrilobular necrosis	0	0	0	0	0	0	1+	0	0	0	0
Hepatocyte mitosis	0	1+	2+	0	0	0	0	0	0	0	0
Average lesions score											
Vacuolar degeneration	0	1+		1+		3+		3+		1.5+	
Cell swelling	0	1+		1+		3+		3+		1.5+	
Cytonecrosis	0	0		1+		1.5+		1.5+		1.5+	
Centrilobular necrosis	0	0		0		0.5+		0		0	
Hepatocyte mitosis	0	1.5+		0		0		0		0	
Total lesion score	**0**	**3.5+**		**3+**		**8+**		**7.5+**		**4.5+**	

UnRx = untreated; NVP = nevirapine; LMS = levamisole

7.3.7 Nevirapine concentrations

Table 7.17 shows nevirapine concentrations of the NVP and NVP+LMS groups, while Figure 7.11 is a graphical illustration of the same. By day 42 nevirapine concentrations had declined (p = 0.0079). Although nevirapine levels in the NVP+LMS group were lower than in the NVP group on day 2, and higher on day 7, this was not statistically different.

Table 7.17: Average (mean ± SD) nevirapine concentrations of the NVP and NVP+LMS groups

Group	NVP	NVP+LMS
	NVP concentration	NVP concentration
(n = 5)	(µg/ml)	(µg/ml)
2 Days	4.292 ± 2.69	2.287 ± 2.59
7 Days	2.008 ± 0.67	4.332 ± 4.98
14 Days	1.983 ± 0.58	1.477 ± 1.63
28 Days	1.721 ± 3.04	1.293 ± 0.73
42 Days	0.497 ± 1.11	0.000 ± 0.00

NVP = nevirapine; LMS = levamisole

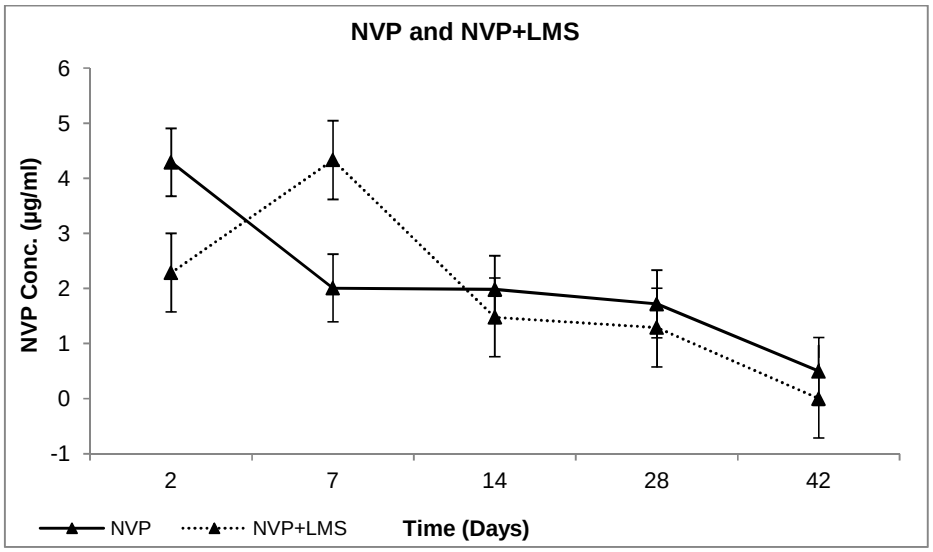

Figure 7.11: Nevirapine concentrations of the NVP and NVP+LMS groups over 42 days

7.3.8 Specific immunology tests

7.3.8.1 Direct observations

Table 7.18 shows changes in body weight of the S, S+LMS, NVP and NVP+LMS groups over the treatment period. All groups showed weight gain at all times, except for the S+LMS group after 2 days (Refer to Appendix H-1 and H-3 for baseline weights).

Table 7.18: Average (mean ± SD) change in rat weights of the S, S+LMS, NVP and NVP+LMS groups

Group (n = 5)	S change in weight (g)	S+LMS change in weight (g)	NVP change in weight (g)	NVP+LMS change in weight (g)
2 Days	9.2±4	-1.4±1	10.6±8	1.3±1
7 Days	35.7±8	19.4±10	28.5±1	21.9±8
14 Days	84.6±5	57.0±26	82.8±2	49.7±5
28 Days	107.8±10	96.1±13	99.7±16	105.6±17
42 Days	171.4±27	153.4±23	105.6±16	113.9±13

S = saline; LMS = levamisole; NVP = nevirapine

7.3.8.2 Cytokines

Table 7.19 shows IL-2 and IL-10 concentrations of the S, S+LMS, NVP and NVP+LMS groups, while Figures 7.12 a – b are graphical illustrations of the same. For the NVP+LMS group, IL-2 increased slightly up to 14 days (p = 0.0500), and then declined (p = 0.0500). Overall, it was lower than with nevirapine alone (p = 0.0500). IL-10 concentrations increased up to day 7, and were lower than with nevirapine alone from day 14 to 42, but this was not statistically different.

Table 7.19: Average (mean ± SD) cytokine concentrations of the S, S+LMS, NVP and NVP+LMS groups

Group	Cytokine		Group	Cytokine	
(n = 3)	IL-2 (pg/ml)	IL-10 (pg/ml)	(n = 3)	IL-2 (pg/ml)	IL-10 (pg/ml)
Untreated					
0 Days	65.46 ± 2.0	31.08 ± 1.2			
S			**NVP**		
2 Days	74.87 ± 6.5	29.96 ± 2.8	2 Days	76.55 ± 1.2	34.77 ± 2.5
7 Days	77.26 ± 5.8	34.57 ± 0.7	7 Days	82.21 ± 4.6	36.63 ± 3.6
14 Days	77.58 ± 6.6	35.69 ± 5.4	14 Days	73.07 ± 7.9	34.87 ± 6.8
28 Days	78.81 ± 4.6	32.46 ± 4.2	28 Days	71.82 ± 10.6	39.64 ± 3.5
42 Days	74.39 ± 5.7	32.03 ± 2.5	42 Days	61.92 ± 4.5	41.89 ± 6.5
S+LMS			**NVP+LMS**		
2 Days	62.89 ± 2.2	30.57 ± 0.9	2 Days	48.00 ± 5.7	37.12 ± 1.6
7 Days	62.89 ± 0.8	31.31 ± 2.0	7 Days	53.83 ± 7.0	39.74 ± 4.7
14 Days	62.61 ± 9.4	32.44 ± 6.5	14 Days	55.17 ± 1.4	35.62 ± 1.8
28 Days	65.82 ± 3.7	29.82 ± 1.7	28 Days	53.17 ± 17.0	35.06 ± 2.9
42 Days	69.45 ± 3.6	29.26 ± 3.1	42 Days	31.67 ± 2.5	35.43 ± 1.7

IL = interleukin; S = saline; NVP = nevirapine; LMS = levamisole

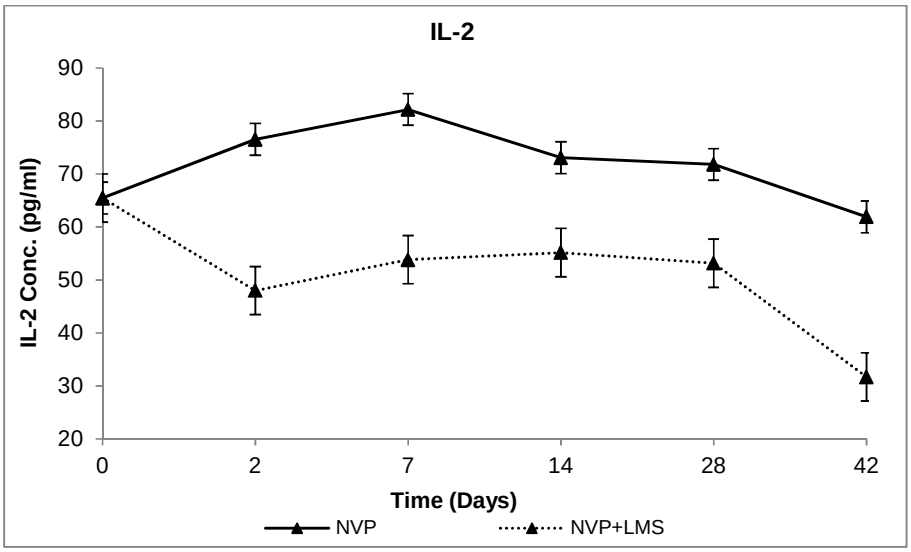

Figure 7.12 a): IL-2 concentrations of the NVP and NVP+LMS groups over 42 days

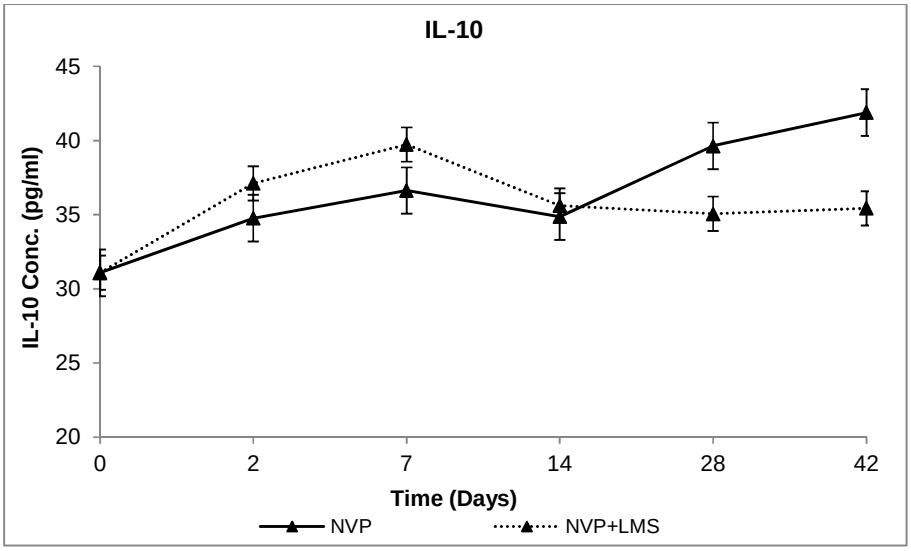

Figure 7.12 b): IL-10 concentrations of the NVP and NVP+LMS groups over 42 days

7.3.8.3 CD4 and CD8 counts

Table 7.20 shows CD4 and CD8 counts of the S, S+LMS, NVP and NVP+LMS groups, while Figures 7.13 a – b are graphical illustrations of the same. In the NVP+LMS group lymphocyte (p = 0.0500), CD4 and CD8 counts (p = 0.0500) increased up to day 14. By day 42, the CD8 count had declined (p = 0.0500), and was also lower than in the NVP group (p = 0.0500).

Table 7.20: Average (mean ± SD) CD4 and CD8 counts of the S, S+LMS, NVP and NVP+LMS groups

Group	**Ly**	**T-Ly**		**Group**	**Ly**	**T-Ly**	
		CD4	**CD8**			**CD4**	**CD8**
(n = 3)	**(x10^9/l)**	**(x10^9/l)**	**(x10^9/l)**	**(n = 3)**	**(x10^9/l)**	**(x10^9/l)**	**(x10^9/l)**
Untreated							
0 Days	4.67±1.8	2.23±1.3	1.42±0.7				
S				**NVP**			
2 Days	5.18±0.7	2.27±0.3	1.35±0.2	2 Days	1.88±0.3	1.07±0.2	0.59±0.1
7 Days	4.07±2.0	1.72±0.8	1.07±0.5	7 Days	3.81±0.9	1.58±0.3	1.07±0.3
14 Days	4.21±0.7	1.69±0.2	1.17±0.2	14 Days	3.38±0.4	1.43±0.1	0.85±0.2
28 Days	6.15±0.8	2.45±0.2	1.58±0.3	28 Days	4.62±0.7	1.78±0.4	1.26±0.2
42 Days	3.23±0.3	1.47±0.1	0.79±0.2	42 Days	3.52±0.6	1.23±0.2	0.81±0.2
S+LMS				**NVP+LMS**			
2 Days	5.99±0.4	2.25±0.1	1.58±0.0	2 Days	3.27±0.0	1.49±0.1	0.88±0.1
7 Days	5.10±0.0	2.09±0.0	1.34±0.0	7 Days	3.12±0.3	1.33±0.4	0.67±0.1
14 Days	3.82±1.8	1.64±0.8	0.97±0.5	14 Days	6.11±2.5	2.35±1.1	1.55±0.7
28 Days	4.27±1.6	1.69±0.6	1.15±0.5	28 Days	4.05±1.0	1.30±0.4	0.92±0.3
42 Days	5.69±0.9	2.26±0.3	0.01±0.0	42 Days	3.32±0.8	1.30±0.3	0.00±0.0

Ly = lymphocytes; CD = cluster of differentiation; S = saline; NVP = nevirapine; LMS = levamisole

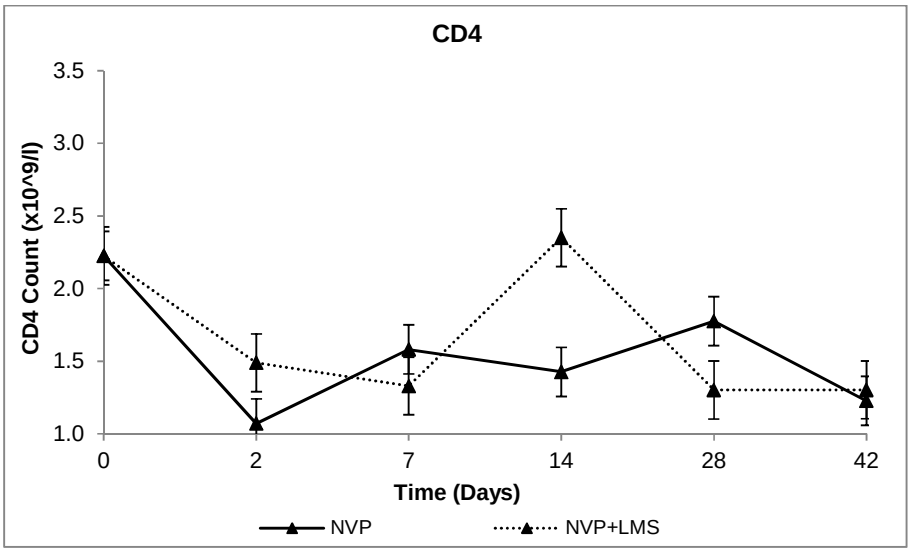

Figure 7.13 a): CD4 counts of the NVP and NVP+LMS groups over 42 days

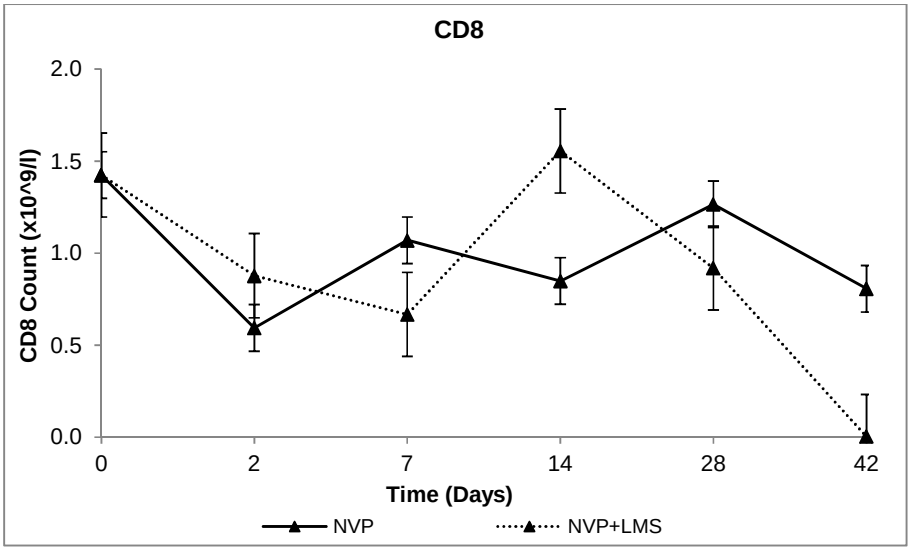

Figure 7.13 b): CD8 counts of the NVP and NVP+LMS groups over 42 days

7.3.8.4 Immunoglobulins

Table 7.21 shows concentrations of IgM and IgG of the S, S+LMS, NVP and NVP+LMS groups, while Figures 7.14 a – b are graphical illustrations of the same. In the NVP+LMS group, IgM concentrations increased up to day 7, after which they declined (p = 0.0500). Up to day 7 they were higher than in the NVP group (p = 0.0500), and this had reversed by day 42 (p = 0.0500). By day 42, IgG concentrations were slightly elevated, but not statistically significant.

Table 7.21: Average (mean ± SD) immunoglobulin concentrations of the S, S+LMS, NVP and NVP+LMS groups

Group	**Immunoglobulin**		**Group**	**Immunoglobulin**	
	IgM	**IgG**		**IgM**	**IgG**
(n = 3)	**(mg/ml)**	**(mg/ml)**	**(n = 3)**	**(mg/ml)**	**(mg/ml)**
Untreated					
0 Days	0.109±0.02	14.434±1.10			
S			**NVP**		
2 Days	0.104±0.04	14.137±0.91	2 Days	0.011±0.01	13.971±1.63
7 Days	0.110±0.04	14.302±0.70	7 Days	0.012±0.00	13.311±0.57
14 Days	0.110±0.03	12.617±0.29	14 Days	0.116±0.03	12.517±1.05
28 Days	0.075±0.03	16.350±1.00	28 Days	0.087±0.02	12.022±0.51
42 Days	0.046±0.01	17.109±0.26	42 Days	0.089±0.04	16.724±2.23
S+LMS			**NVP+LMS**		
2 Days	0.040±0.00	9.486±0.95	2 Days	0.045±0.00	11.222±0.91
7 Days	0.045±0.02	12.487±1.21	7 Days	0.080±0.01	13.290±1.16
14 Days	0.059±0.02	11.961±1.72	14 Days	0.028±0.01	10.433±0.29
28 Days	0.053±0.02	12.095±1.15	28 Days	0.014±0.01	11.897±1.70
42 Days	0.046±0.01	13.013±1.30	42 Days	0.028±0.02	12.997±1.50

IgM = immunoglobulin M; IgG = immunoglobulin G; S = saline; NVP = nevirapine; LMS = levamisole

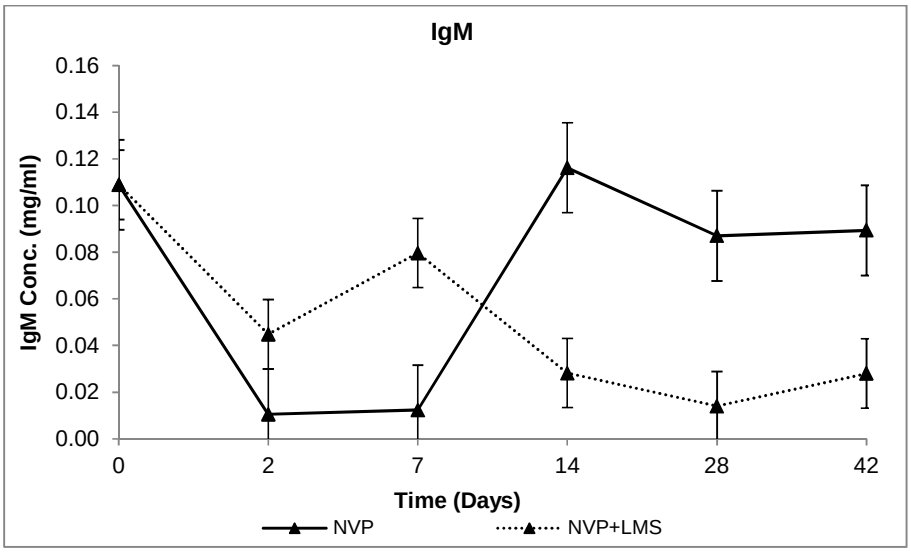

Figure 7.14 a): IgM concentrations of the NVP and NVP+LMS groups over 42 days

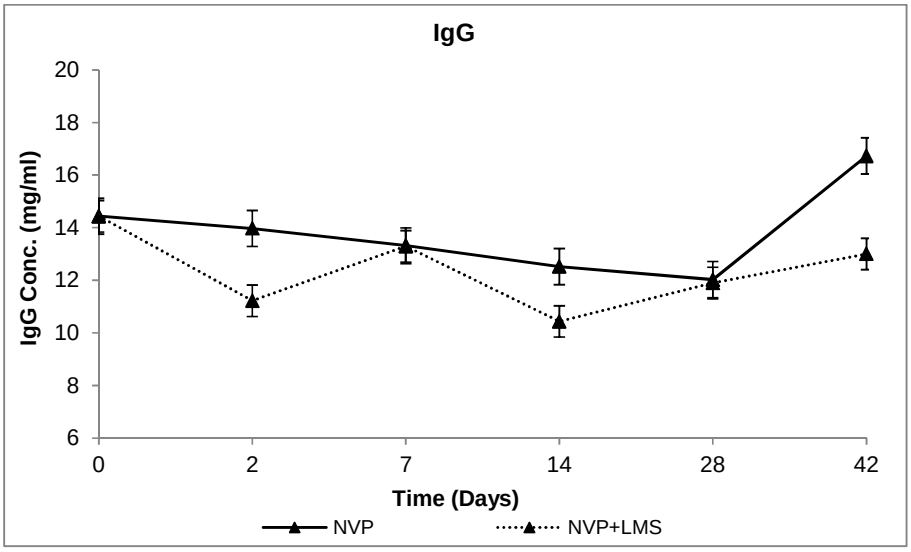

Figure 7.14 b): IgG concentrations of the NVP and NVP+LMS groups over 42 days

7.3.9 Main observations

- From the histopathology, co-treatment with levamisole caused liver injury up to day 14 and by day 28 it started to improve.
- Nevirapine concentrations increased up to day 7 and were lower than with nevirapine alone thereafter.
- IL-10 concentrations increased up to day 7, and were lower than with nevirapine alone from day 14 and onwards.
- The CD4 and CD8 counts increased up to day 14 and thereafter were similar to nevirapine alone.

C. Phase III: Co-treatment with a CYP450 inducer

7.3.10 Physiological observations (function tests)

7.3.10.1 Full blood count

Table 7.22 shows results of the full blood count of the S, S+CBZ, NVP and NVP+CBZ groups. The changes of red blood count parameters as observed for concomitant nevirapine and carbamazepine treatment, are similar to those of nevirapine alone (Section 7.3.1.1), and no statistical differences were observed between the two groups. For white blood count parameters no statistical differences were seen in the NVP+CBZ group, nor between the two groups.

Table 7.22: Average (mean ± SD) full blood count and platelets results of the S, S+CBZ, NVP and NVP+CBZ groups

Group (n = 3)	RCC ($x10^{12}$/l)	Hb (g/dl)	Hct (l/l)	MCV (fl)	MCH (pg)	MCHC (g/dl)	Plt ($x10^9$/l)	WCC ($x10^9$/l)	Neu ($x10^9$/l)	Ly ($x10^9$/l)	Mo ($x10^9$/l)	Eos ($x10^9$/l)	Bas ($x10^9$/l)
Untreated													
0 Days	6.28±0.2	12.9±0.3	0.398±0.01	63.5±2.5	20.5±0.4	32.3±0.8	860±221.1	6.95±2.7	0.77±0.2	4.67±1.8	0.19±0.1	0.02±0.0	0.00±0.0
S													
2 Days	6.67±0.2	13.7±0.1	0.422±0.01	63.3±2.3	20.6±0.6	32.4±0.6	849±81.6	6.50±0.9	0.60±0.2	5.18±0.7	0.21±0.0	0.50±0.2	0.01±0.0
7 Days	7.53±0.9	15.3±1.7	0.451±0.04	60.1±2.5	20.3±0.2	33.9±1.2	1033±79.8	5.44±2.4	1.03±0.8	4.07±2.0	0.30±0.3	0.04±0.0	0.01±0.0
14 Days	6.77±0.6	13.9±1.1	0.417±0.03	61.8±2.8	20.5±0.6	33.2±0.6	721±196.4	5.22±1.2	0.63±0.5	4.21±0.7	0.18±0.1	0.18±0.1	0.05±0.0
28 Days	7.07±0.7	13.9±1.3	0.390±0.04	55.1±1.0	19.7±0.1	35.8±0.6	961±172.5	7.38±1.0	0.91±0.2	6.15±0.8	0.24±0.1	0.07±0.0	0.01±0.0
42 Days	6.93±0.8	13.4±1.8	0.374±0.05	53.9±1.0	19.3±0.4	35.8±0.2	839±166.0	3.93±0.3	0.54±0.1	3.23±0.3	0.11±0.0	0.04±0.0	0.01±0.0
S+CBZ													
2 Days	6.74±0.2	13.2±0.1	0.406±0.00	60.4±2.0	19.6±0.6	28.8±6.5	953±95.9	6.48±1.0	0.51±0.0	5.46±1.1	0.24±0.3	0.02±0.0	0.01±0.0
7 Days	6.62±0.4	13.1±0.6	0.414±0.02	62.5±1.3	19.8±0.4	31.7±0.2	779±187.7	7.32±2.4	0.59±0.2	6.20±2.1	0.27±0.1	0.27±0.3	0.01±0.0
14 Days	7.32±0.1	14.7±0.3	0.442±0.01	60.4±2.8	20.1±0.5	33.3±0.8	861±149.2	8.41±0.7	0.76±0.0	7.28±0.5	0.33±0.2	0.05±0.0	0.01±0.0
28 Days	7.27±0.9	14.5±1.7	0.424±0.05	58.4±0.8	19.9±0.3	34.1±0.9	637±79.9	5.48±1.3	0.84±0.3	4.32±1.0	0.23±0.3	0.08±0.0	0.00±0.0
42 Days	7.90±0.1	15.4±0.4	0.451±0.01	57.1±1.4	19.5±0.4	34.2±0.2	727±48.7	6.47±0.6	0.66±0.1	5.55±0.5	0.21±0.0	0.05±0.0	0.01±0.0
NVP													
2 Days	6.51±0.2	13.6±0.4	0.408±0.01	62.7±0.8	20.9±0.1	33.2±0.3	854±209.3	3.48±0.8	1.02±0.1	1.88±0.3	0.10±0.0	0.48±0.4	0.00±0.0
7 Days	7.14±0.3	14.2±0.5	0.424±0.02	59.5±1.8	19.9±0.6	33.5±0.5	779±141.3	5.54±1.3	0.76±0.3	3.81±0.9	0.49±0.2	0.48±0.2	0.01±0.0
14 Days	6.97±0.3	13.9±0.4	0.415±0.01	59.5±2.2	19.9±0.6	33.5±0.3	1013±73.0	4.86±0.7	1.08±0.2	3.38±0.4	0.37±0.1	0.02±0.0	0.01±0.0
28 Days	7.36±0.6	14.2±1.1	0.404±0.03	55.0±0.7	19.3±0.3	35.0±0.6	876±17.2	6.07±0.6	1.09±0.2	4.62±0.7	0.28±0.2	0.08±0.1	0.01±0.0
42 Days	7.73±0.3	14.5±0.3	0.405±0.00	52.5±2.3	18.8±0.5	35.8±0.8	1052±115.6	5.42±0.1	1.52±0.6	3 .52±0.6	0.33±0.0	0.04±0.0	0.01±0.0
NVP+CBZ													
2 Days	6.25±0.3	12.8±0.7	0.395±0.02	63.1±0.6	20.4±0.1	32.3±0.2	893±35.5	4.99±1.5	1.91±0.6	2.80±0.7	0.26±0.2	0.02±0.0	0.00±0.0
7 Days	6.73±0.2	13.4±0.4	0.427±0.01	63.5±1.7	19.9±0.3	31.4±1.0	838±126.5	4.65±0.7	0.97±0.3	3.23±0.8	0.42±0.0	0.01±0.0	0.01±0.0
14 Days	7.32±0.3	14.2±0.6	0.426±0.02	58.2±0.9	19.3±0.4	33.3±0.8	851±32.3	5.77±1.1	0.83±0.1	4.54±0.9	0.28±0.2	0.12±0.2	0.00±0.0
28 Days	7.26±0.2	14.6±0.6	0.429±0.02	59.1±1.4	20.1±0.4	34.3±1.1	732±97.2	5.36±1.2	1.04±0.4	4.10±0.7	0.20±0.2	0.02±0.0	0.00±0.0
42 Days	7.88±0.3	14.7±0.9	0.407±0.02	51.6±1.5	18.6±0.9	36.1±0.8	900±131.9	5.29±0.8	1.50±0.5	3.20±0.9	0.34±0.1	0.24±0.4	0.01±0.0

RCC = red cell count; Hb = haemoglobin; Hct = haematocrit; MCV = mean corpuscular volume; MCH = mean corpuscular haemoglobin; MCHC = mean corpuscular haemoglobin concentration; Plt = platelets; WCC = white cell count; Neu = neutrophils; Ly = lymphocytes; Mo = monocytes; Eos = eosinophils; Bas = basophils; S = saline; CBZ = carbamazepine; NVP = nevirapine

7.3.10.2 Renal function tests

Table 7.23 shows the changes of BUN and Cr of the S, S+CBZ, NVP and NVP+CBZ groups. In all groups BUN and Cr levels were normal. Here, Cr levels also spiked on day 28, but were still within the normal range (p = 0.0500).

Table 7.23: Average (mean ± SD) renal function test results of the S, S+CBZ, NVP and NVP+CBZ groups

Group	**RFT**		**Group**	**RFT**	
	BUN	**Cr**		**BUN**	**Cr**
(n = 3)	**(mmol/l)**	**(µmol/l)**	**(n = 3)**	**(mmol/l)**	**(µmol/l)**
Untreated					
0 Days	7.2 ± 1	37 ± 8			
S			**NVP**		
2 Days	7.3 ± 1	39 ± 2	2 Days	7.9 ± 1	36 ± 1
7 Days	8.1 ± 0	46 ± 7	7 Days	8.7 ± 0	46 ± 2
14 Days	7.5 ± 1	39 ± 3	14 Days	9.1 ± 1	41 ± 9
28 Days	10.6 ± 2	73 ± 17	28 Days	8.5 ± 1	63 ± 7
42 Days	5.8 ± 1	38 ± 9	42 Days	7.0 ± 0	27 ± 3
S+CBZ			**NVP+CBZ**		
2 Days	6.5 ± 0	36 ± 2	2 Days	7.2 ± 1	37 ± 2
7 Days	6.8 ± 1	33 ± 6	7 Days	7.1 ± 1	30 ± 7
14 Days	6.4 ± 1	36 ± 6	14 Days	7.8 ± 0	35 ± 3
28 Days	7.2 ± 1	64 ± 12	28 Days	8.0 ± 1	64 ± 11
42 Days	7.2 ± 1	8 ± 6	42 Days	8.0 ± 1	18 ± 5

RFT = renal function test; BUN = blood urea nitrogen; Cr = creatinine; S = saline; NVP = nevirapine; CBZ = carbamazepine

7.3.10.3 Liver function tests

Table 7.24 shows the changes of ALT, AST and ALP of the S, S+CBZ, NVP and NVP+CBZ groups. Over the 42 days, the results were similar in all groups.

Table 7.24: Average (mean ± SD) liver function test results of the S, S+CBZ, NVP and NVP+CBZ groups

Group	LFT			Group	LFT		
	ALT	AST	ALP		ALT	AST	ALP
(n = 3)	(U/l)	(U/l)	(U/l)	(n = 3)	(U/l)	(U/l)	(U/l)
Untreated							
0 Days	50±5	88±14	352±76				
S				**NVP**			
2 Days	46±2	90±7	400±7	2 Days	63±7	107±10	359±43
7 Days	49±10	103±25	304±13	7 Days	87±36	169±115	447±78
14 Days	58±4	127±37	508±37	14 Days	72±3	109±33	443±43
28 Days	47±2	115±44	216±19	28 Days	53±4	128±44	166±37
42 Days	46±6	76±28	109±76	42 Days	54±2	70±4	14±9
S+CBZ				**NVP+CBZ**			
2 Days	52±4	90±12	332±18	2 Days	54±10	146±40	399±67
7 Days	43±6	86±8	341±28	7 Days	57±13	135±10	316±15
14 Days	47±2	85±2	356±1	14 Days	48±4	94±18	264±18
28 Days	52±5	97±7	152±78	28 Days	53±3	91±15	171±22
42 Days	45±2	91±20	104±74	42 Days	57±7	123±65	151±30

LFT = liver function test; ALT = alanine aminotransferase; AST = aspartate aminotransferase; ALP = alkaline phosphatase; S = saline; NVP = nevirapine; CBZ = carbamazepine

7.3.10.4 Liver histopathology

(a) Liver histopathology reports

Liver sections for histopathology (Figures 6.15 a – j and 7.15 a – j) were randomly selected and the main histopathology lesions are summarised in the tally tables (Tables 7.25 a and b). The following report is a summary of the features of the lesion:

i. Figures 6.15 a and b: Liver sections A and B from the S+CBZ group after 2 days of saline and carbamazepine co-treatment

The report is the same as in Chapter 6, Section 6.3.10.4 i.

ii. Figures 6.15 c and d: Liver sections A and B from the S+CBZ group after 7 days of saline and carbamazepine co-treatment

The report is the same as in Chapter 6, Section 6.3.10.4 ii.

iii. Figures 6.15 e and f: Liver sections A and B from the S+CBZ group after 14 days of saline and carbamazepine co-treatment

The report is the same as in Chapter 6, Section 6.3.10.4 iii.

iv. Figures 6.15 g and h: Liver sections A and B from the S+CBZ group after 28 days of saline and carbamazepine co-treatment

The report is the same as in Chapter 6, Section 6.3.10.4 iv.

v. Figures 6.15 i and j: Liver sections A and B from the S+CBZ group after 42 days of saline and carbamazepine co-treatment

The report is the same as in Chapter 6, Section 6.3.10.4 v.

vi. Figures 7.15 a and b: Liver sections A and B from the NVP+CBZ group after 2 days of nevirapine and carbamazepine co-treatment

Representative photographs of rat livers, after 2 days of daily nevirapine and carbamazepine co-treatment. The report: “In liver section A the liver parenchyma reveals minimal vacuolar degeneration as well as minimal cytonecrosis. A moderate degree of mitosis could be confirmed within the hepatic parenchyma. Degenerative changes and cytonecrosis are minimal, while a mitotic figure in metaphase is visible in liver section B.”

vii. Figures 7.15 c and d: Liver sections A and B from the NVP+CBZ group after 7 days of nevirapine and carbamazepine co-treatment

Representative photographs of rat livers, after 7 days of daily nevirapine and carbamazepine co-treatment. The report: “The hepatic parenchymal cells show minimal vacuolar degeneration and cytonecrosis in liver section A. Moderate granular vacuolar degeneration and cell swelling as well as mild cytonecrosis are present within the parenchyma of liver section B.”

viii. Figures 7.15 e and f: Liver sections A and B from the NVP+CBZ group after 14 days of nevirapine and carbamazepine co-treatment

Representative photographs of rat livers, after 14 days of daily nevirapine and carbamazepine co-treatment. The report: “Mild to moderate cellular swelling and granular vacuolar degeneration were present in both liver sections A and B. Cytonecrosis was graded as mild, while there was no centrilobular zonal necrosis or hepatocyte present.”

ix. Figures 7.15 g and h: Liver sections A and B from the NVP+CBZ group after 28 days of nevirapine and carbamazepine co-treatment

Representative photographs of rat livers, after 28 days of daily nevirapine and carbamazepine co-treatment. The report: “Granular vacuolar degeneration and cell swelling were mild in both liver sections A and B, as was cytonecrosis. No centrilobular zonal necrosis or hepatocyte mitosis was observed.”

x. Figures 7.15 i and j: Liver sections A and B from the NVP+CBZ group after 42 days of nevirapine and carbamazepine co-treatment

Representative photographs of rat livers, after 42 days of daily nevirapine and carbamazepine co-treatment. The report: “The degeneration and cytonecrosis are graded as mild in the parenchyma of liver section A. In liver section B, vacuolar degeneration and cell swelling are moderate, while mild single cell necrosis as well as minimal centrilobular necrosis could be detected.”

In view of the histopathology photographs (Figures 6.15 a – j and 7.15 a – j), reports and tally tables (Tables 7.25 a and b), it appeared that co-treatment with nevirapine and carbamazepine caused a lesser degree of liver injury than with nevirapine alone.

(b) Liver histopathology photographs

Histopathology photographs of the S+CBZ group (Figures 6.15 a – j) are presented in Chapter 6, Section 6.3.10.4 b. Figures 7.15 a – j are representative of randomly selected liver sections of the NVP+CBZ group.

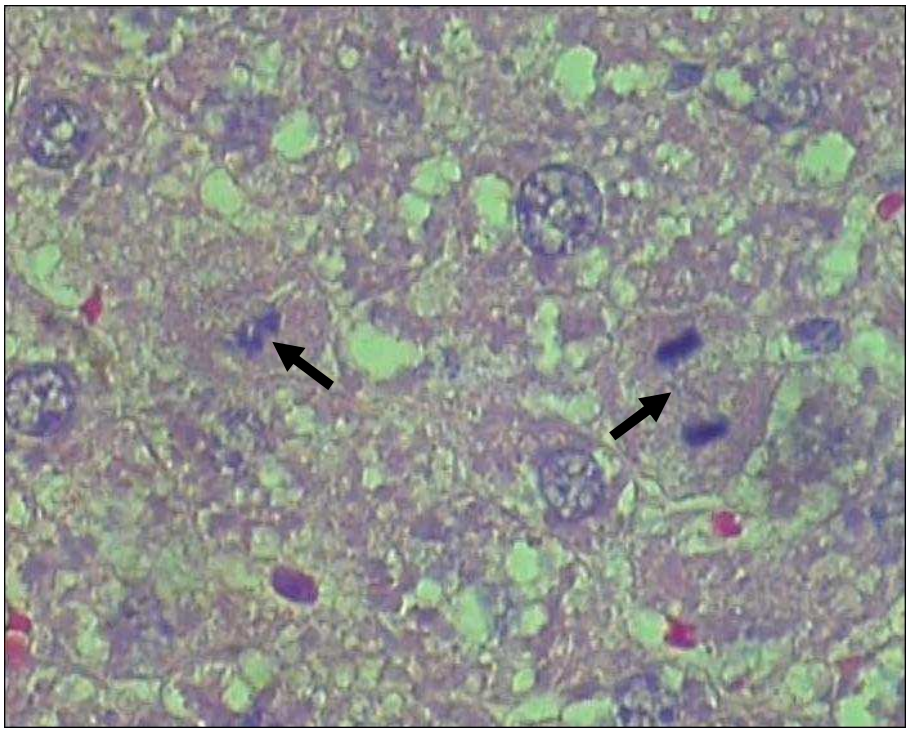

Figure 7.15 a): Liver section A from the NVP+CBZ group after 2 days of treatment, showing minimal vacuolar degeneration, minimal cytonecrosis, and a moderate degree of mitosis within the hepatic parenchyma

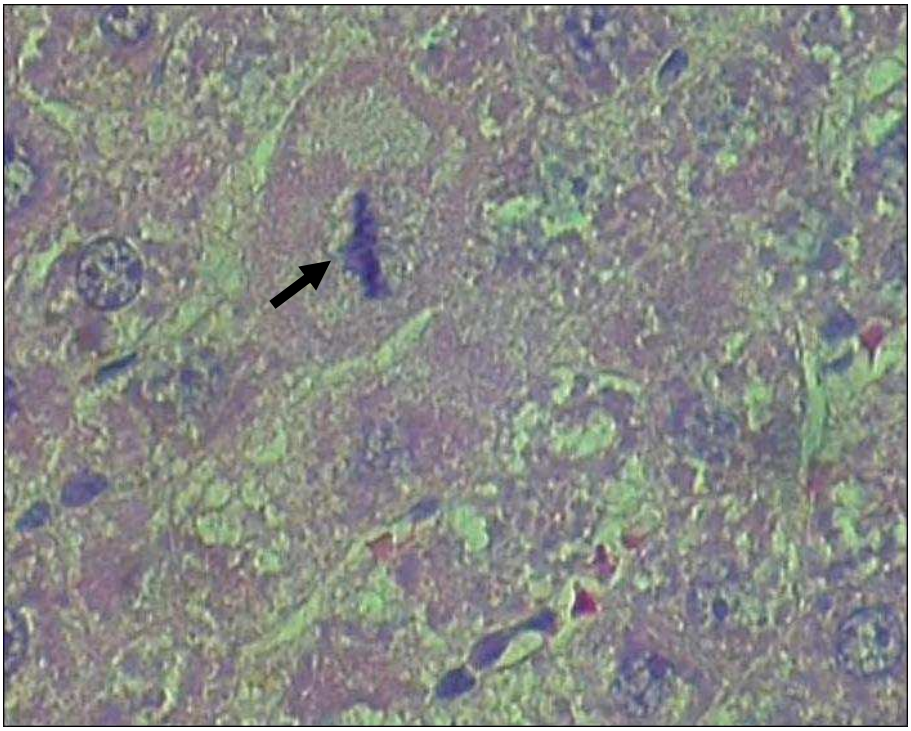

Figure 7.15 b): Liver section B from the NVP+CBZ group after 2 days of treatment, showing minimal degenerative changes and cytonecrosis, and a mitotic figure in metaphase

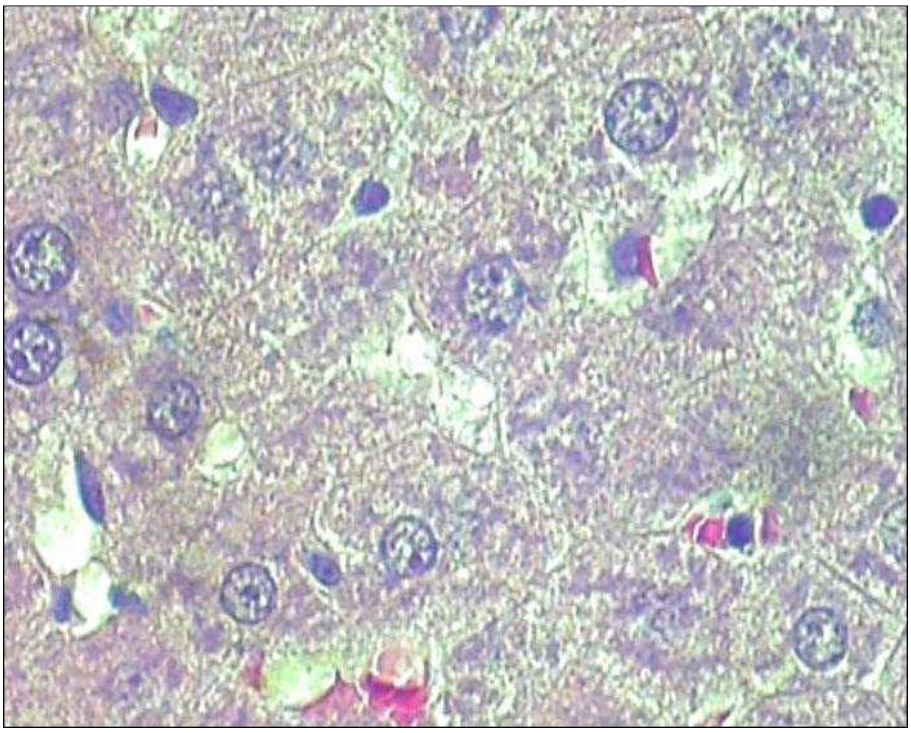

Figure 7.15 c): Liver section A from the NVP+CBZ group after 7 days of treatment, showing hepatic parenchymal cells with minimal vacuolar degeneration and cytonecrosis

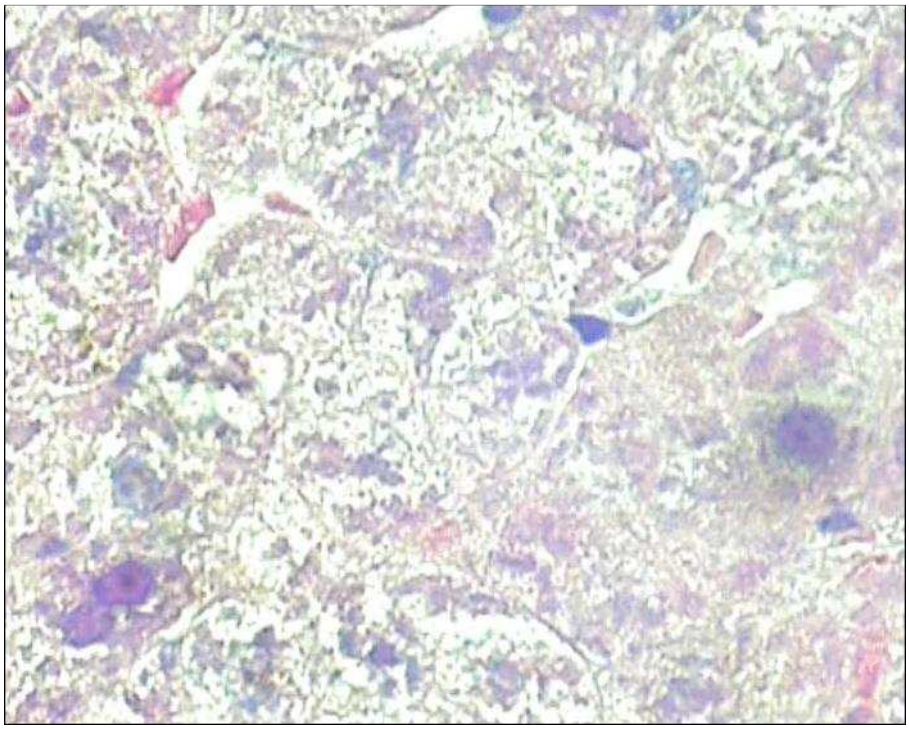

Figure 7.15 d): Liver section B from the NVP+CBZ group after 7 days of treatment, showing moderate granular vacuolar degeneration and cell swelling, as well as mild cytonecrosis

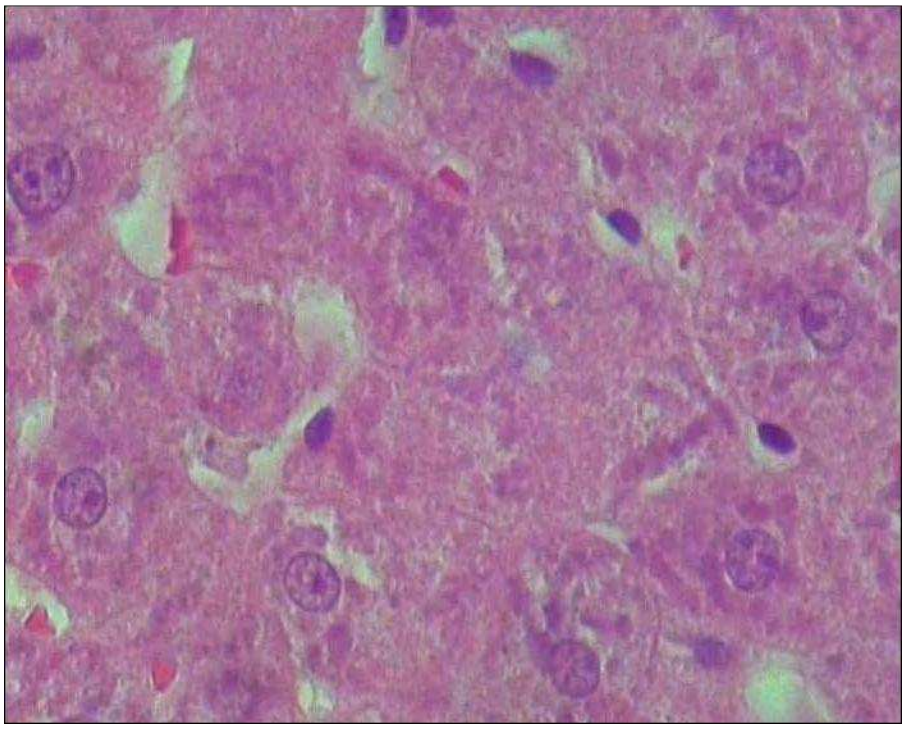

Figure 7.15 e): Liver section A from the NVP+CBZ group after 14 days of treatment, showing hepatic parenchymal cells with moderate vacuolar degeneration, cell swelling and mild cytonecrosis

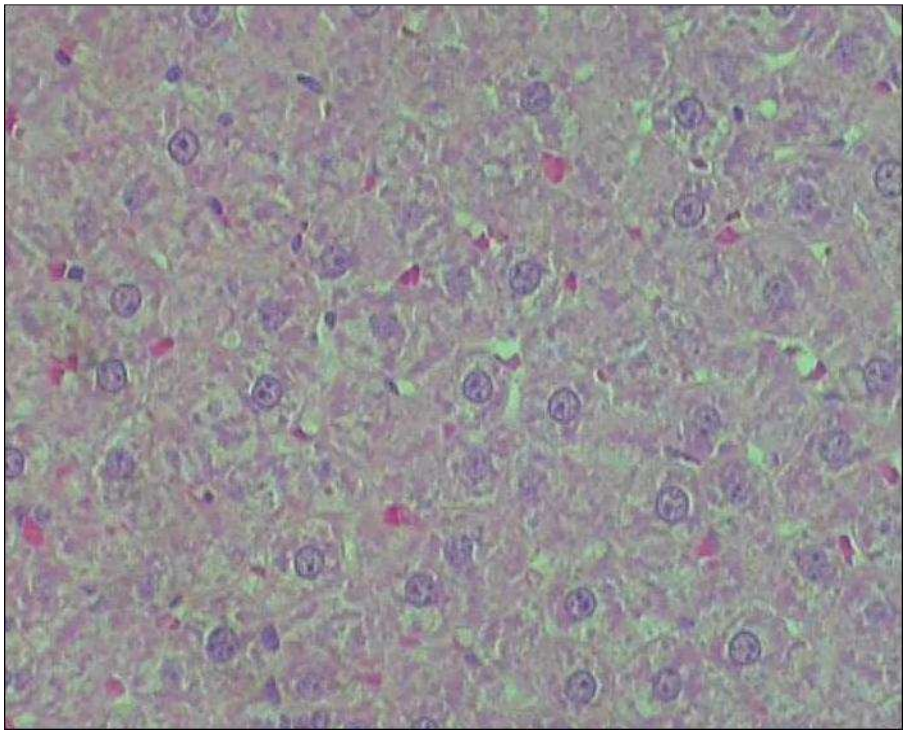

Figure 7.15 f): Liver section B from the NVP+CBZ group after 14 days of treatment, showing mild vacuolar degeneration with cell swelling, as well as mild cell necrosis, and some poorly stained nuclei suggesting cytonecrosis

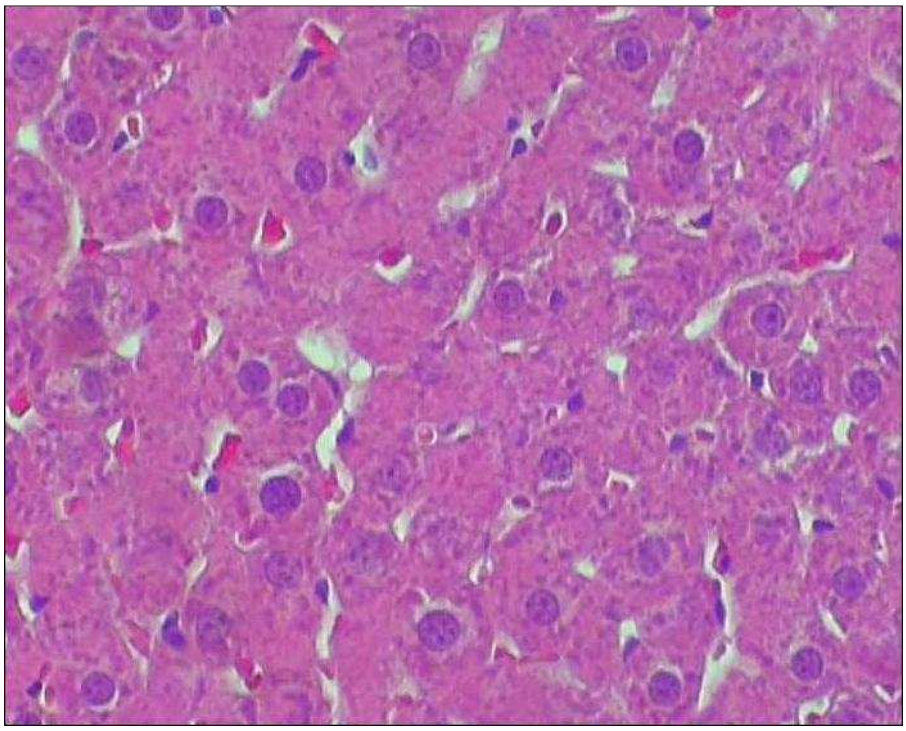

Figure 7.15 g): Liver section A from the NVP+CBZ group after 28 days of treatment, showing visible liver cords, mild degeneration and cytonecrosis

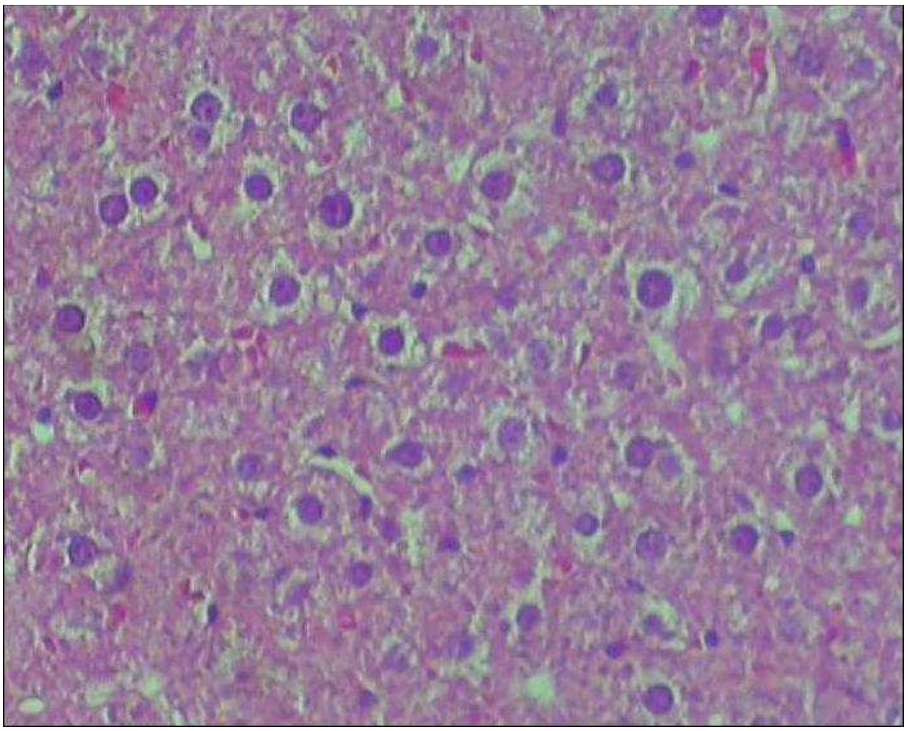

Figure 7.15 h): Liver section B from the NVP+CBZ group after 28 days of treatment, showing mild vacuolar degeneration with cell swelling and mild cytonecrosis in the liver parenchyma

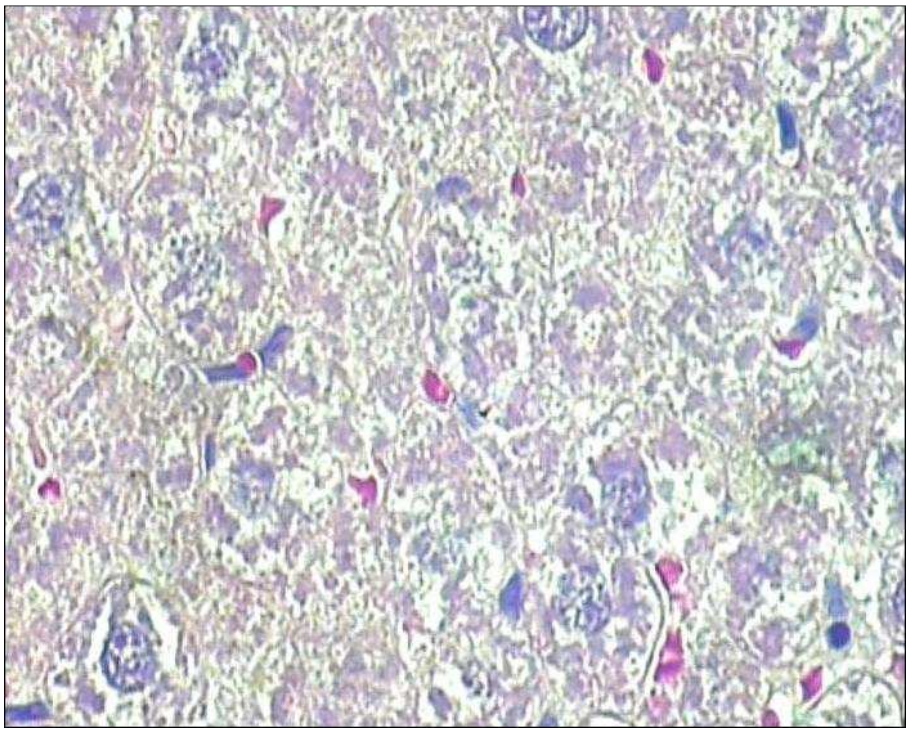

Figure 7.15 i): Liver section A from the NVP+CBZ group after 42 days of treatment, showing mild degeneration and cytonecrosis in the parenchyma

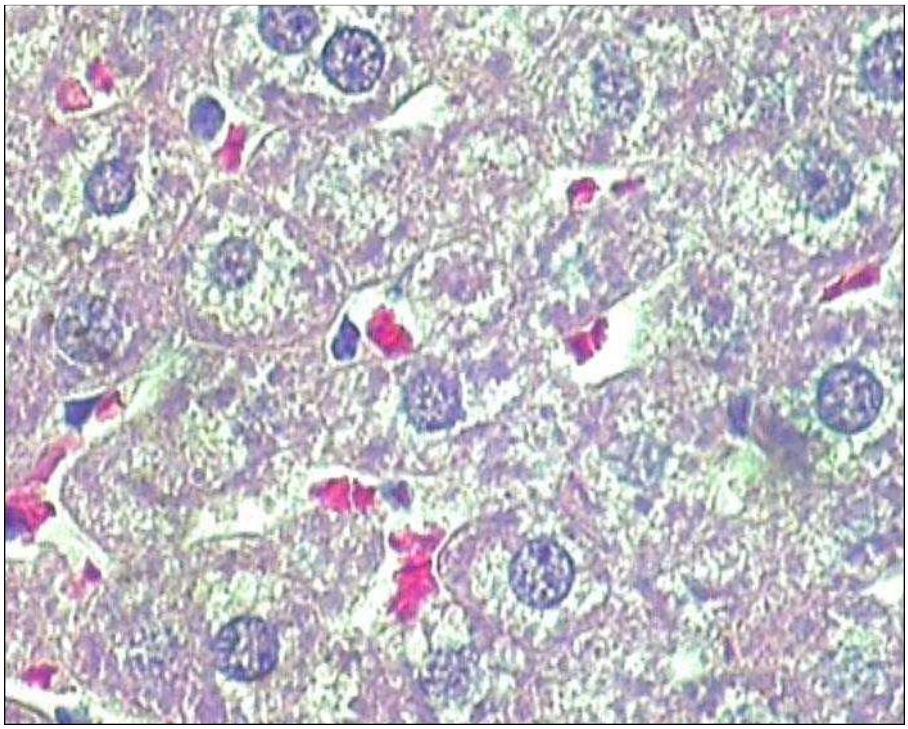

Figure 7.15 j): Liver section B from the NVP+CBZ group after 42 days of treatment, showing moderate vacuolar degeneration and cell swelling, mild single cell necrosis and minimal centrilobular necrosis

Table 7.25 a): Tally of main pathology lesions (lesions score) in livers of untreated rats and the S+CBZ group

Group	UnRx	S+CBZ									
		2 Days		7 Days		14 Days		28 Days		42 Days	
(n = 2)	Fig.6.3a	Fig.6.15a	Fig.6.15b	Fig.6.15c	Fig.6.15d	Fig.6.15e	Fig.6.15f	Fig.6.15g	Fig.6.15h	Fig.6.15i	Fig.6.15j
Histopathological lesions											
Vacuolar degeneration	0	0	1+	1+	1+	2+	2+	1+	1+	1+	1+
Cell swelling	0	0	1+	1+	1+	2+	2+	1+	1+	1+	1+
Cytonecrosis	0	0	0	0	0	2+	2+	0	1+	1+	1+
Centrilobular necrosis	0	0	0	0	0	0	0	0	0	0	0
Hepatocyte mitosis	0	0	0	0	0	0	0	0	0	0	0
Average lesions score											
Vacuolar degeneration	0	0.5+		1+		2+		1+		1+	
Cell swelling	0	0.5+		1+		2+		1+		1+	
Cytonecrosis	0	0		0		2+		0.5+		1+	
Centrilobular necrosis	0	0		0		0		0		0	
Hepatocyte mitosis	0	0		0		0		0		0	
Total lesion score	**0**	**1+**		**2+**		**6+**		**2.5+**		**3+**	

UnRx = untreated; S = saline; CBZ = carbamazepine

Table 7.25 b): Tally of main pathology lesions (lesions score) in livers of untreated rats and the NVP+CBZ group

Group	UnRx	NVP+CBZ									
		2 Days		7 Days		14 Days		28 Days		42 Days	
(n = 2)	Fig.6.3a	Fig.7.15a	Fig.7.15b	Fig.7.15c	Fig.7.15d	Fig.7.15e	Fig.7.15f	Fig.7.15g	Fig.7.15h	Fig.7.15i	Fig.7.15j
Histopathological lesions											
Vacuolar degeneration	0	1+	1+	1+	3+	3+	2+	2+	2+	2+	3+
Cell swelling	0	1+	1+	1+	3+	3+	2+	2+	2+	2+	3+
Cytonecrosis	0	1+	1+	1+	2+	2+	2+	2+	2+	2+	2+
Centrilobular necrosis	0	0	0	0	0	0	0	0	0	0	1+
Hepatocyte mitosis	0	3+	2+	0	0	0	0	0	0	0	0
Average lesions score											
Vacuolar degeneration	0	1+		2+		2.5+		2+		2.5+	
Cell swelling	0	1+		2+		2.5+		2+		2.5+	
Cytonecrosis	0	1+		1.5+		2+		2+		2+	
Centrilobular necrosis	0	0		0		0		0		0.5+	
Hepatocyte mitosis	0	2.5+		0		0		0		0	
Total lesion score	**0**	**5.5+**		**5.5+**		**7+**		**6+**		**7.5+**	

UnRx = untreated; NVP = nevirapine; CBZ = carbamazepine

7.3.11 Nevirapine concentrations

Table 7.26 shows nevirapine concentrations of the NVP and NVP+CBZ groups, while Figure 7.16 is a graphical illustration of the same. For the NVP+CBZ group, nevirapine levels showed some fluctuations, but were consistently lower than in the NVP group until day 28 (p = 0.0476).

Table 7.26: Average (mean ± SD) nevirapine concentrations of the NVP and NVP+CBZ groups

Group	NVP	NVP+CBZ
	NVP concentration	NVP concentration
(n = 5)	(µg/ml)	(µg/ml)
2 Days	4.292 ± 2.69	0.353 ± 0.17
7 Days	2.008 ± 0.67	0.769 ± 0.06
14 Days	1.983 ± 0.58	0.851 ± 0.74
28 Days	1.721 ± 3.04	0.195 ± 0.39
42 Days	0.497 ± 1.11	0.729 ± 1.38

NVP = nevirapine; CBZ = carbamazepine

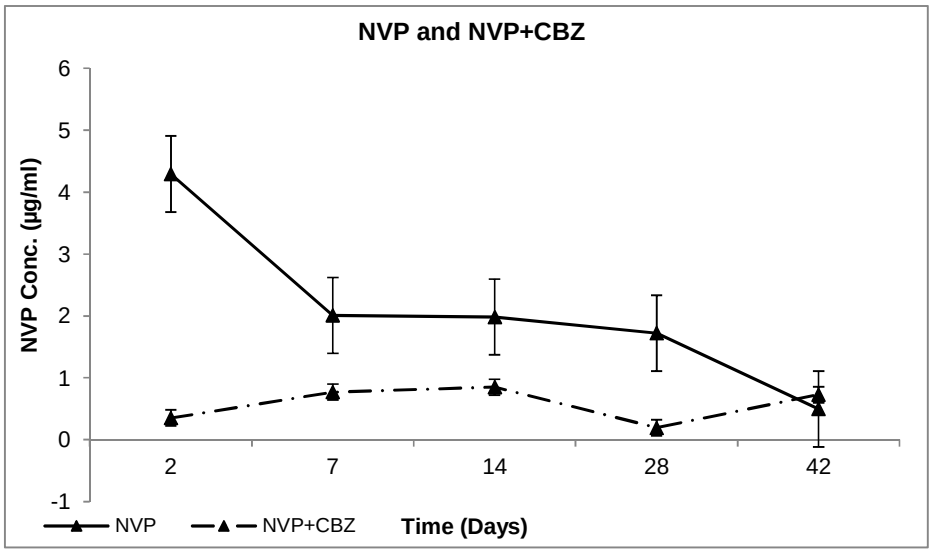

Figure 7.16: Nevirapine concentrations of the NVP and NVP+CBZ groups over 42 days

7.3.12 Specific immunology tests

7.3.12.1 Direct observations

Table 6.27 shows changes in body weight of the S, S+CBZ, NVP and NVP+CBZ groups over the 42 day treatment period. All groups showed weight gain at all times, except for the S+CBZ group after 2 days (Refer to Appendix H-1 and H-3 for baseline weights).

Table 7.27: Average (mean ± SD) change in rat weights of the S, S+CBZ, NVP and NVP+CBZ groups

Group (n = 5)	S change in weight (g)	S+CBZ change in weight (g)	NVP change in weight (g)	NVP+CBZ change in weight (g)
2 Days	9.2±4	-3.9±2	10.6±8	8.2±2
7 Days	35.7±8	34.0±4	28.5±1	11.0±11
14 Days	84.6±5	66.2±4	82.8±2	51.7±8
28 Days	107.8±10	97.3±30	99.7±16	87.3±21
42 Days	171.4±27	142.5±18	105.6±16	101.0±23

S = saline; CBZ = carbamazepine; NVP = nevirapine

7.3.12.2 Cytokines

Table 7.28 shows IL-2 and IL-10 concentrations of the S, S+CBZ, NVP and NVP+CBZ groups, while Figures 7.17 a – b are graphical illustrations of the same. By day 42 IL-2 levels were elevated (p = 0.0500), and also higher than in the NVP group (p = 0.0500). IL-10 concentrations increased up to day 14, but this carried no statistical significance. Although IL-10 concentrations were mostly higher in the NVP group, this was not statistically different from the NVP+CBZ group.

Table 7.28: Average (mean ± SD) cytokine concentrations of the S, S+CBZ, NVP and NVP+CBZ groups

Group	Cytokine		Group	Cytokine	
(n = 3)	IL-2 (pg/ml)	IL-10 (pg/ml)	(n = 3)	IL-2 (pg/ml)	IL-10 (pg/ml)
Untreated					
0 Days	65.46 ± 2.0	31.08 ± 1.2			
S			**NVP**		
2 Days	74.87 ± 6.5	29.96 ± 2.8	2 Days	76.55 ± 1.2	34.77 ± 2.5
7 Days	77.26 ± 5.8	34.57 ± 0.7	7 Days	82.21 ± 4.6	36.63 ± 3.6
14 Days	77.58 ± 6.6	35.69 ± 5.4	14 Days	73.07 ± 7.9	34.87 ± 6.8
28 Days	78.81 ± 4.6	32.46 ± 4.2	28 Days	71.82 ± 10.6	39.64 ± 3.5
42 Days	74.39 ± 5.7	32.03 ± 2.5	42 Days	61.92 ± 4.5	41.89 ± 6.5
S+CBZ			**NVP+CBZ**		
2 Days	52.33 ± 14.6	37.31 ± 2.3	2 Days	70.50 ± 3.5	34.12 ± 3.5
7 Days	112.50 ± 7.8	40.11 ± 4.7	7 Days	62.50 ± 7.8	36.12 ± 4.2
14 Days	120.83 ± 12.8	40.11 ± 3.1	14 Days	91.17 ± 27.8	37.55 ± 6.2
28 Days	117.75 ± 23.7	40.67 ± 6.0	28 Days	94.17 ± 48.8	31.47 ± 4.9
42 Days	92.50 ± 46.0	36.00 ± 4.9	42 Days	147.00 ± 4.2	31.69 ± 6.2

IL = interleukin; S = saline; NVP = nevirapine; CBZ = carbamazepine

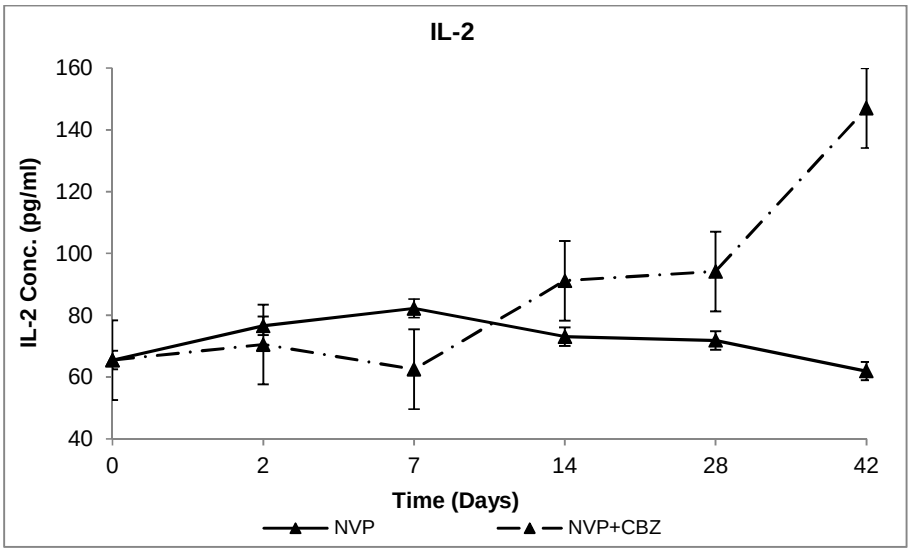

Figure 7.17 a): IL-2 concentrations of the NVP and NVP+CBZ groups over 42 days

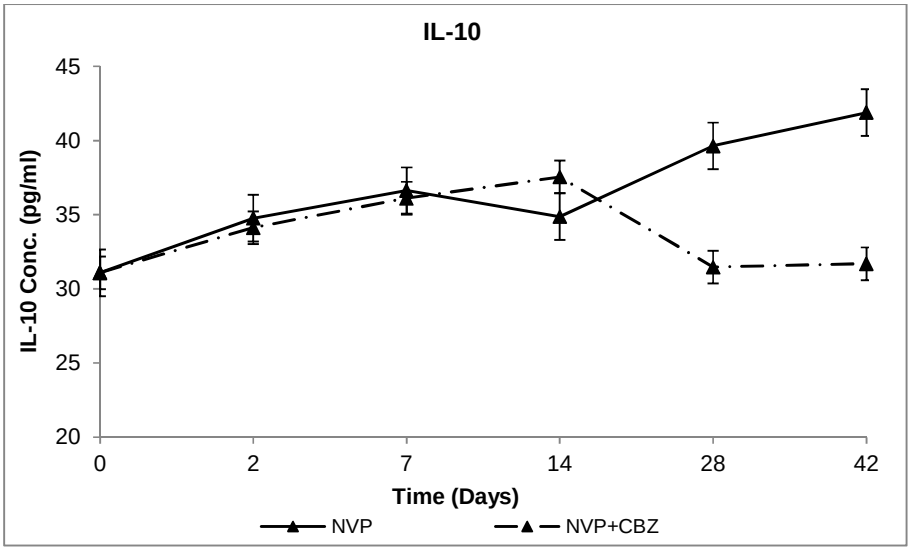

Figure 7.17 b): IL-10 concentrations of the NVP and NVP+CBZ groups over 42 days

7.3.12.3 CD4 and CD8 counts

Table 7.29 shows CD4 and CD8 counts of the S, S+CBZ, NVP and NVP+CBZ groups, while Figures 7.18 a – b are graphical illustrations of the same. In the NVP+CBZ group, the CD4 and CD8 counts were normal. Although the CD4 and CD8 counts in the NVP group were higher than in the NVP+CBZ group on days 7 and 28, this was not statistically different.

Table 7.29: Average (mean ± SD) CD4 and CD8 counts of the S, S+CBZ, NVP and NVP+CBZ groups

Group	**Ly**	**T-Ly**		**Group**	**Ly**	**T-Ly**	
		CD4	**CD8**			**CD4**	**CD8**
(n = 3)	**(x10^9/l)**	**(x10^9/l)**	**(x10^9/l)**	**(n = 3)**	**(x10^9/l)**	**(x10^9/l)**	**(x10^9/l)**
Untreated							
0 Days	4.67±1.8	2.23±1.3	1.42±0.7				
S				**NVP**			
2 Days	5.18±0.7	2.27±0.3	1.35±0.2	2 Days	1.88±0.3	1.07±0.2	0.59±0.1
7 Days	4.07±2.0	1.72±0.8	1.07±0.5	7 Days	3.81±0.9	1.58±0.3	1.07±0.3
14 Days	4.21±0.7	1.69±0.2	1.17±0.2	14 Days	3.38±0.4	1.43±0.1	0.85±0.2
28 Days	6.15±0.8	2.45±0.2	1.58±0.3	28 Days	4.62±0.7	1.78±0.4	1.26±0.2
42 Days	3.23±0.3	1.47±0.1	0.79±0.2	42 Days	3.52±0.6	1.23±0.2	0.81±0.2
S+CBZ				**NVP+CBZ**			
2 Days	5.46±1.1	1.96±0.3	1.39±0.1	2 Days	2.80±0.7	1.40±0.3	0.79±0.2
7 Days	6.20±2.1	2.33±0.8	1.41±0.4	7 Days	3.23±0.8	1.37±0.3	0.75±0.2
14 Days	7.28±0.5	2.70±0.2	1.59±0.2	14 Days	4.54±0.9	1.57±0.3	0.90±0.2
28 Days	4.32±1.0	1.79±0.4	0.00±0.0	28 Days	4.10±0.7	1.45±0.1	0.91±0.0
42 Days	5.55±0.5	1.73±0.2	1.54±0.2	42 Days	3.20±0.9	1.21±0.2	0.78±0.3

Ly = lymphocytes; CD = cluster of differentiation; S = saline; NVP = nevirapine; CBZ = carbamazepine

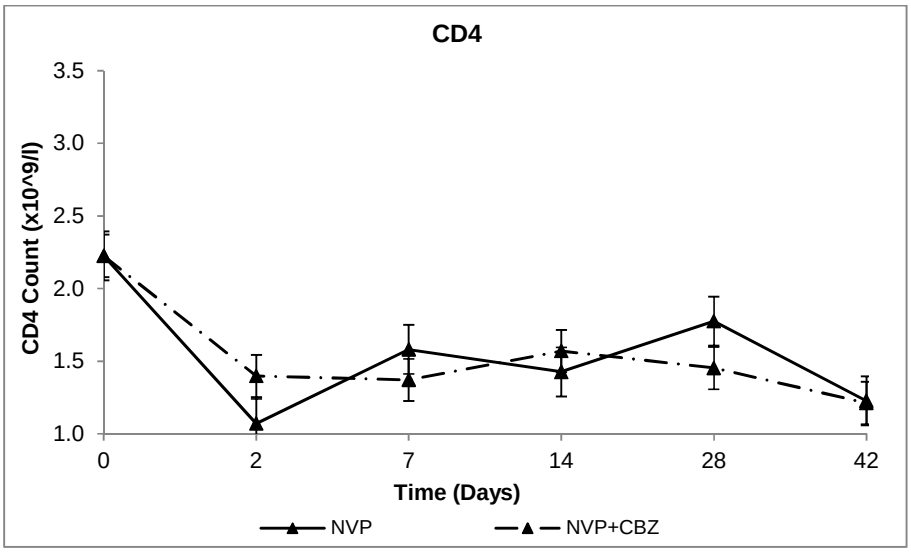

Figure 7.18 a): CD4 counts of the NVP and NVP+CBZ groups over 42 days

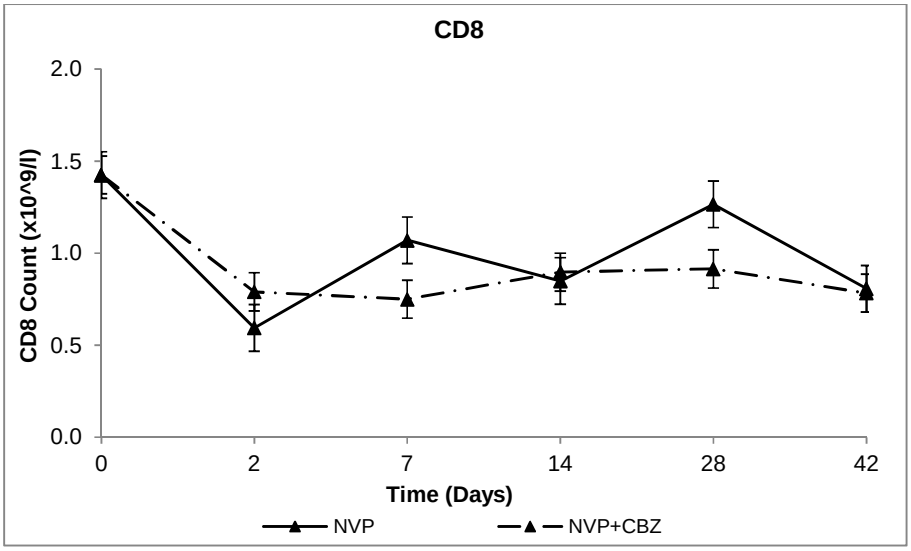

Figure 7.18 b): CD8 counts of the NVP and NVP+CBZ groups over 42 days

7.3.12.4 Immunoglobulins

Table 7.30 shows concentrations of IgM and IgG of the S, S+CBZ, NVP and NVP+CBZ groups, while Figures 7.19 a – b are graphical illustrations of the same. In the NVP+CBZ group, IgM concentrations increased up to day 7 (p = 0.0500), and this was higher than in the NVP group on the same day (p = 0.0500). IgG concentrations were low in the NVP+CBZ group on day 7 (p = 0.0500), and lower than in the NVP group on all occasions (p = 0.0500).

Table 7.30: Average (mean ± SD) immunoglobulin concentrations of the S, S+CBZ, NVP and NVP+CBZ groups

Group	**Immunoglobulin**		**Group**	**Immunoglobulin**	
	IgM	**IgG**		**IgM**	**IgG**
(n = 3)	**(mg/ml)**	**(mg/ml)**	**(n = 3)**	**(mg/ml)**	**(mg/ml)**
Untreated					
0 Days	0.109±0.02	14.434±1.10			
S			**NVP**		
2 Days	0.104±0.04	14.137±0.91	2 Days	0.011±0.01	13.971±1.63
7 Days	0.110±0.04	14.302±0.70	7 Days	0.012±0.00	13.311±0.57
14 Days	0.110±0.03	12.617±0.29	14 Days	0.116±0.03	12.517±1.05
28 Days	0.075±0.03	16.350±1.00	28 Days	0.087±0.02	12.022±0.51
42 Days	0.046±0.01	17.109±0.26	42 Days	0.089±0.04	16.724±2.23
S+CBZ			**NVP+CBZ**		
2 Days	0.107±0.01	9.823±1.19	2 Days	0.031±0.01	12.827±1.11
7 Days	0.108±0.00	8.483±0.63	7 Days	0.147±0.04	7.651±1.39
14 Days	0.083±0.01	9.751±2.10	14 Days	0.096±0.02	8.392±1.28
28 Days	0.084±0.01	11.525±2.12	28 Days	0.072±0.01	10.135±2.44
42 Days	0.071±0.01	12.140±1.83	42 Days	0.074±0.04	9.023±1.61

IgM = immunoglobulin M; IgG = immunoglobulin G; S = saline; NVP = nevirapine; CBZ = carbamazepine

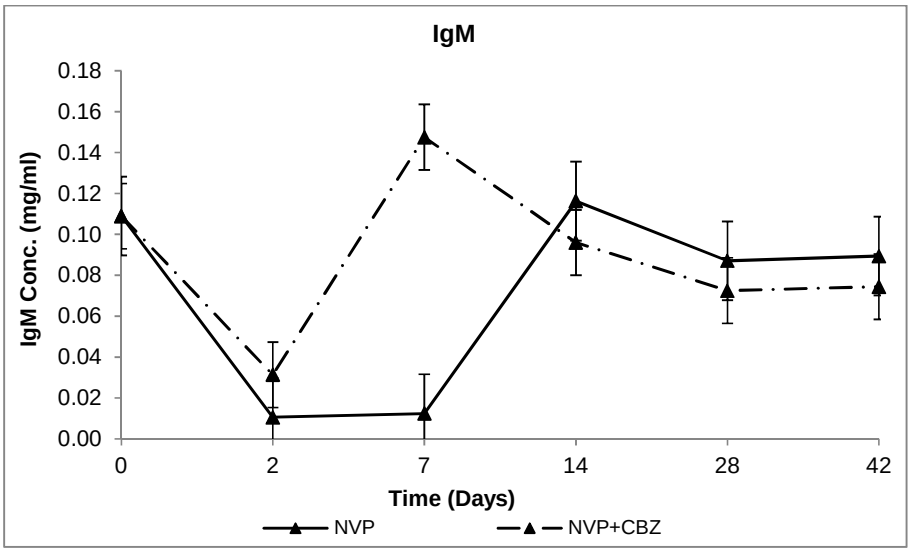

Figure 7.19 a): IgM concentrations of the NVP and NVP+CBZ groups over 42 days

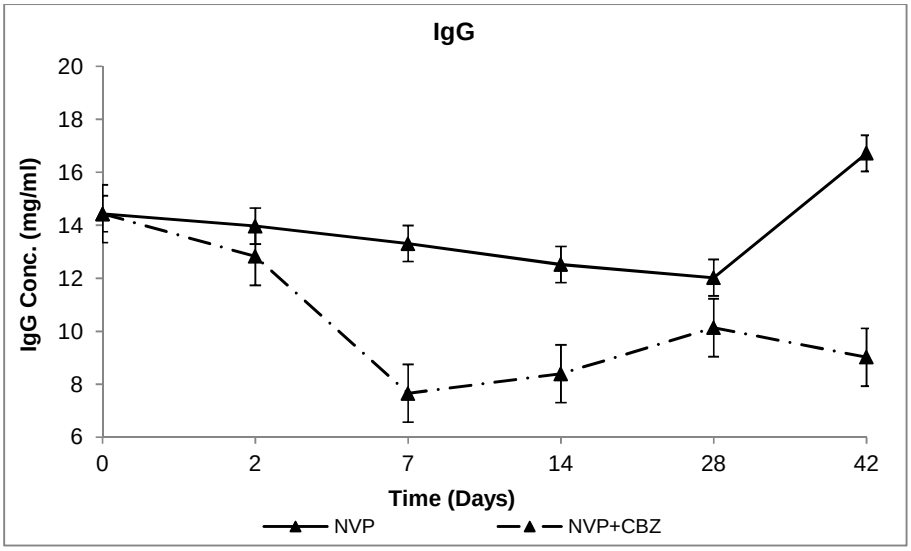

Figure 7.19 b): IgG concentrations of the NVP and NVP+CBZ groups over 42 days

7.3.13 Activity of rat CYP1A2, CYP2E1 and CYP3A2 *in vivo*

7.3.13.1 Protein concentrations

The results of BSA calibration samples are the same as shown in Section 7.3.4.1, Table 7.10 and Figure 7.8. Table 7.31 shows microsomal protein concentrations of untreated rats, and the NVP and NVP+CBZ groups.

Table 7.31: Average (mean ± SD) microsomal protein concentrations of the untreated, NVP and NVP+CBZ groups

Selected liver (n = 3)	Prot. conc. (mg/ml)	Abs. (nm)	Selected liver (n = 3)	Prot. conc. (mg/ml)	Abs. (nm)
Untreated					
Rat 1	49.40 ± 6.8	0.132 ± 0.02			
Rat 2	55.02 ± 1.1	0.146 ± 0.00			
Rat 4	51.00 ± 5.1	0.136 ± 0.01			
NVP-2D			**NVP+CBZ-2D**		
Rat 2	44.01 ± 3.5	0.087 ± 0.01	Rat 2	62.60 ± 2.5	0.130 ± 0.00
Rat 3	55.32 ± 10.7	0.124 ± 0.03	Rat 3	74.05 ± 2.1	0.147 ± 0.00
Rat 4	62.63 ± 8.3	0.139 ± 0.02	Rat 5	54.02 ± 0.0	0.107 ± 0.00
NVP-7D			**NVP+CBZ-7D**		
Rat 2	43.95 ± 5.5	0.078 ± 0.01	Rat 2	73.36 ± 7.8	0.151 ± 0.02
Rat 3	54.70 ± 4.3	0.098 ± 0.01	Rat 3	84.13 ± 1.8	0.173 ± 0.00
Rat 5	50.02 ± 5.5	0.089 ± 0.01	Rat 4	65.90 ± 1.6	0.116 ± 0.00
NVP-14D			**NVP+CBZ-14D**		
Rat 2	56.85 ± 3.8	0.115 ± 0.01	Rat 1	54.66 ± 2.8	0.110 ± 0.01
Rat 3	54.48 ± 5.2	0.112 ± 0.01	Rat 2	69.28 ± 2.8	0.140 ± 0.01
Rat 5	50.52 ± 3.1	0.102 ± 0.01	Rat 5	61.57 ± 6.2	0.128 ± 0.01

Prot. conc. = protein concentration; Abs. = absorption; NVP = nevirapine; CBZ = carbamazepine; D = days

7.3.13.2 CYP1A2, CYP2E1 and CYP3A2 activity in vivo

Table 7.32 shows CYP1A2, CYP2E1 and CYP3A2 activity after 2, 7 and 14 days of nevirapine alone (NVP) and concomitant nevirapine and carbamazepine (NVP+CBZ) treatment, while Figures 7.20 a – c are graphical illustrations of the same. The combination of nevirapine and carbamazepine increased CYP1A2 activity on each occasion ($p = 0.0286$), and although this was higher than with nevirapine alone on days 7 and 14, it was not statistically significant. CYP2E1 activity was only slightly increased and different from the normal on day 7 ($p = 0.0119$), which was similar to nevirapine alone. CYP3A2 activity was elevated on each occasion, and different

from the normal on day 14 (p = 0.0333). Although CYP3A2 induction was higher with nevirapine alone, this was not statistically different.

Table 7.32: Average (mean ± SD) CYP1A2, CYP2E1 and CYP3A2 activity

Group (n = 3)	CYP1A2 (pmol/min*mg)	CYP2E1 (nmol/min*mg)	CYP3A2 (pmol/min*mg)
Untreated			
0 Days	4.40 ± 0.8	0.77 ± 0.1	84.63 ± 6.9
NVP			
2 Days	13.63 ± 1.5	0.87 ± 0.0	107.17 ± 8.7
7 Days	7.35 ± 1.1	0.91 ± 0.0	94.28 ± 5.2
14 Days	5.31 ± 1.6	1.15 ± 0.6	105.79 ± 11.0
NVP+CBZ			
2 Days	11.94 ± 1.4	0.81 ± 0.0	86.77 ± 15.6
7 Days	9.54 ± 1.4	0.91 ± 0.0	82.19 ± 10.9
14 Days	8.01 ± 1.1	0.93 ± 0.3	94.69 ± 3.7

RR = reaction rate; NVP = nevirapine; CBZ = carbamazepine

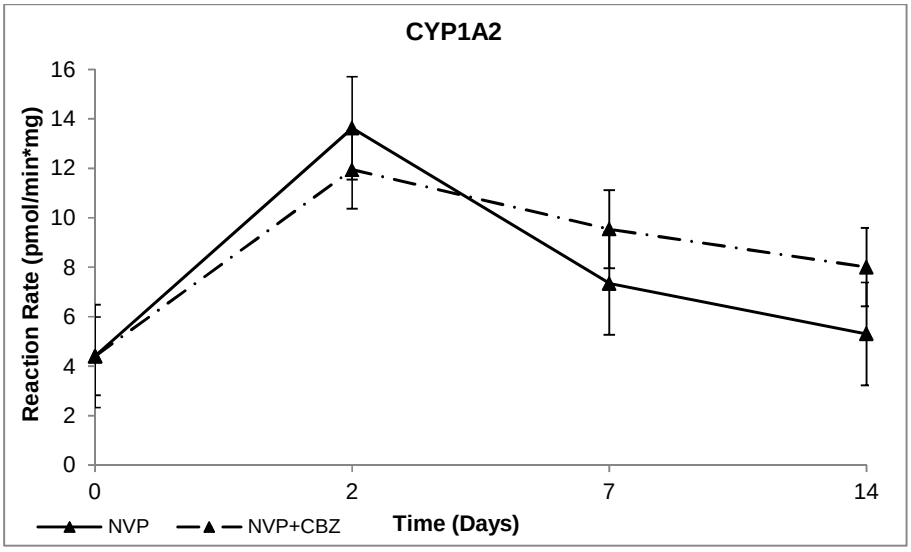

Figure 7.20 a): CYP1A2 activity after nevirapine alone, and carbamazepine co-treatment

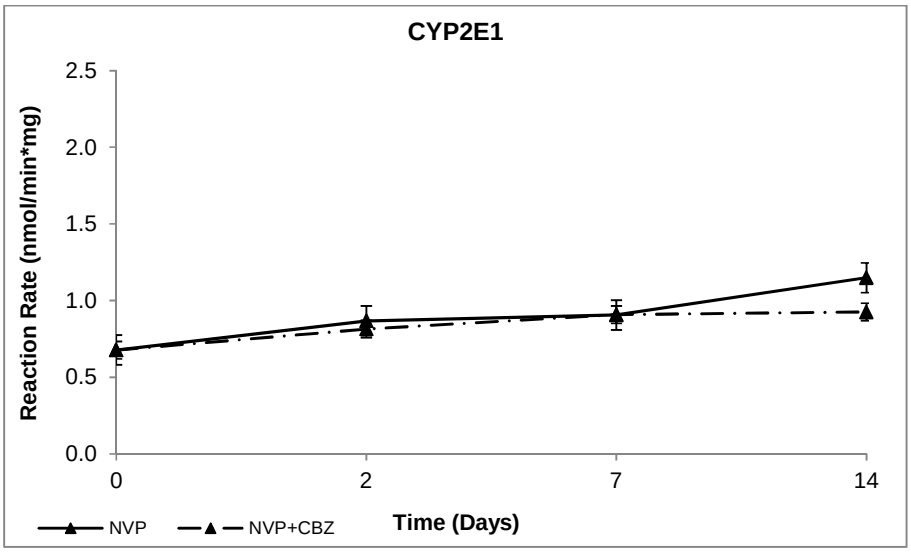

Figure 7.20 b): CYP2E1 activity after nevirapine alone, and carbamazepine co-treatment

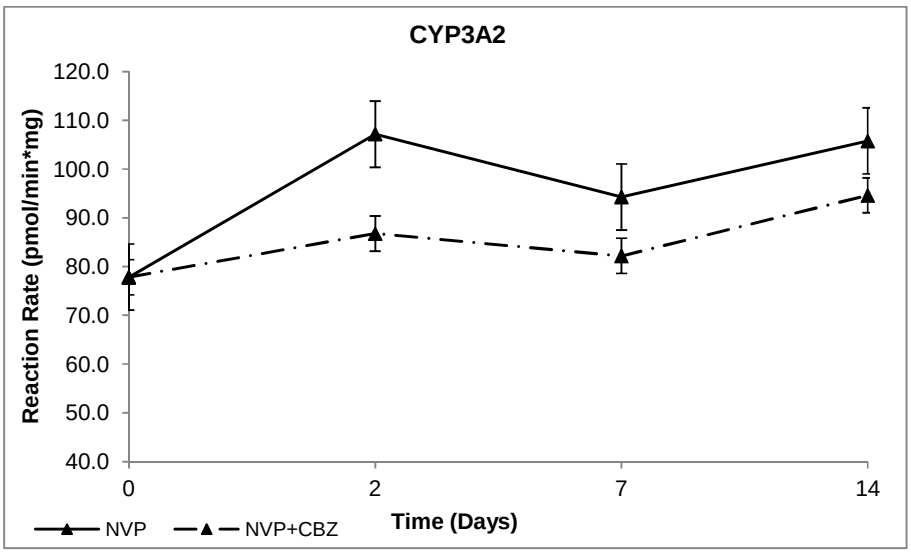

Figure 7.20 c): CYP3A2 activity after nevirapine alone, and carbamazepine co-treatment

7.3.14 Main observations

- Co-treatment with carbamazepine caused liver injury up to day 42.
- Nevirapine concentrations were consistently lower with carbamazepine co-administration.
- By day 42 IL-2 levels were elevated and higher than with nevirapine alone, while IL-10 concentrations were unchanged.
- IgM concentrations increased up to day 7, and were higher than with nevirapine alone.
- Co-administration with carbamazepine increased CYP1A2 activity, had no effect on CYP2E1 activity, and increased CYP3A2 activity, although lower than with nevirapine alone.

7.4 SUMMARY OF THE RESULTS

Since the immune system and CYP450 enzymes are described in the hypothesised mechanism of nevirapine-induced hepatotoxicity, this study aimed at demonstrating physiological and pathological changes during daily nevirapine administration at normal doses in animals that did not develop clinical hepatotoxicity. As described in Section 6.4, these changes occurred within their normal concentration ranges. Specifically, the liver injury was subclinical and the concentrations for nevirapine and chemokines were within their normal ranges. Therefore, it was concluded that changes or responses between the different groups do not have to be statistically significant in order to make sense.

Furthermore, these changes were interpreted as premonitory symptoms of the major pathological process, *i.e.*, during treatment at a normal dose the body is able to respond to drug insults by increasing or decreasing chemokines or enzyme activity, but within the respective normal range. As such, a better understanding of these activities may help to determine the mechanism of nevirapine-induced hepatotoxicity and to develop treatment strategies to prevent this unfortunate adverse event.

Table 7.33 is a summary of the changes observed between the different groups, while Table 7.34 shows the relation between lesions scores and nevirapine concentrations, and Figure 7.21 is a graphical illustration of the same. Prolonged administration of nevirapine alone caused subclinical liver injury in the first 14 days, and subsequent recovery by day 28 (Table 7.34; Figure 7.21). The injury was associated with a moderate increase in IL-2 up to day 7 and CD4 count up to day 28 (Th1 response) followed by an increase in IgG from day 28 and IL-10 from day 14 onwards (Th2 response), low nevirapine concentrations by day 7 and a progressive increase in CYP3A2 activity (Table 7.33; NVP alone). Here, the Th1 response, low nevirapine concentrations and CYP3A2 induction correlated with the liver injury, and the Th2 response with the recovery.

Co-treatment with levamisole, an immune stimulant, caused the same severity of liver injury, and was associated with lower nevirapine concentrations, lower IL-2 (Th1 response) and the same IgG and IL-10 (Th2 response; Table 7.33; NVP+LMS).

Here, the same Th2 response along with the lower nevirapine concentrations (Table 7.34) implies that probably more reactive metabolites were produced and could not be removed as quickly with the same magnitude Th2 response. Treatment with levamisole alone did not cause liver injury. Therefore, immune stimulation led to the same degree of nevirapine-induced liver injury.

Co-treatment with carbamazepine, an enzyme inducer, caused mild liver injury up to day 42 (Table 7.34; Figure 7.21). It was associated with increased CYP3A2 activity, lower nevirapine concentrations, sharp increase in IL-2 up to day 42 (Th1 response) versus an increase in IL-10 from day 2 to day 28 (Th2 response; Table 7.33). Unfortunately, treatment with carbamazepine alone caused mild liver injury up to day 14. Therefore, the strong Th1 response was counteracted by the strong Th2 response, and led to less severe nevirapine-induced liver injury.

Table 7.33: Description of the changes or responses in the test group relative to those in the control or NVP group

NVP alone

	Period 1 0 – 14 days of treatment	**Period 2 14 – 42 days of treatment**
	NVP vs. S	**NVP vs. S**
Liver injury (score)	Increased, 9+ (d14)	Decreased, 5+ (d42)
NVP conc. (µg/ml)	Peak at d2 (4.3 µg/ml)	Lower than phase 1
IL-2 conc. (pg/ml)	Increased (higher), peaked d7	Decreased, lower than S group by d42
IL-10 conc. (pg/ml)	Moderate increase up to d7	Increase from d14 up to d42, higher than S group
CYP3A2(pmol/min*mg)	Increased CYP3A2 activity by d2 and continued so	

NVP+LMS

	Period 1 0 – 14 days of treatment	**Period 2 14 – 42 days of treatment**
	NVP+LMS vs. NVP alone	**NVP+LMS vs. NVP alone**
Liver injury (score)	Increased, 8+ (d14)	Decreased, 4.5+ (d42)
NVP conc. (µg/ml)	Peak at d7 (4.3 µg/ml)	Lower
IL-2 conc. (pg/ml)	Lower	Lower
IL-10 conc. (pg/ml)	Moderate increase up to d7	Lower

NVP+CBZ

	Period 1 0 – 14 days of treatment	**Period 2 14 – 42 days of treatment**
	NVP+CBZ vs. NVP alone	**NVP+CBZ vs. NVP alone**
Liver injury (score)	Increased, 7+ (d14)	Similar, 7.5+ (d42)
NVP conc. (µg/ml)	Peak at d14 (0.9 µg/ml)	Lower
IL-2 conc. (pg/ml)	Moderate increase from d7 to d14	Sharp increase by d42
IL-10 conc. (pg/ml)	Not different	

NVP = nevirapine; LMS = levamisole; CBZ = carbamazepine; d = day

Table 7.34: The relation between nevirapine concentrations and histopathological lesions in the NVP, NVP+LMS and NVP+CBZ groups

Group	Lesions score (score)	NVP concentration (µg/ml)
Untreated		
0 Days	0+	0.000
NVP		
2 Days	8.5+	4.292
7 Days	9+	2.008
14 Days	9+	1.983
28 Days	5+	1.721
42 Days	5+	0.497
NVP+LMS		
2 Days	3.5+	2.287
7 Days	3+	4.332
14 Days	8+	1.477
28 Days	7.5+	1.293
42 Days	4.5+	0.000
NVP+CBZ		
2 Days	5.5+	0.353
7 Days	5.5+	0.769
14 Days	7+	0.851
28 Days	6+	0.195
42 Days	7.5+	0.729

NVP = nevirapine; LMS = levamisole; CBZ = carbamazepine

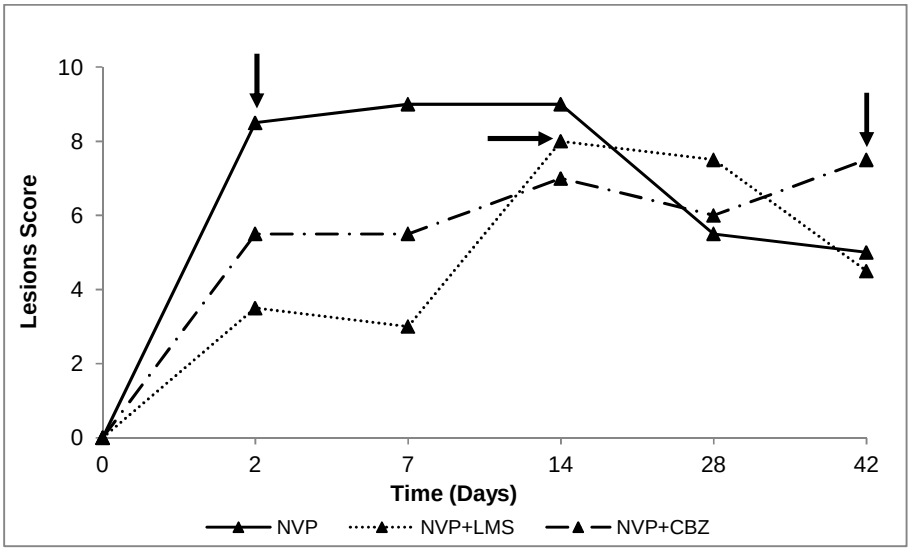

Figure 7.21: Lesions score chart of the NVP, NVP+LMS and NVP+CBZ groups over 42 days

In the treatment phases with nevirapine alone and co-administration with a CYP450 inducer, there was immune activation of the Th1 and Th2 responses and subsequent liver recovery (for nevirapine alone; Figure 7.21). This is indicative of a counter regulatory immune mechanism to prevent progression to overt hepatotoxicity, and suggests that the immune system is involved in nevirapine-induced liver injury. However, further studies are required to evaluate the role of CYP450, as both nevirapine and carbamazepine are metabolised by, and responsible for, autoinduction of CYP3A2. Therefore, the drugs might have competed for metabolism by CYP3A2 and this led to interference of the enzyme inducing effects of carbamazepine.

In conclusion, the data suggest that the immune system is involved in initiating nevirapine-induced liver injury, but may also be implemented to prevent the progression to overt hepatotoxicity.

CHAPTER 8

CYTOCHROME P450 AND THE IMMUNE RESPONSE TO PROLONGED ADMINISTRATION OF PARACETAMOL

8.0 SUMMARY

Introduction: Although paracetamol-induced liver injury is initiated by NAPQI, a product of CYP450 metabolism, involvement of the immune system has also been implicated. Therefore the effect of prolonged paracetamol administration on the CYP450 and immune response was investigated here.

Methods: Ethical approval (Animal Experiment NR 09/2012) was obtained from the Animal Ethics Committee of the University of the Free State, and male SPD rats (200 – 250 g) were used. The animal experiment was divided into three phases. During phase I, two groups of 25 rats each were administered daily S or PAR (500 mg/kg), while for phase II, two groups of 25 rats each received daily S or PAR in combination with an immune stimulant, LMS (2.5 mg/kg), and lastly, during phase III, two groups of 25 rats each received daily S or PAR along with a CYP450 inducer, CBZ (60 mg/kg). For each phase, five rats per group were sacrificed after 2, 7, 14, 28 and 42 days. Blood was analysed for full blood count, CD4 and CD8 counts, liver function, renal function, IL-2, IL-10, IgG, IgM and paracetamol concentrations. A piece of liver was sent for histopathology testing, and rat liver microsomes were analysed for CYP1A2, CYP2E1 and CYP3A2 activity.

Results: Paracetamol alone caused mild liver injury up to day 14 and was associated with increased CYP450 activity and low paracetamol concentrations. The healing by day 28 was probably due to an anti-oxidant mechanism which delayed the immune response, as this was lower than in the control. Co-administration with levamisole or carbamazepine caused only minimal liver injury, and was associated with an early Th2 immune response.

Conclusion: Paracetamol did not show a distinct pattern of immune response by which to associate it with the liver injury, most probably because the concentrations were too low for generation of toxic metabolites.

8.1 INTRODUCTION

It is well known that paracetamol-induced liver injury is initiated by its metabolite, NAPQI, after paracetamol overdose (Liu and Kaplowitz, 2007). It was also postulated that the immune system may further contribute to the injury (James *et al*., 2003; Liu and Kaplowitz, 2006), but this remains to be proven. This chapter describes the role of the CYP450 and immune response in paracetamol-induced liver injury. The results are reported under the following parameters: physiological observations (function tests), *i.e.*, full blood count, renal function tests, liver function tests and liver histopathology; paracetamol concentrations; specific immune tests, *i.e.*, direct observations, cytokines, CD4 and CD8 counts and immunoglobulins; and activity of rat CYP1A2, CYP2E1 and CYP3A2.

8.2 METHODS

A. Materials

8.2.1 Apparatus

All apparatus used are the same as described in Chapter 6, Section 6.2.1.

8.2.2 Chemicals and reagents

Panado® (paracetamol) paediatric syrup (120 mg/5 ml) and tablets (500 mg; Adcock Ingram Limited, Midrand, Gauteng, South Africa) were purchased from a local pharmacy, while the paracetamol standard was obtained from Sigma-Aldrich™ (St. Louis, MO, U.S.A). All other chemicals were of analytical grade, and the same as discussed in Chapter 6, Section 6.2.2.

8.2.3 Preparation of drugs for oral administration

The paracetamol preparation (500 mg/kg) was a mixture of Panado® paediatric syrup and tablets (Walubo *et al.*, 2004), since the tablets have limited solubility in water. Levamisole and carbamazepine were prepared as described in Chapter 6, Section 6.2.3.

8.2.4 Buffers and reagents

All buffers and reagents used are the same as discussed in Chapter 6, Section 6.2.4.

B. Methods

8.2.5 Experimental design

Here, a total of 155 rats were used. Five rats were not treated with any drug, and used for baseline data. Furthermore, the study was divided into three phases.

8.2.5.1 Phase I – Treatment with paracetamol alone

Rats were weighed and divided into two groups of 25 animals each, namely the S group (control) and the PAR group (test). Rats received saline solution or paracetamol once daily for 2, 7, 14, 28, and 42 days, respectively. At each time frame, five rats were sacrificed on the day following the last day of dosing. Saline solution and paracetamol were administered orally on a daily basis as follows (Figure 8.1):

- S group: saline solution (1 ml, orally)
- PAR group: 500 mg/kg paracetamol (1 ml, orally)

The dose of paracetamol was from a previous departmental study (Walubo *et al.*, 2004).

8.2.5.2 Phase II – Co-treatment with an immune stimulant

Rats were weighed and divided into two groups of 25 animals each, namely the S+LMS group (control) and the PAR+LMS group (test). Rats received saline solution plus levamisole or paracetamol plus levamisole for 2, 7, 14, 28, and 42 days, respectively. Levamisole administration was started on the day before the start of saline or paracetamol dosing, thereafter saline and paracetamol were administered daily in the morning, and levamisole daily in the afternoon to avoid a potential drug interaction. At each time frame, five rats were sacrificed on the day following the last day of dosing. Saline solution, paracetamol and levamisole were administered orally as follows (Figure 8.1):

- S+LMS group: saline solution (1 ml, orally) and 2.5 mg/kg levamisole (500 µl, orally)
- PAR+LMS group: 500 mg/kg paracetamol (1 ml, orally) and 2.5 mg/kg levamisole (500 µl, orally)

The dose of levamisole was from a report by Gautam *et al*. (2009).

8.2.5.3 Phase III – Co-treatment with a CYP450 inducer

Rats were weighed and divided into two groups of 25 animals each, namely the S+CBZ group (control) and the PAR+CBZ group (test). Rats received saline solution plus carbamazepine or paracetamol plus carbamazepine for 2, 7, 14, 28, and 42 days, respectively. Carbamazepine administration was started on the day before the start of saline or paracetamol dosing, thereafter saline and paracetamol were administered daily in the morning, and carbamazepine daily in the afternoon to avoid a potential drug interaction. At each time frame, five rats were sacrificed on the day following the last day of dosing. Saline solution, paracetamol and carbamazepine were administered orally as follows (Figure 8.1):

- S+CBZ group: saline solution (1 ml, orally) and 60 mg/kg carbamazepine (500 µl, orally)
- PAR+CBZ group: 500 mg/kg paracetamol (1 ml, orally) and 60 mg/kg carbamazepine (500 µl, orally)

The dose of carbamazepine was from a report by Tateishi and co-workers (1999).

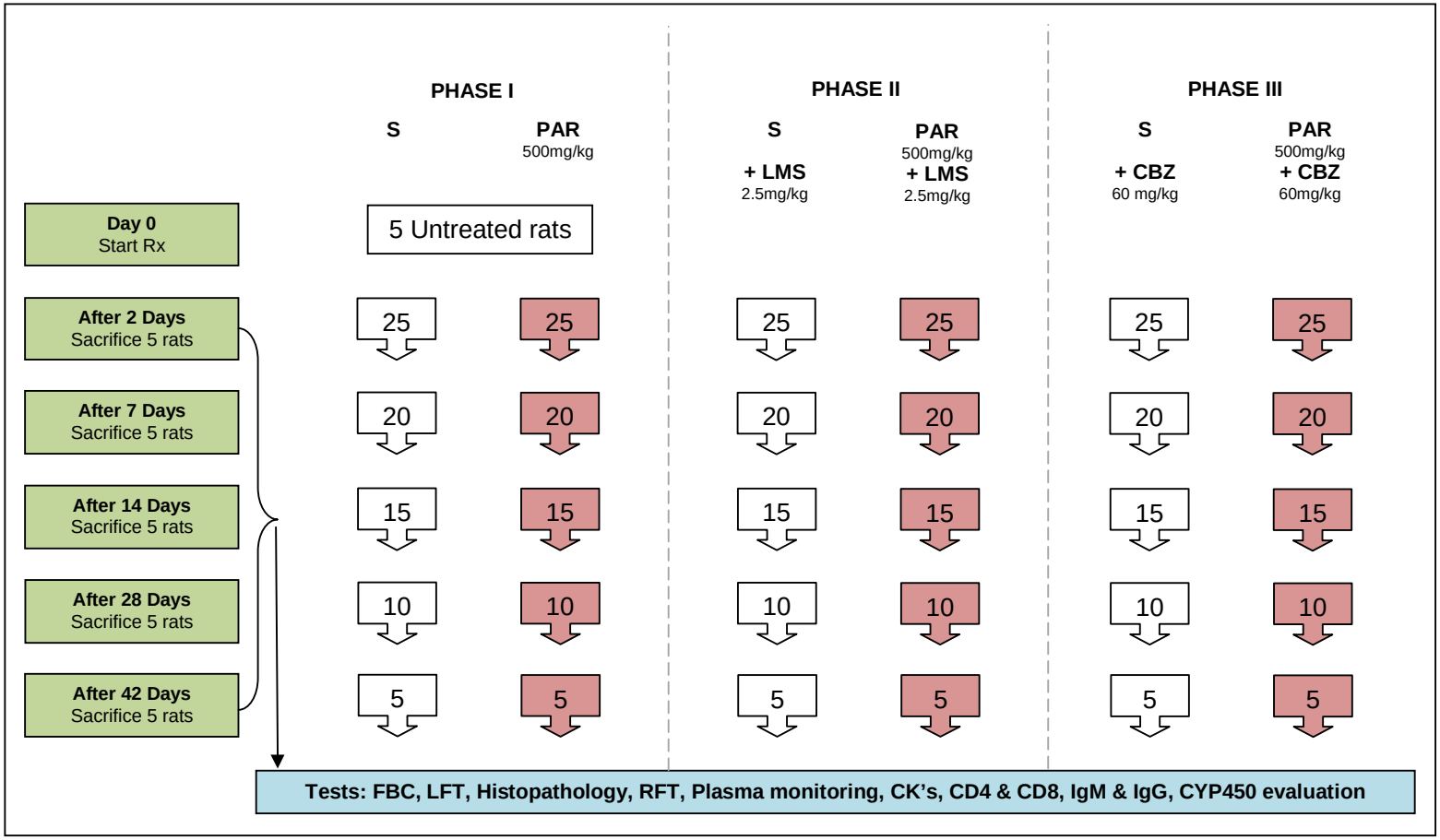

Figure 8.1: A schematic illustration of the experimental design of Phase I, II and III of prolonged paracetamol administration

8.2.6 Animal care

Ethical approval and animal care are as discussed in Chapter 6, Section 6.2.6.

8.2.7 Animal weighing, blood collection and liver removal

The process of animal weighing, blood collection and liver removal, is as discussed in Chapter 6, Section 6.2.7.

8.2.8 Analysis of function tests

The analysis of full blood count, CD4 and CD8 counts, liver and renal function tests, and histopathology of the rat livers, are as described in Chapter 6, Section 6.2.8.

8.2.9 Analysis of cytokines by enzyme-linked immunosorbent assay

The analysis of IL-2 and IL-10 by ELISA, is as described in Chapter 6, Section 6.2.9.

8.2.10 Analysis of immunoglobulins by enzyme-linked immunosorbent assay

The analysis of IgM and IgG by ELISA, is as discussed in Chapter 6, Section 6.2.10.

8.2.11 Analysis of paracetamol concentrations in rat plasma

Plasma concentrations of paracetamol in rats were monitored using the HPLC assay as developed and described in Chapter 5. For paracetamol, a standard curve was generated from five known calibration standards, from which paracetamol concentrations in rat plasma were derived.

8.2.12 Preparation of rat liver microsomes

Microsomes were prepared as discussed in Chapter 6, Section 6.2.12.

8.2.13 Protein assay

The protein assay was conducted as described in Chapter 6, Section 6.2.13.

8.2.14 Determination of rat CYP1A2, CYP2E1 and CYP3A2 activity *in vivo*

The assays which were used to determine rat CYP1A2, CYP2E1 and CYP3A2 activity *in vivo*, are as described in Chapter 6, Section 6.2.14.

8.2.15 Statistical analysis of results

Data were analysed by non-parametric methods using the GraphPad Instat statistical program. Accordingly, parameters were reported as mean and standard deviation (SD), and the Mann-Whitney Test was used for data comparison with the level of significance set at $p < 0.05$.

8.3 RESULTS

The results are divided into three phases: Phase I – treatment with paracetamol alone, Phase II – co-treatment with an immune stimulant, and Phase III – co-treatment with a CYP450 inducer, and all parameters are reported as such.

A. Phase I: Treatment with paracetamol alone

8.3.1 Physiological observations (function tests)

8.3.1.1 Full blood count

Table 8.1 shows results of the full blood count of the S and PAR groups. By day 42 in the PAR group, the red cell count, MCHC and platelet count increased ($p = 0.0500$), while the haematocrit, MCV and MCH decreased ($p = 0.0500$). Although a similar pattern of change was observed for the same parameters in the S group, it was mostly higher in the PAR group ($p = 0.0500$). The elevated red cell count and haemoglobin signify higher oxygen requirements, while ageing of red blood cells was associated with a small MCV, and subsequent increased MCHC. Regarding white blood cell changes, the white cell count and lymphocytes had decreased by day 42 ($p = 0.0500$).

Table 8.1: Average (mean ± SD) full blood count and platelets results of the S and PAR groups

Group (n = 3)	RCC ($x10^{12}$/l)	Hb (g/dl)	Hct (l/l)	MCV (fl)	MCH (pg)	MCHC (g/dl)	Plt ($x10^9$/l)	WCC ($x10^9$/l)	Neu ($x10^9$/l)	Ly ($x10^9$/l)	Mo ($x10^9$/l)	Eos ($x10^9$/l)	Bas ($x10^9$/l)
Untreated													
0 Days	6.28±0.2	12.9±0.3	0.398±0.01	63.5±2.5	20.5±0.4	32.3±0.8	860±221.1	6.95±2.7	0.77±0.2	4.67±1.8	0.19±0.1	0.02±0.0	0.01±0.0
S													
2 Days	6.67±0.2	13.7±0.1	0.422±0.01	63.3±2.3	20.6±0.6	32.4±0.6	849±81.6	6.50±0.9	0.60±0.2	5.18±0.7	0.21±0.0	0.50±0.2	0.01±0.0
7 Days	7.53±0.9	15.3±1.7	0.451±0.04	60.1±2.5	20.3±0.2	33.9±1.2	1033±79.8	5.44±2.4	1.03±0.8	4.07±2.0	0.30±0.3	0.04±0.0	0.01±0.0
14 Days	6.77±0.6	13.9±1.1	0.417±0.03	61.8±2.8	20.5±0.6	33.2±0.6	721±196.4	5.22±1.2	0.63±0.5	4.21±0.7	0.18±0.1	0.18±0.1	0.05±0.0
28 Days	7.07±0.7	13.9±1.3	0.390±0.04	55.1±1.0	19.7±0.1	35.8±0.6	961±172.5	7.38±1.0	0.91±0.2	6.15±0.8	0.24±0.1	0.07±0.0	0.01±0.0
42 Days	6.93±0.8	13.4±1.8	0.374±0.05	53.9±1.0	19.3±0.4	35.8±0.2	839±166.0	3.93±0.3	0.54±0.1	3.23±0.3	0.11±0.0	0.04±0.0	0.01±0.0
PAR													
2 Days	6.77±0.0	14.8±0.0	0.461±0.00	68.1±0.0	21.9±0.0	32.1±0.0	419±0.0	7.54±0.0	0.75±0. 0	5.96±0.0	0.37±0.0	0.46±0.0	0.01±0.0
7 Days	6.75±0.2	13.9±0.1	0.426±0.00	63.2±2.4	20.7±0.8	32.7±0.1	807±22.8	7.32±0.8	0.80±0.2	6.22±0.8	0.23±0.0	0.07±0.0	0.01±0.0
14 Days	6.66±0.2	13.8±0.3	0.411±0.01	61.7±0.5	20.7±0.1	33.6±0.4	834±43.0	4.19±0.4	0.55±0.0	3.42±0.6	0.09±0.1	0.13±0.2	0.00±0.0
28 Days	7.10±0.2	14.1±0.2	0.397±0.00	56.0±2.2	19.8±0.8	35.4±0.4	880±87.0	5.32±1.2	0.88±0.1	4.19±1.1	0.21±0.1	0.04±0.0	0.01±0.0
42 Days	7.33±0.3	14.5±0.3	0.401±0.01	54.8±1.8	19.8±0.5	36.2±0.3	961±85.8	4.91±0.3	0.80±0.2	3.86±0.4	0.20±0.0	0.05±0.0	0.01±0.0

RCC = red cell count; Hb = haemoglobin; Hct = haematocrit; MCV = mean corpuscular volume; MCH = mean corpuscular haemoglobin; MCHC = mean corpuscular haemoglobin concentration; Plt = platelets; WCC = white cell count; Neu = neutrophils; Ly = lymphocytes; Mo = monocytes; Eos = eosinophils; Bas = basophils; S = saline; PAR = paracetamol

8.3.1.2 Renal function tests

Table 8.2 shows the changes of BUN and of the S and PAR groups. In both groups BUN and Cr levels were normal, in spite of the spike on day 28 (p = 0.0500), as this was still within the normal range.

Table 8.2: Average (mean ± SD) renal function test results of the S and PAR groups

Group	**RFT**	
	BUN	**Cr**
(n = 3)	**(mmol/l)**	**(µmol/l)**
Untreated		
0 Days	7.2 ± 1	37 ± 8
S		
2 Days	7.3 ± 1	39 ± 2
7 Days	8.1 ± 0	46 ± 7
14 Days	7.5 ± 1	39 ± 3
28 Days	10.6 ± 2	73 ± 17
42 Days	5.8 ± 1	38 ± 9
PAR		
2 Days	6.7 ± 1	36 ± 6
7 Days	7.8 ± 1	37 ± 5
14 Days	7.3 ± 1	36 ± 2
28 Days	7.3 ± 1	73 ± 4
42 Days	6.9 ± 1	24 ± 5

RFT = renal function test; BUN = blood urea nitrogen; Cr = creatinine; S = saline; PAR = paracetamol

8.3.1.3 Liver function tests

Table 8.3 shows the changes of ALT, AST and ALP of the S and PAR groups. Liver function was normal as there were no differences in ALT, AST and ALP between the two groups.

Table 8.3: Average (mean ± SD) liver function test results of the S and PAR groups

Group	LFT		
	ALT	AST	ALP
(n = 3)	(U/l)	(U/l)	(U/l)
Untreated			
0 Days	50 ± 5	88 ± 14	352 ± 76
S			
2 Days	46 ± 2	90 ± 7	400 ± 7
7 Days	49 ± 10	103 ± 25	304 ± 13
14 Days	58 ± 4	127 ± 37	508 ± 37
28 Days	47 ± 2	115 ± 44	216 ± 19
42 Days	46 ± 6	76 ± 28	109 ± 76
PAR			
2 Days	54 ± 4	109 ± 6	402 ± 64
7 Days	58 ± 2	104 ± 30	349 ± 27
14 Days	53 ± 2	95 ± 12	364 ± 38
28 Days	49 ± 4	122 ± 35	224 ± 46
42 Days	48 ± 5	64 ± 3	55 ± 64

LFT = liver function test; ALT = alanine aminotransferase; AST = aspartate aminotransferase; ALP = alkaline phosphatase; S = saline; PAR = paracetamol

8.3.1.4 Liver histopathology

(a) Liver histopathology reports

Liver sections for histopathology (Figures 8.2 a – k) were randomly selected, and the main histopathology lesions are summarised in the tally table (Table 8.4). The following report is a summary of the features of the lesion:

i. Figure 8.2 a: Liver section from an untreated rat at day 0

A representative photograph of a rat liver from an untreated rat. The report: “No pathology appears to be present in the two control (untreated) animals.”

ii. Figures 8.2 b and c: Liver sections A and B from the PAR group after 2 days of paracetamol alone treatment

Representative photographs of rat livers, after 2 days of daily paracetamol alone treatment. The report: “Moderate granular vacuolar degeneration and cell swelling were observed in both liver sections. The hepatocytes appeared swollen with granular appearance and this is regarded as a degenerative change, which may be reversible when the toxic insult is withdrawn. Mild cytonecrosis was noted, as characterised by loss of cell boundaries, severe disruption of the cytoplasm with

severe granular appearance of cytoplasm or eosinophilic staining and nuclear pyknosis or complete nuclear loss. Centrilobular zonal necrosis was minimal in liver section B."

iii. Figures 8.2 d and e: Liver sections A and B from the PAR group after 7 days of paracetamol alone treatment

Representative photographs of rat livers, after 7 days of daily paracetamol alone treatment. The report: "Cellular swelling and vacuolar degeneration were mild in sections A and B. Cytonecrosis was only minimal in both sections. No centrilobular necrosis or mitotic figures were observed."

iv. Figures 8.2 f and g: Liver sections A and B from the PAR group after 14 days of paracetamol alone treatment

Representative photographs of rat livers, after 14 days of daily paracetamol alone treatment. The report: "Moderate cellular swelling and vacuolar hepatopathy as well as a loss of coordinated and well-organized hepatocytic cords were seen in both liver sections. Mild cytonecrosis was visible in liver section A, whilst liver section B showed eosinophilic staining and nuclear pyknosis. Mild cell proliferation and hepatocellular mitosis was found in both liver sections."

v. Figures 8.2 h and i: Liver sections A and B from the PAR group after 28 days of paracetamol alone treatment

Representative photographs of rat livers, after 28 days of daily paracetamol alone treatment. The report: "Liver pathology had improved by day 28, as only minimal granular vacuolar degeneration and cell swelling were observed in both liver sections. There were no signs of cytonecrosis, centrilobular zonal necrosis, or hepatocyte mitosis."

vi. Figures 8.2 j and k: Liver sections A and B from the PAR group after 42 days of paracetamol alone treatment

Representative photographs of rat livers, after 42 days of daily paracetamol alone treatment. The report: "Minimal to mild granular vacuolar degeneration and cell swelling were observed in both liver sections. Cytonecrosis was also only minimally visible, whilst no centrilobular zonal necrosis or hepatocyte mitosis was present."

In view of the histopathology photographs (Figures 8.2 a – k), reports and tally table (Table 8.4), it was concluded that treatment with paracetamol alone caused liver injury up to 14 days, and by day 28 this had improved.

(b) Liver histopathology photographs

Figures 8.2 a – k are representative of randomly selected liver sections of untreated rats, as well as the PAR group after paracetamol alone treatment.

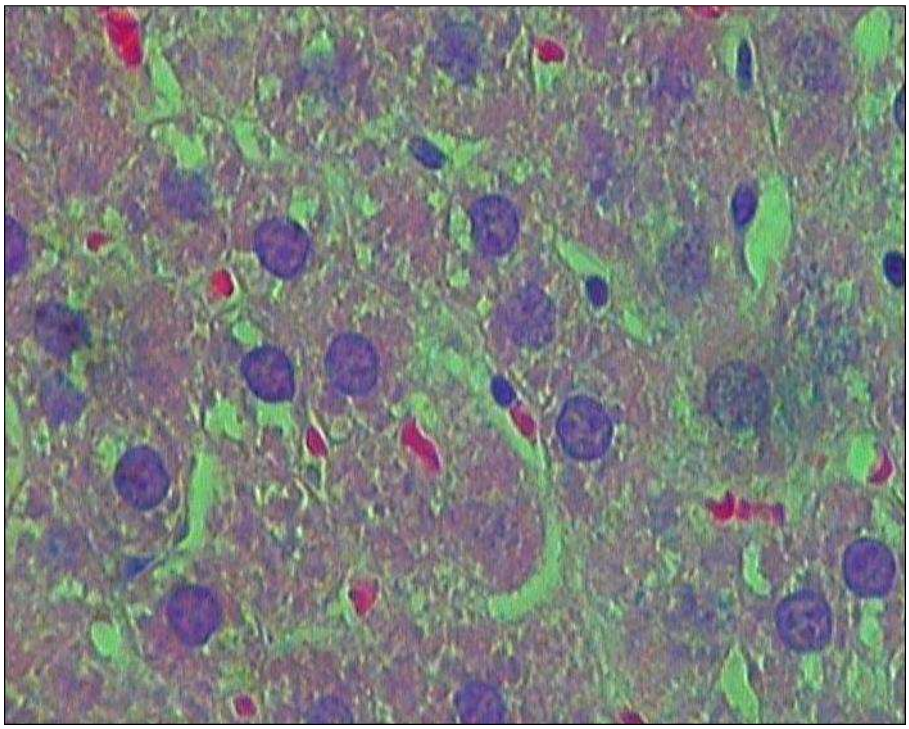

Figure 8.2 a): Liver section from an untreated rat at day 0, showing a normal liver with no inflammation

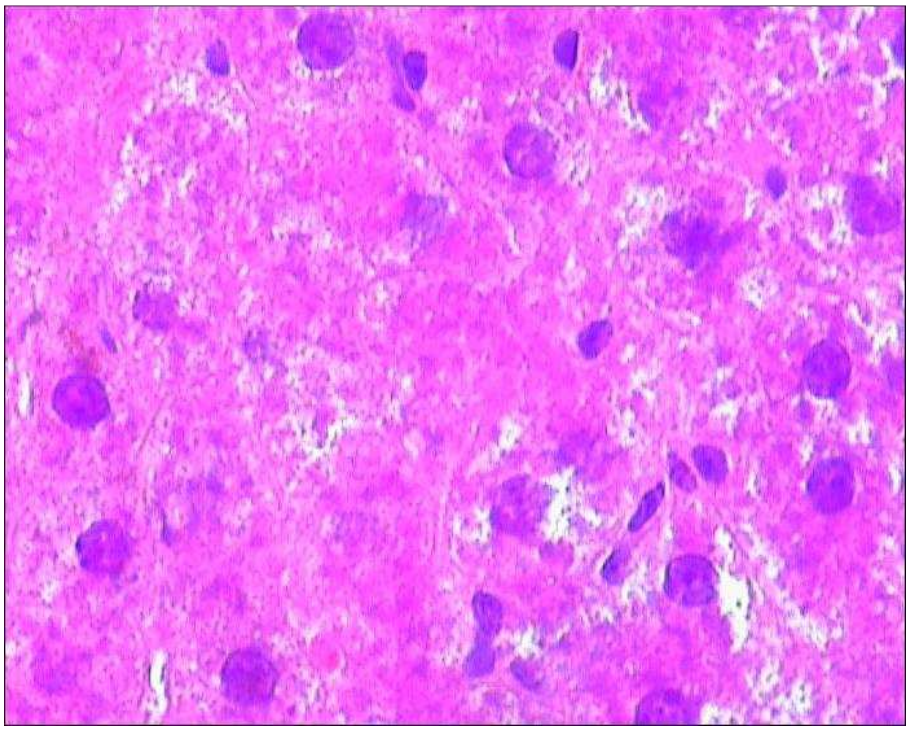

Figure 8.2 b): Liver section A from the PAR group after 2 days of treatment, showing mild cytonecrosis with eosinophilic staining and nuclear pyknosis

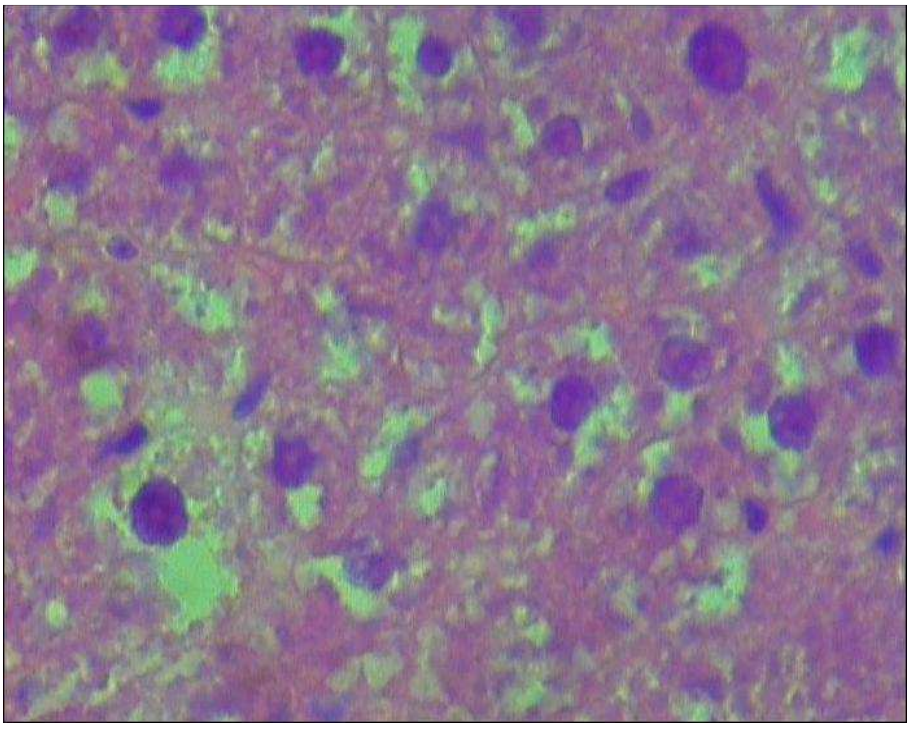

Figure 8.2 c): Liver section B from the PAR group after 2 days of treatment, showing moderate granular vacuolar degeneration and hepatocyte swelling

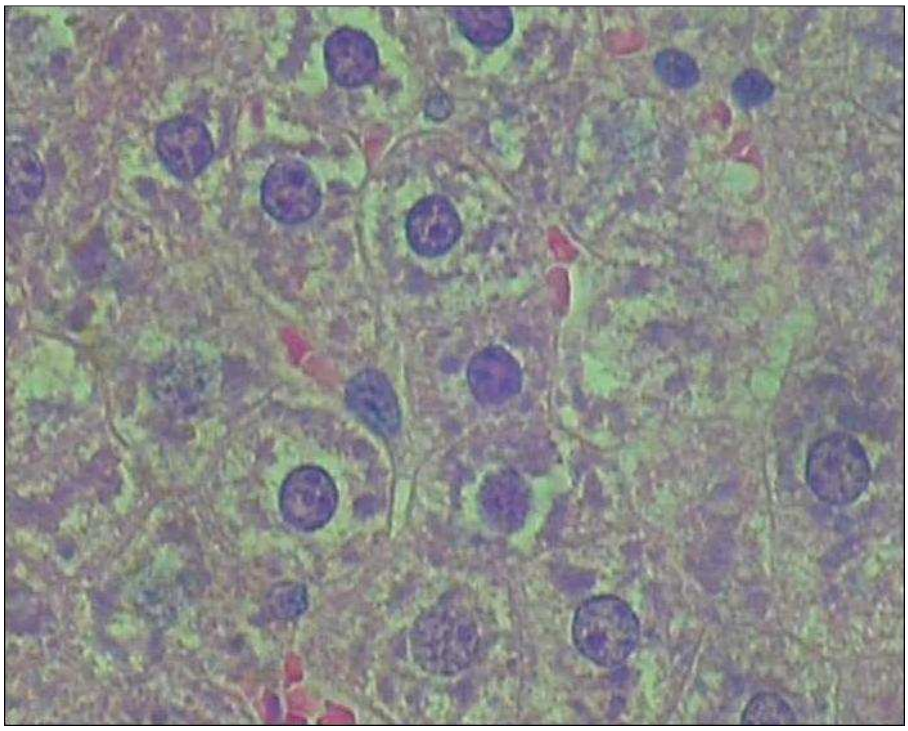

Figure 8.2 d): Liver section A from the PAR group after 7 days of treatment, showing mild hepatopathy and minimal cytonecrosis

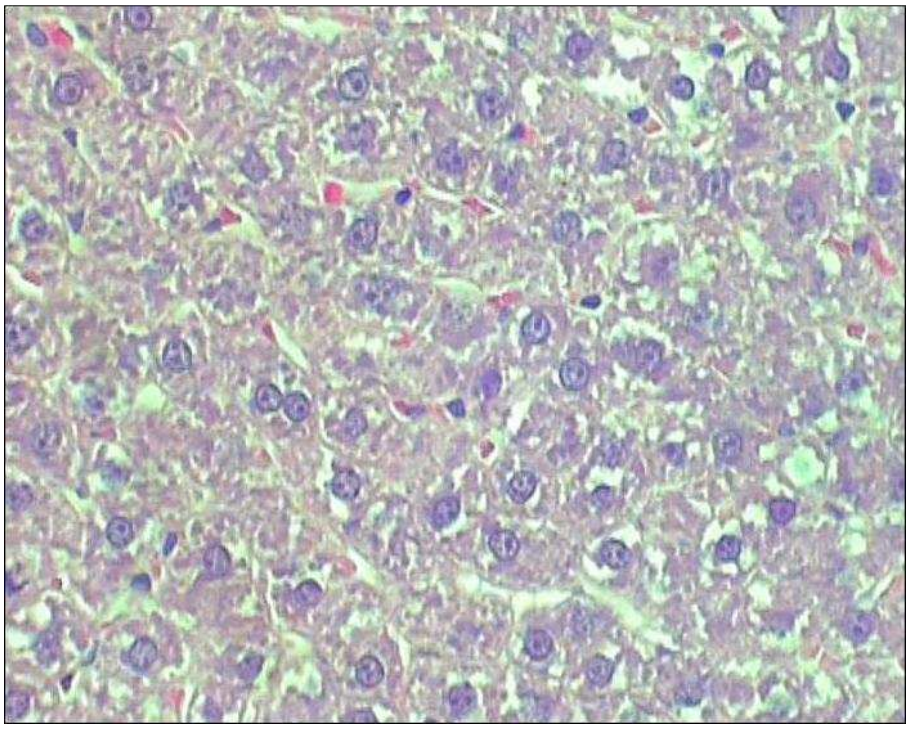

Figure 8.2 e): Liver section B from the PAR group after 7 days of treatment, showing mild degeneration and minimal cytonecrosis

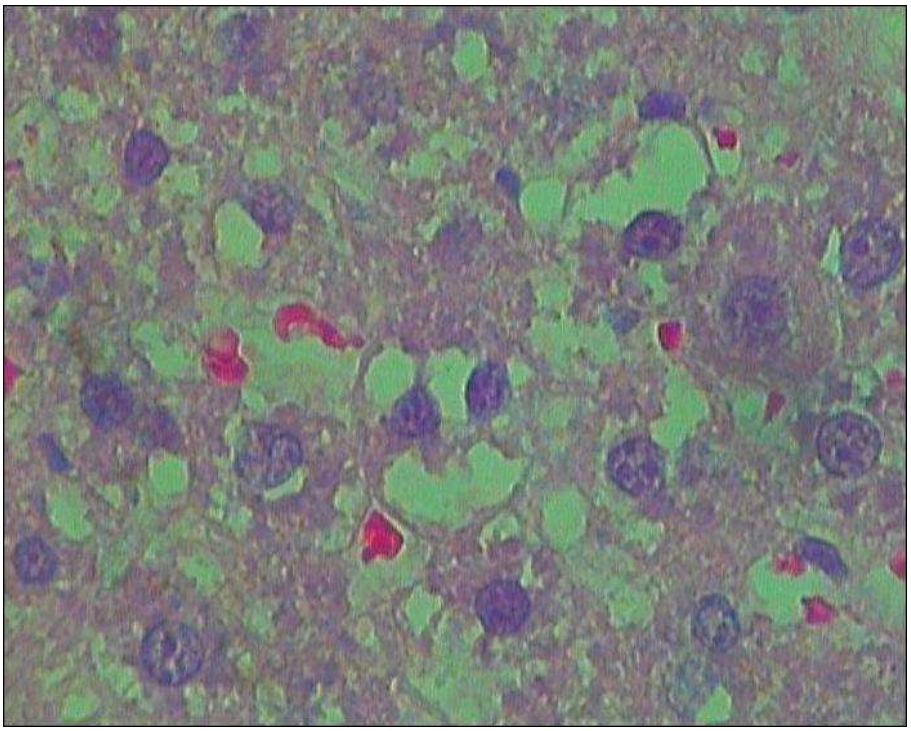

Figure 8.2 f): Liver section A from the PAR group after 14 days of treatment, showing moderate cellular swelling and vacuolar hepatopathy with loss of coordinated hepatocytic cords

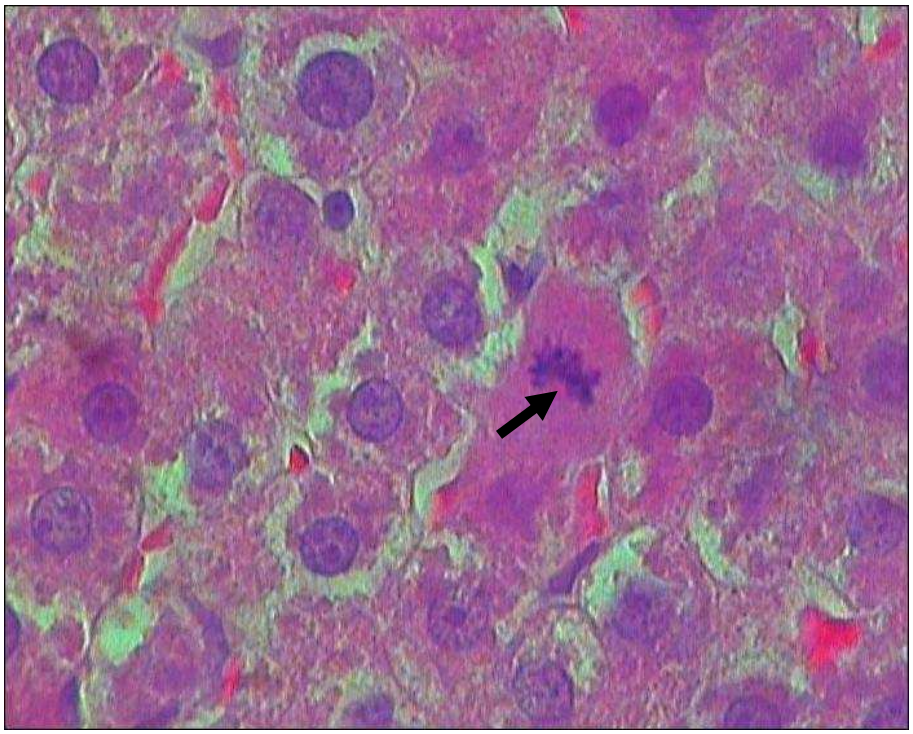

Figure 8.2 g): Liver section B from the PAR group after 14 days of treatment, showing cytonecrosis with eosinophilic staining and nuclear pyknosis, and hepatocellular mitosis

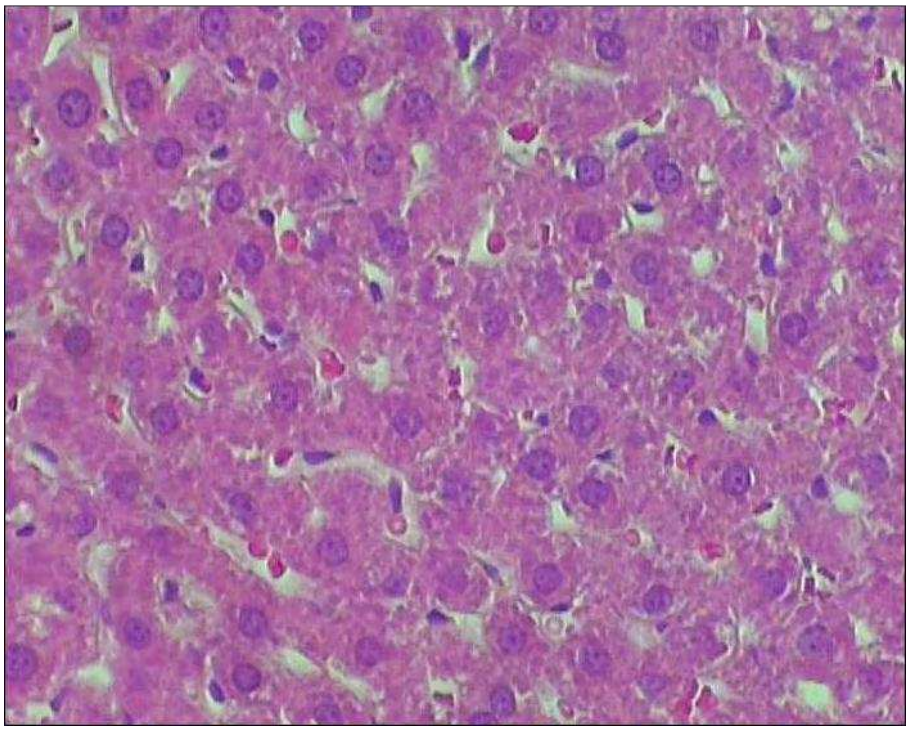

Figure 8.2 h): Liver section A from the PAR group after 28 days of treatment, showing hepatocytic cords with minimal vacuolar degeneration, and no cytonecrosis

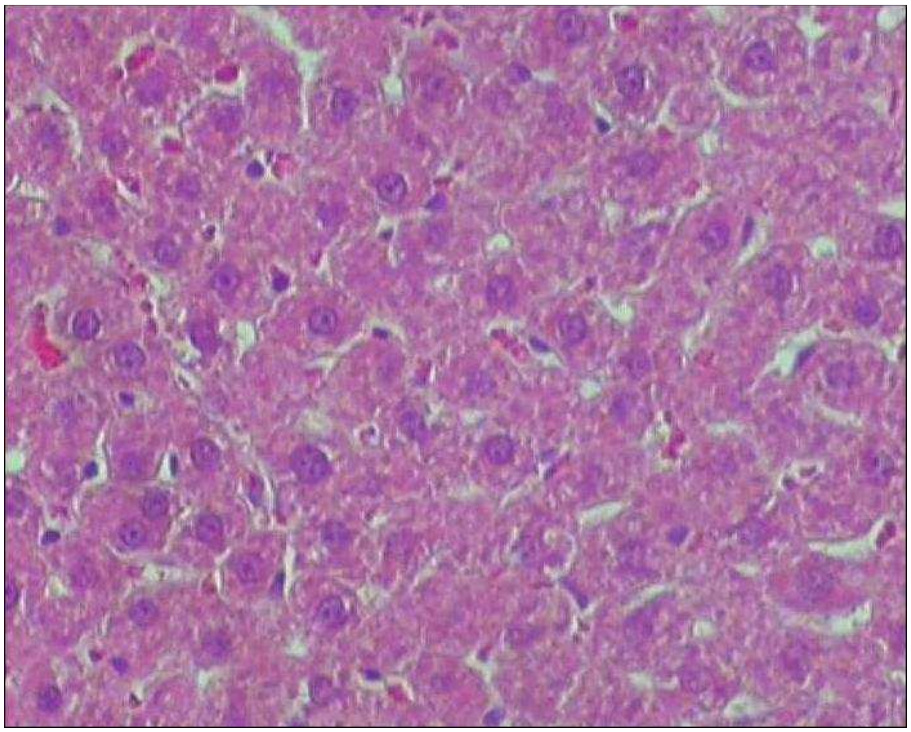

Figure 8.2 i): Liver section B from the PAR group after 28 days of treatment, showing hepatic cords with minimal vacuolar degenerative changes

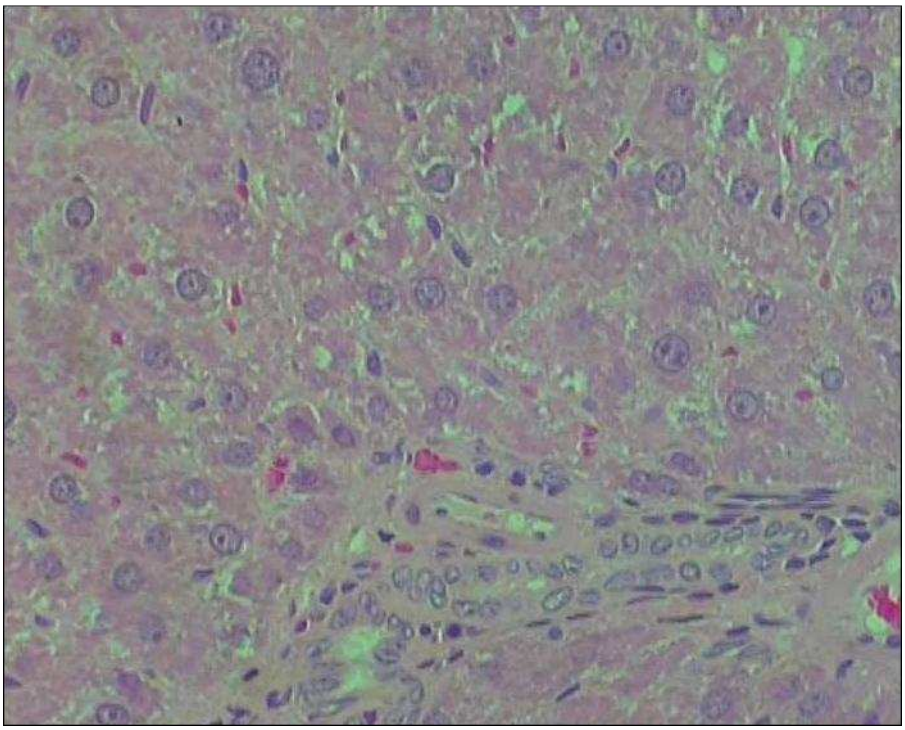

Figure 8.2 j): Liver section A from the PAR group after 42 days of treatment, showing periportal hepatocytes with minimal degeneration

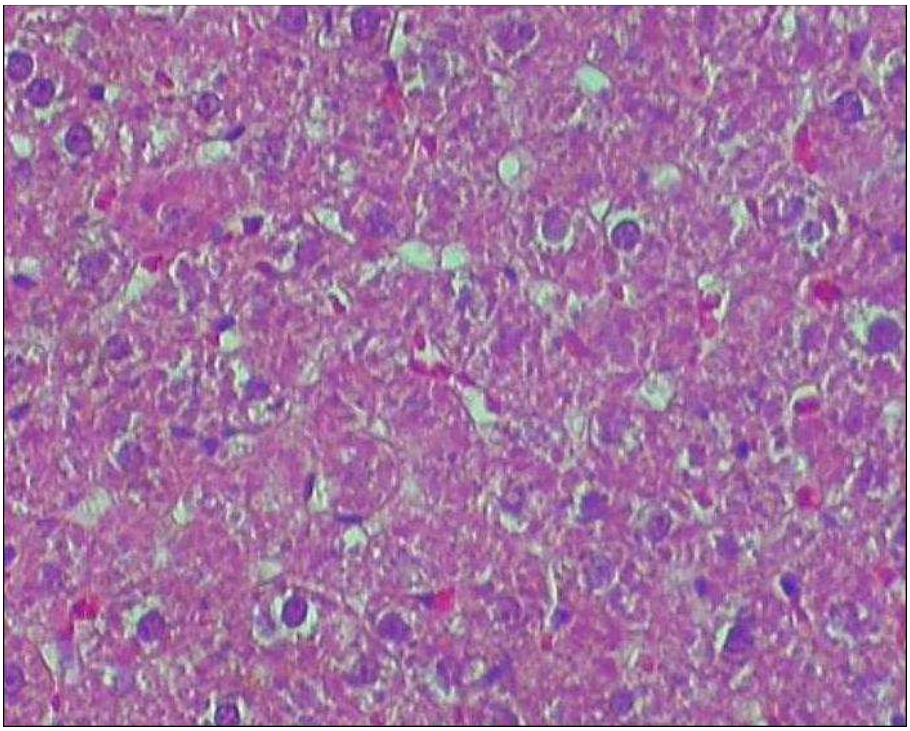

Figure 8.2 k): Liver section B from the PAR group after 42 days of treatment, showing mild vacuolar degeneration and cell swelling, as well as minimal cytonecrosis

Table 8.4: Tally of main pathology lesions (lesions score) in livers of untreated rats and the PAR group

Group	UnRx	PAR									
		2 Days		7 Days		14 Days		28 Days		42 Days	
(n = 2)	Fig.8.2a	Fig.8.2b	Fig.8.2c	Fig.8.2d	Fig.8.2e	Fig.8.2f	Fig.8.2g	Fig.8.2h	Fig.8.2i	Fig.8.2j	Fig.8.2k
Histopathological lesions											
Vacuolar degeneration	0	3+	3+	2+	2+	3+	3+	1+	1+	1+	2+
Cell swelling	0	3+	3+	2+	2+	3+	3+	1+	1+	1+	2+
Cytonecrosis	0	2+	2+	1+	1+	2+	1+	0	0	1+	1+
Centrilobular necrosis	0	0	1+	0	0	0	0	0	0	0	0
Hepatocyte mitosis	0	0	0	0	0	1+	1+	0	0	0	0
Average lesions score											
Vacuolar degeneration	0	3+		2+		3+		1+		1.5+	
Cell swelling	0	3+		2+		3+		1+		1.5+	
Cytonecrosis	0	2+		1+		1.5+		0		1+	
Centrilobular necrosis	0	0.5+		0		0		0		0	
Hepatocyte mitosis	0	0		0		1+		0		0	
Total lesion score	**0**	**8.5+**		**5+**		**8.5+**		**2+**		**4+**	

UnRx = untreated; PAR = paracetamol

8.3.2 Paracetamol concentrations

Table 8.5 shows paracetamol concentrations of the PAR group, while Figure 8.3 is a graphical illustration of the same. By day 42 paracetamol concentrations had declined (p = 0.0040).

Table 8.5: Average (mean ± SD) paracetamol concentrations of the PAR group

Group	**PAR**
	PAR concentration
(n = 5)	**(µg/ml)**
2 Days	0.786 ± 0.22
7 Days	0.721 ± 0.28
14 Days	0.535 ± 0.12
28 Days	0.637 ± 0.49
42 Days	0.046 ± 0.10

PAR = paracetamol

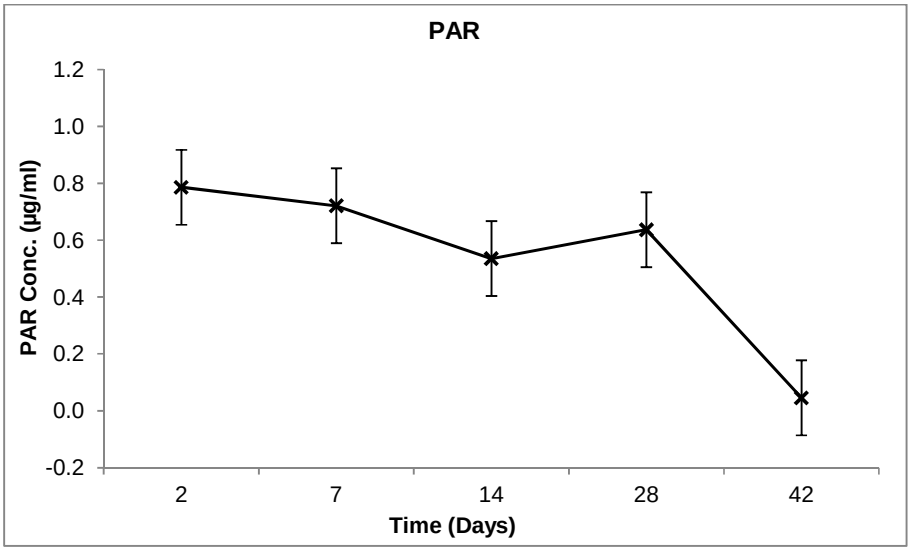

Figure 8.3: Paracetamol concentrations of the PAR group over 42 days

8.3.3 Specific immunology tests

8.3.3.1 Direct observations

Table 8.6 shows changes in body weight of the S and PAR groups over the treatment period. Both groups showed weight gain at all times, but the weight gain was greater in the S group from days 14 to 42 (Refer to Appendix H-1 and H-4 for baseline weights).

Table 8.6: Average (mean ± SD) change in rat weights of the S and PAR groups

Group (n = 5)	S change in weight (g)	PAR change in weight (g)
2 Days	9.2±4	9.7±6
7 Days	35.6±8	34.6±4
14 Days	84.6±5	62.6±13
28 Days	107.8±10	103.5±14
42 Days	171.4±27	147.5±20

S = saline; PAR = paracetamol

8.3.3.2 Cytokines

Table 8.7 shows IL-2 and IL-10 concentrations of the S and PAR groups, while Figures 8.4 a – b are graphical illustrations of the same. For the PAR group, IL-2 concentrations gradually declined till day 14, and had normalised by day 42 ($p = 0.0500$). In the S group, IL-2 concentrations were stable over the 42 days, and always higher than in the PAR group ($p = 0.0500$). IL-10 concentrations in both groups were stable over the 42 days, and higher in the S group on days 7 and 14 ($p = 0.0500$).

Table 8.7: Average (mean ± SD) cytokine concentrations of the S and PAR groups

Group	Cytokine	
(n = 3)	IL-2 (pg/ml)	IL-10 (pg/ml)
Untreated		
0 Days	65.46 ± 2.0	31.08 ± 1.2
S		
2 Days	74.87 ± 6.5	29.96 ± 2.8
7 Days	77.26 ± 5.8	34.58 ± 0.7
14 Days	77.85 ± 6.6	35.69 ± 5.4
28 Days	78.81 ± 4.6	32.46 ± 4.2
42 Days	74.39 ± 5.7	32.03 ± 2.5
PAR		
2 Days	66.66 ± 0.7	28.51 ± 2.4
7 Days	60.66 ± 4.6	28.33 ± 5.6
14 Days	58.01 ± 1.9	29.17 ± 2.3
28 Days	59.83 ± 2.9	29.35 ± 2.4
42 Days	65.40 ± 6.5	28.79 ± 3.1

IL = interleukin; S = saline; PAR = paracetamol

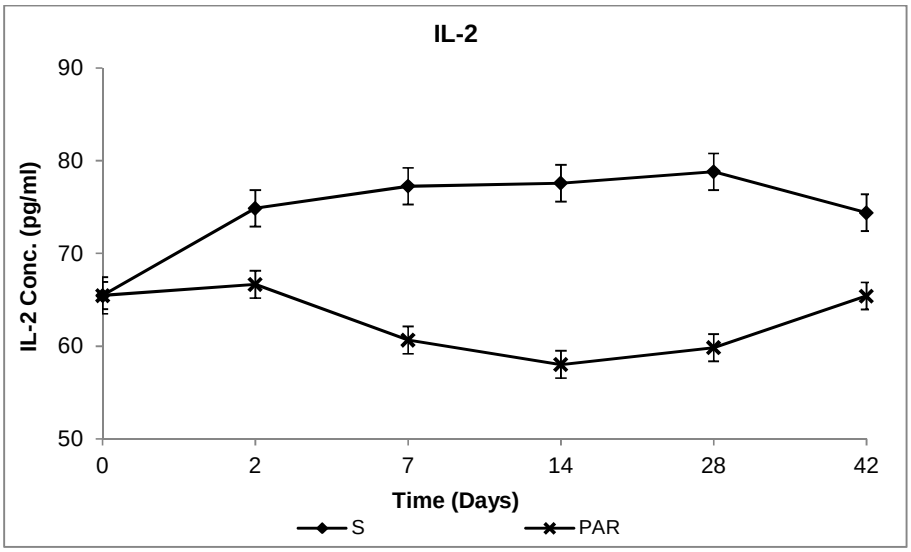

Figure 8.4 a): IL-2 concentrations of the S and PAR groups over 42 days

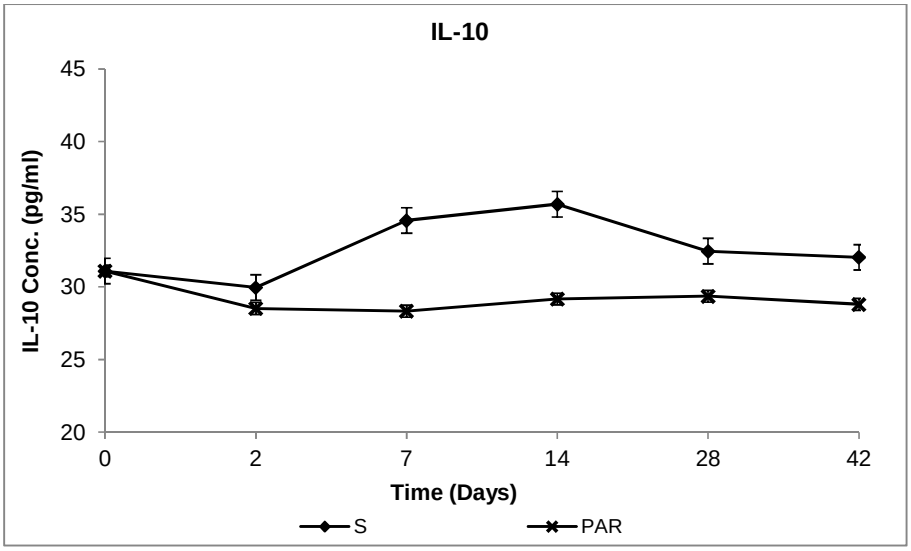

Figure 8.4 b): IL-10 concentrations of the S and PAR groups over 42 days

8.3.3.3 CD4 and CD8 counts

Table 8.8 shows CD4 and CD8 counts of the S and PAR groups, while Figures 8.5 a – b are graphical illustrations of the same. By day 42 in the PAR group, CD4 and CD8 counts had declined (p = 0.0500).

Table 8.8: Average (mean ± SD) CD4 and CD8 counts of the S and PAR groups

Group	**Ly**	**T-Ly**	
		CD4	**CD8**
(n = 3)	**(x10^9/l)**	**(x10^9/l)**	**(x10^9/l)**
Untreated			
0 Days	4.67±1.8	2.23±1.3	1.42±0.7
S			
2 Days	5.18±0.7	2.27±0.3	1.35±0.2
7 Days	4.07±2.0	1.72±0.8	1.07±0.5
14 Days	4.21±0.7	1.69±0.2	1.17±0.2
28 Days	6.15±0.8	2.45±0.2	1.58±0.3
42 Days	3.23±0.3	1.47±0.1	0.79±0.2
PAR			
2 Days	5.96±0.0	3.04±0.0	1.51±0.0
7 Days	6.22±0.8	2.53±0.3	1.66±0.3
14 Days	3.42±0.6	1.66±0.5	0.92±0.1
28 Days	4.19±1.1	1.84±0.1	1.01±0.5
42 Days	3.86±0.4	1.53±0.2	1.14±0.1

Ly = lymphocytes; CD = cluster of differentiation; S = saline; PAR = paracetamol

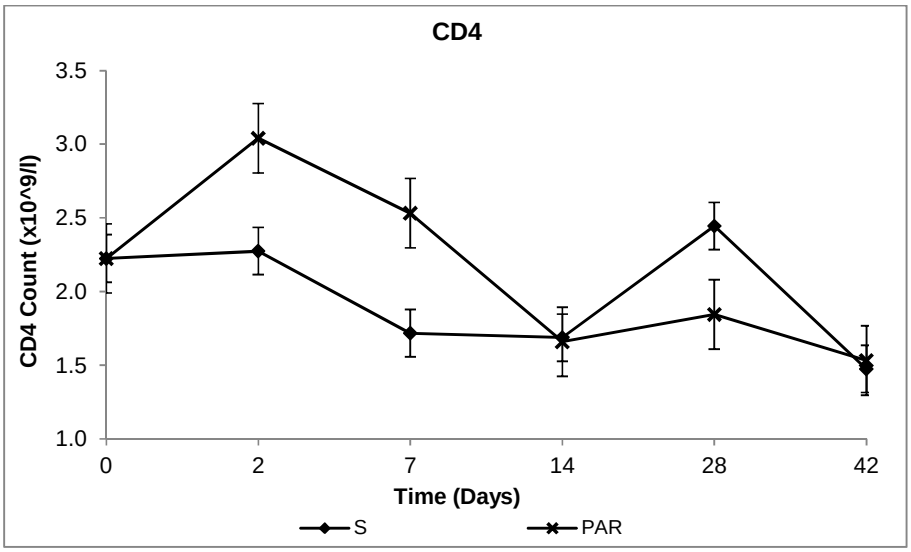

Figure 8.5 a): CD4 counts of the S and PAR groups over 42 days

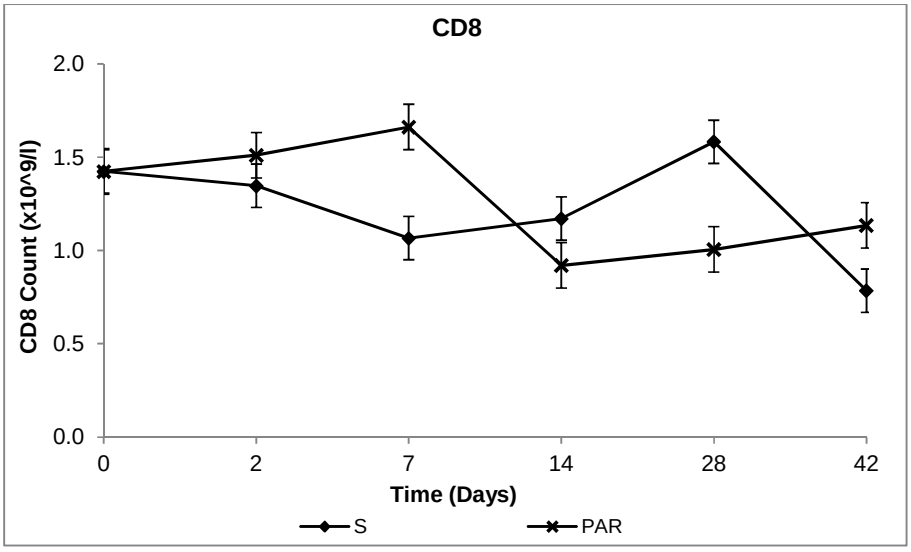

Figure 8.5 b): CD8 counts of the S and PAR groups over 42 days

8.3.3.4 Immunoglobulins

Table 8.9 shows concentrations of IgM and IgG of the S and PAR groups, while Figures 8.6 a – b are graphical illustrations of the same. By day 42 in the PAR group, IgM concentrations had declined (p = 0.0500). This was also seen in the S group, but was not statistically significant. IgG concentrations were stable in the PAR group over the 42 days, while in the S group they were elevated by day 42 (p = 0.0500), and higher than in the PAR group (p = 0.0500).

Table 8.9: Average (mean ± SD) immunoglobulin concentrations of the S and PAR groups

Group	**Immunoglobulin**	
	IgM	**IgG**
(n = 3)	**(mg/ml)**	**(mg/ml)**
Untreated		
0 Days	0.109 ± 0.02	14.434 ± 1.10
S		
2 Days	0.104 ± 0.04	14.137 ± 0.91
7 Days	0.110 ± 0.04	14.302 ± 0.70
14 Days	0.110 ± 0.03	12.617 ± 0.29
28 Days	0.075 ± 0.03	16.350 ± 1.00
42 Days	0.046 ± 0.01	17.109 ± 0.26
PAR		
2 Days	0.123 ± 0.02	11.745 ± 0.27
7 Days	0.088 ± 0.01	11.837 ± 2.16
14 Days	0.091 ± 0.02	12.919 ± 1.86
28 Days	0.074 ± 0.01	13.199 ± 2.46
42 Days	0.057 ± 0.01	13.135 ± 0.62

IgM = immunoglobulin M; IgG = immunoglobulin G; S = saline; PAR = paracetamol

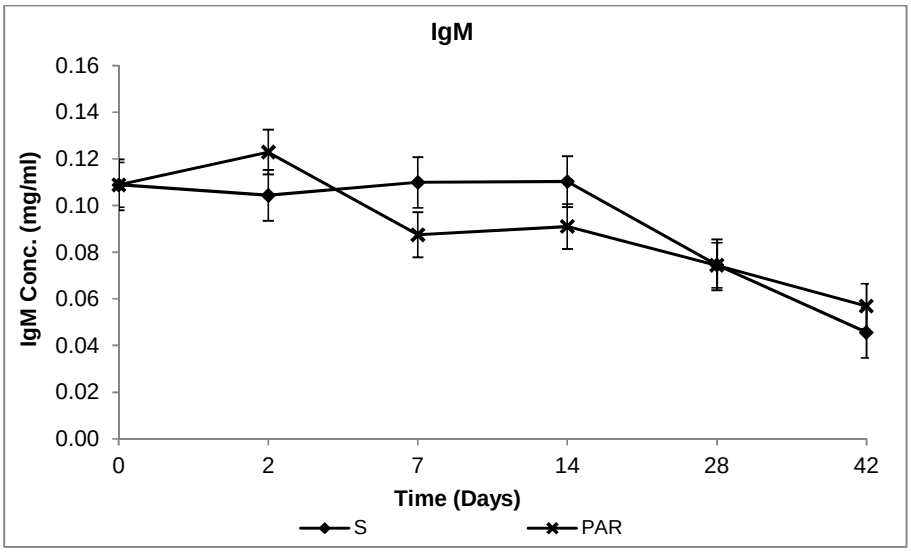

Figure 8.6 a): IgM concentrations of the S and PAR groups over 42 days

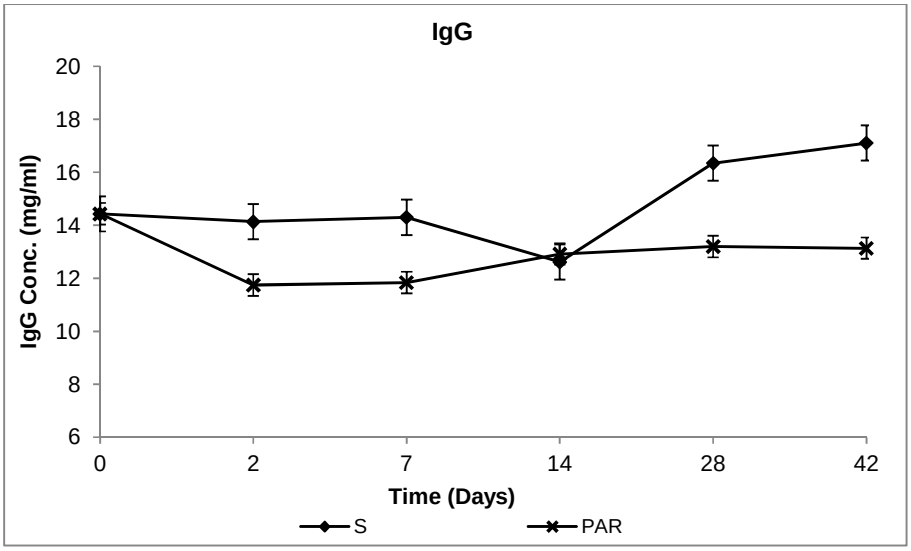

Figure 8.6 b): IgG concentrations of the S and PAR groups over 42 days

8.3.4 Activity of rat CYP1A2, CYP2E1 and CYP3A2 *in vivo*

8.3.4.1 Protein concentrations

Table 8.10 and Figure 8.7 show the results of BSA calibration samples, used for the protein assay, while Table 8.11 is that of microsomal protein concentrations of untreated rats and the PAR group. The absorption plot (Figure 8.7) is linear with a correlation coefficient (r^2) of 0.9914 and regression equation of y = 0.02x – 0.007. Final protein concentrations of microsomal liver samples from selected rats from the untreated and PAR groups were calculated as indicated in Table 8.11.

Table 8.10: Protein assay calibration data

BSA concentration (mg/ml)	Absorption (nm)
0.5	0.00
1	0.01
2.5	0.05
5	0.12
10	0.21

BSA = bovine serum albumin

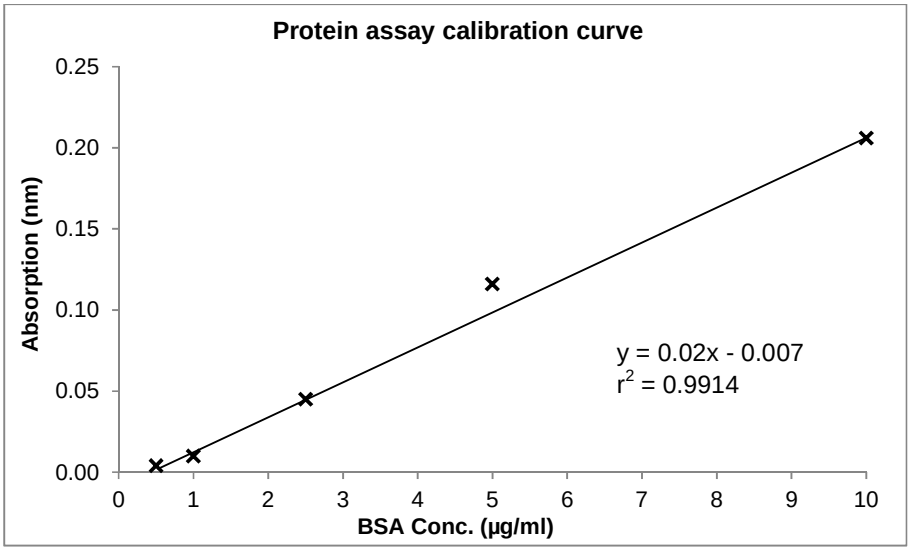

Figure 8.7: Calibration curve of BSA standards

Table 8.11: Average (mean ± SD) microsomal protein concentrations of the untreated and PAR groups

Selected liver (n = 3)	Protein concentration (mg/ml)	Absorption (nm)
Untreated		
Rat 1	49.40 ± 6.8	0.13 ± 0.0
Rat 2	55.02 ± 1.1	0.15 ± 0.0
Rat 4	51.00 ± 5.1	0.14 ± 0.0
PAR-2D		
Rat 1	20.05 ± 1.3	0.04 ± 0.0
Rat 2	44.32 ± 3.2	0.09 ± 0.0
Rat 4	28.98 ± 2.3	0.06 ± 0.0
PAR-7D		
Rat 1	39.70 ± 4.0	0.07 ± 0.0
Rat 2	29.54 ± 3.7	0.05 ± 0.0
Rat 5	30.24 ± 4.7	0.05 ± 0.0
PAR-14D		
Rat 1	33.47 ± 9.4	0.07 ± 0.0
Rat 3	36.06 ± 3.7	0.08 ± 0.0
Rat 4	28.98 ± 5.0	0.07 ± 0.0

PAR = paracetamol; D = days

8.3.4.2 CYP1A2, CYP2E1 and CYP3A2 activity in vivo

Table 8.12 shows CYP1A2, CYP2E1 and CYP3A2 reaction rates after 2, 7 and 14 days of paracetamol alone treatment, while Figures 8.8 a – c are graphical illustrations of the same. Treatment with paracetamol alone increased CYP1A2 activity on all occasions and this was different from the normal ($p = 0.0286$). CYP2E1 activity was elevated on day 2 ($p = 0.0119$), after which it declined but remained higher than the normal until day 7 ($p = 0.0119$). The effect of paracetamol alone on CYP3A2 activity was minimal and had no statistical significance.

Table 8.12: Average (mean ± SD) CYP1A2, CYP2E1 and CYP3A2 activity

Group (n = 3)	CYP1A2 (pmol/min*mg)	CYP2E1 (nmol/min*mg)	CYP3A2 (pmol/min*mg)
Untreated			
0 Days	4.40 ± 0.8	0.77 ± 0.1	84.63 ± 6.9
PAR			
2 Days	7.08 ± 1.1	2.02 ± 0.3	83.60 ± 6.5
7 Days	9.72 ± 0.6	1.23 ± 0.1	68.40 ± 7.5
14 Days	12.63 ± 1.4	0.74 ± 0.0	72.23 ± 5.2

PAR = paracetamol

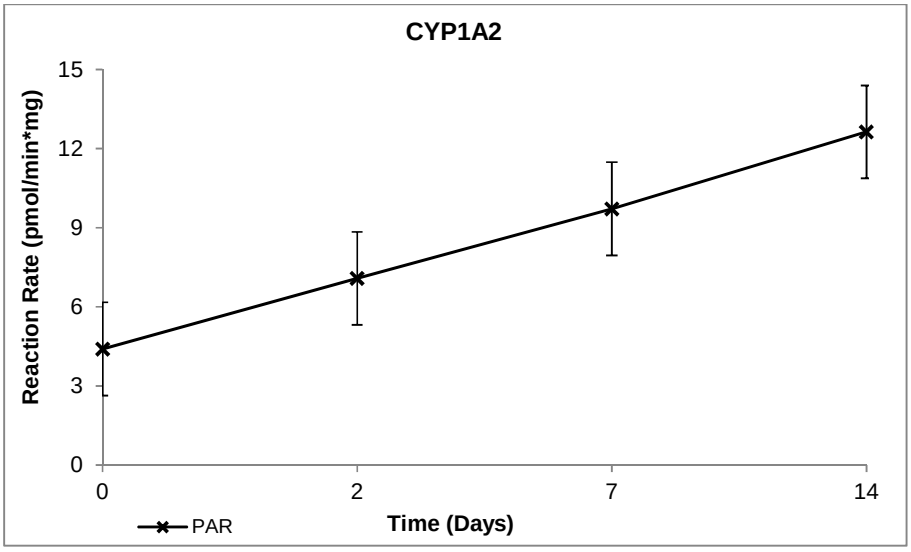

Figure 8.8 a): CYP1A2 activity after paracetamol alone treatment

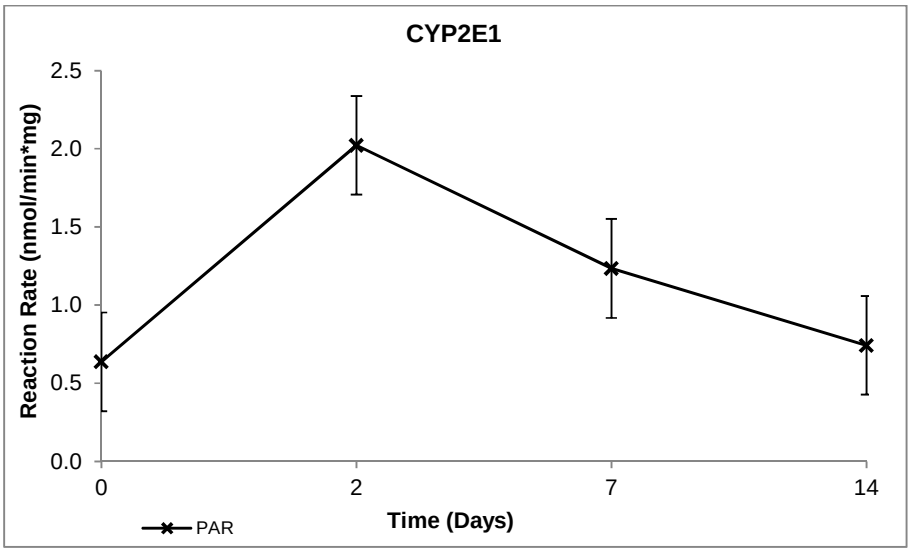

Figure 8.8 b): CYP2E1 activity after paracetamol alone treatment

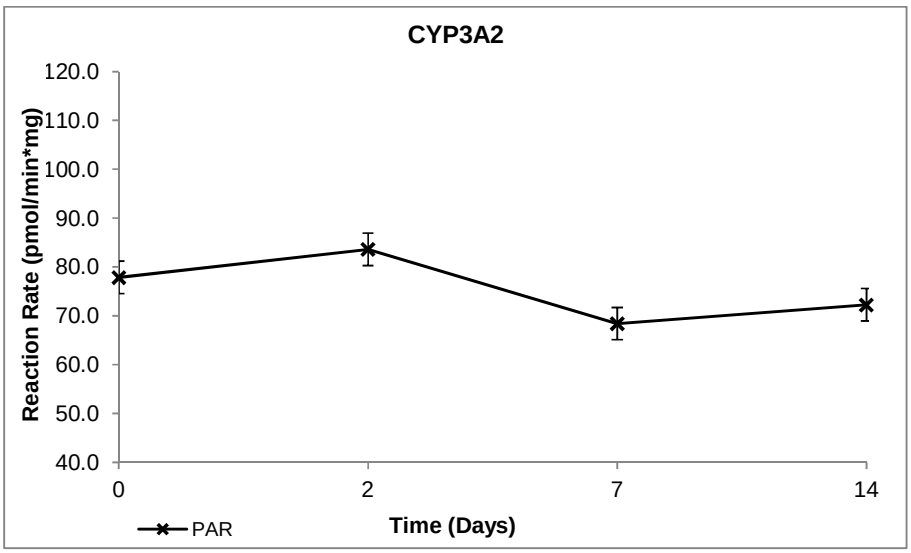

Figure 8.8 c): CYP3A2 activity after paracetamol alone treatment

8.3.5 Main observations

- ALT observations did not correlate with the histopathology changes and this implies that the liver injury was subclinical.
- From the histopathology, paracetamol alone caused liver injury up to 14 days, and by day 28 this had improved.
- By day 42 paracetamol concentrations had declined.
- On a cytokine signalling level, treatment with paracetamol alone did not stimulate IL-2 or IL-10 production.
- The lowered CD4 and CD8 counts correlate with the equally low IL-2 and IL-10 concentrations.
- Treatment with paracetamol alone had no profound effect on immunoglobulins.
- Paracetamol increased CYP1A2 and CYP2E1 activity.

B. Phase II: Co-treatment with an immune stimulant

8.3.6 Physiological observations (function tests)

8.3.6.1 Full blood count

Table 8.13 shows results of the full blood count of the S, S+LMS, PAR and PAR+LMS groups. By day 42 in the PAR+LMS group, the red cell count and haemoglobin were increased but not statistically significant, whilst the MCHC had increased significantly ($p = 0.0500$). On the other hand, the haematocrit, MCV and MCH had decreased ($p = 0.0500$). Also on day 42, the platelet count was higher in the PAR group than in the PAR+LMS group ($p = 0.0500$). For the white blood count, no significant changes were observed.

Table 8.13: Average (mean ± SD) full blood count and platelets results of the S, S+LMS, PAR and PAR+LMS groups

Group (n = 3)	RCC ($x10^{12}$/l)	Hb (g/dl)	Hct (l/l)	MCV (fl)	MCH (pg)	MCHC (g/dl)	Plt ($x10^9$/l)	WCC ($x10^9$/l)	Neu ($x10^9$/l)	Ly ($x10^9$/l)	Mo ($x10^9$/l)	Eos ($x10^9$/l)	Bas ($x10^9$/l)
Untreated													
0 Days	6.28±0.2	12.9±0.3	0.398±0.01	63.5±2.5	20.5±0.4	32.3±0.8	860±221.1	6.95±2.7	0.77±0.2	4.67±1.8	0.19±0.1	0.02±0.0	0.01±0.0
S													
2 Days	6.67±0.2	13.7±0.1	0.422±0.01	63.3±2.3	20.6±0.6	32.4±0.6	849±81.6	6.50±0.9	0.60±0.2	5.18±0.7	0.21±0.0	0.50±0.2	0.01±0.0
7 Days	7.53±0.9	15.3±1.7	0.451±0.04	60.1±2.5	20.3±0.2	33.9±1.2	1033±79.8	5.44±2.4	1.03±0.8	4.07±2.0	0.30±0.3	0.04±0.0	0.01±0.0
14 Days	6.77±0.6	13.9±1.1	0.417±0.03	61.8±2.8	20.5±0.6	33.2±0.6	721±196.4	5.22±1.2	0.63±0.5	4.21±0.7	0.18±0.1	0.18±0.1	0.05±0.0
28 Days	7.07±0.7	13.9±1.3	0.390±0.04	55.1±1.0	19.7±0.1	35.8±0.6	961±172.5	7.38±1.0	0.91±0.2	6.15±0.8	0.24±0.1	0.07±0.0	0.01±0.0
42 Days	6.93±0.8	13.4±1.8	0.374±0.05	53.9±1.0	19.3±0.4	35.8±0.2	839±166.0	3.93±0.3	0.54±0.1	3.23±0.3	0.11±0.0	0.04±0.0	0.01±0.0
S+LMS													
2 Days	6.53±0.4	13.2±0.6	0.408±0.03	62.5±0.5	20.3±0.5	32.4±0.7	755±239.0	7.33±0.2	0.66±0.0	5.99±0.4	0.35±0.0	0.34±0.1	0.01±0.0
7 Days	6.91±0.0	13.4±0.0	0.408±0.00	59.0±0.0	19.4±0.0	32.8±0.0	850±00.0	6.75±0.0	0.93±0.0	5.10±0.0	0.38±0.0	0.33±0.0	0.01±0.0
14 Days	6.99±0.4	13.8±0.8	0.427±0.02	61.1±1.6	19.8±0.1	32.4±0.8	718±204.1	4.65±1.7	0.53±0.3	3.82±1.8	0.12±0.1	0.18±0.3	0.00±0.0
28 Days	7.41±0.2	14.9±0.4	0.436±0.01	58.8±0.2	20.1±0.4	34.1±0.8	578±62.1	5.13±1.8	0.71±0.1	4.27±1.6	0.10±0.1	0.04±0.0	0.01±0.0
42 Days	7.91±0.2	15.6±0.4	0.455±0.02	57.5±0.7	19.7±0.2	34.2±0.5	701±116.7	6.76±0.8	0.85±0.1	5.69±0.9	0.16±0.1	0.04±0.0	0.01±0.0
PAR													
2 Days	6.77±0.0	14.8±0.0	0.461±0.00	68.1±0.0	21.9±0.0	32.1±0.0	419±0.0	7.54±0.0	0.75±0. 0	5.96±0.0	0.37±0.0	0.46±0.0	0.01±0.0
7 Days	6.75±0.2	13.9±0.1	0.426±0.00	63.2±2.4	20.7±0.8	32.7±0.1	807±22.8	7.32±0.8	0.80±0.2	6.22±0.8	0.23±0.0	0.07±0.0	0.01±0.0
14 Days	6.66±0.2	13.8±0.3	0.411±0.01	61.7±0.5	20.7±0.1	33.6±0.4	834±43.0	4.19±0.4	0.55±0.0	3.42±0.6	0.09±0.1	0.13±0.2	0.00±0.0
28 Days	7.10±0.2	14.1±0.2	0.397±0.00	56.0±2.2	19.8±0.8	35.4±0.4	880±87.0	5.32±1.2	0.88±0.1	4.19±1.1	0.21±0.1	0.04±0.0	0.01±0.0
42 Days	7.33±0.3	14.5±0.3	0.401±0.01	54.8±1.8	19.8±0.5	36.2±0.3	961±85.8	4.91±0.3	0.80±0.2	3.86±0.4	0.20±0.0	0.05±0.0	0.01±0.0
PAR+LMS													
2 Days	6.95±0.3	14.2±0.1	0.442±0.01	63.6±4.4	20.4±1.0	32.2±0.9	670±413.2	6.89±1.4	0.84±0.2	5.46±1.4	0.33±0.1	0.24±0.4	0.01±0.0
7 Days	6.79±0.4	13.9±0.8	0.444±0.02	65.4±1.0	20.4±0.7	31.2±0.5	862±143.1	6.35±1.7	0.69±0.1	5.30±1.6	0.30±0.1	0.04±0.0	0.01±0.0
14 Days	6.98±0.3	14.3±0.7	0.429±0.02	61.5±0.5	20.5±0.1	33.3±0.4	702±29.1	7.22±0.5	0.72±0.1	6.13±0.4	0.33±0.1	0.04±0.0	0.01±0.0
28 Days	7.22±0.2	14.6±0.3	0.433±0.01	60.0±0.8	20.3±0.2	33.8±0.3	611±84.6	5.45±1.4	0.61±0.1	4.67±1.3	0.15±0.1	0.03±0.0	0.00±0.0
42 Days	7.14±0.1	14.3±0.5	0.408±0.01	57.2±0.9	20.0±0.3	34.9±0.1	609±22.5	4.88±2.3	0.74±0.2	3.90±2.0	0.21±0.1	0.02±0.0	0.01±0.0

RCC = red cell count; Hb = haemoglobin; Hct = haematocrit; MCV = mean corpuscular volume; MCH = mean corpuscular haemoglobin; MCHC = mean corpuscular haemoglobin concentration; Plt = platelets; WCC = white cell count; Neu = neutrophils; Ly = lymphocytes; Mo = monocytes; Eos = eosinophils; Bas = basophils; S = saline; LMS = levamisole; PAR = paracetamol

8.3.6.2 Renal function tests

Table 8.14 shows the changes of BUN and Cr of the S, S+LMS, PAR and PAR+LMS groups. In all groups BUN and Cr levels were normal. Here, Cr levels also spiked on day 28, but were still within the normal range (p = 0.0500).

Table 8.14: Average (mean ± SD) renal function test results of the S, S+LMS, PAR and PAR+LMS groups

Group	**RFT**		**Group**	**RFT**	
	BUN	**Cr**		**BUN**	**Cr**
(n = 3)	**(mmol/l)**	**(µmol/l)**	**(n = 3)**	**(mmol/l)**	**(µmol/l)**
Untreated					
0 Days	7.2 ± 1	37 ± 8			
S			**PAR**		
2 Days	7.3 ± 1	39 ± 2	2 Days	6.7 ± 1	36 ± 6
7 Days	8.1 ± 0	46 ± 7	7 Days	7.8 ± 1	37 ± 5
14 Days	7.5 ± 1	39 ± 3	14 Days	7.3 ± 1	36 ± 2
28 Days	10.6 ± 2	73 ± 17	28 Days	7.3 ± 1	73 ± 4
42 Days	5.8 ± 1	38 ± 9	42 Days	6.9 ± 1	24 ± 5
S+LMS			**PAR+LMS**		
2 Days	8.3 ±1	38 ± 5	2 Days	6.2 ± 1	27 ± 9
7 Days	7.8 ± 1	40 ± 18	7 Days	5.8 ± 0	33 ± 11
14 Days	6.5 ± 1	35 ± 5	14 Days	6.9 ± 1	35 ± 3
28 Days	6.1 ± 0	60 ± 3	28 Days	5.4 ± 0	63 ± 2
42 Days	6.5 ± 1	21 ± 1	42 Days	6.6 ± 1	13 ± 7

RFT = renal function test; BUN = blood urea nitrogen; Cr = creatinine; S = saline; LMS = levamisole; PAR = paracetamol

8.3.6.3 Liver function tests

Table 8.15 shows the changes of ALT, AST and ALP of the S, S+LMS, PAR and PAR+LMS. Over the 42 days, the results were similar in all groups.

Table 8.15: Average (mean ± SD) liver function test results of the S, S+LMS, PAR and PAR+LMS groups

Group	LFT			Group	LFT		
	ALT	AST	ALP		ALT	AST	ALP
(n = 3)	(U/l)	(U/l)	(U/l)	(n = 3)	(U/l)	(U/l)	(U/l)
Untreated							
0 Days	50±5	88±14	352±76				
S				**PAR**			
2 Days	46±2	90±7	400±7	2 Days	54±4	109±6	402±64
7 Days	49±10	103±25	304±13	7 Days	58±2	104±30	349±27
14 Days	58±4	127±37	508±37	14 Days	53±2	95±12	364±38
28 Days	47±2	115±44	216±19	28 Days	49±4	122±35	224±46
42 Days	46±6	76±28	109±76	42 Days	48±5	64±3	55±64
S+LMS				**PAR+LMS**			
2 Days	40±3	113±53	541±9	2 Days	53±11	138±19	322±33
7 Days	52±15	90±27	483±130	7 Days	58±8	99±19	407±59
14 Days	48±12	73±16	478±105	14 Days	48±1	93±18	366±14
28 Days	50±7	75±19	127±63	28 Days	45±4	61±1	32±14
42 Days	46±2	75±6	24±11	42 Days	46±3	74±9	12±4

LFT = liver function test; ALT = alanine aminotransferase; AST = aspartate aminotransferase; ALP = alkaline phosphatase; S = saline; LMS = levamisole; PAR = paracetamol

8.3.6.4 Liver histopathology

(a) Liver histopathology reports

Liver sections for histopathology (Figures 6.10 a – j and 8.9 a – j) were randomly selected, and the main histopathology lesions are summarised in the tally tables (Tables 8.16 a and b). The following report is a summary of the features of the lesion:

i. Figures 6.10 a and b: Liver sections A and B from the S+LMS group after 2 days of saline and levamisole co-treatment

The report is the same as in Chapter 6, Section 6.3.6.4 i.

ii. Figures 6.10 c and d: Liver sections A and B from the S+LMS group after 7 days of saline and levamisole co-treatment

The report is the same as in Chapter 6, Section 6.3.6.4 ii.

iii. Figures 6.10 e and f: Liver sections A and B from the S+LMS group after 14 days of saline and levamisole co-treatment

The report is the same as in Chapter 6, Section 6.3.6.4 iii.

iv. Figures 6.10 g and h: Liver sections A and B from the S+LMS group after 28 days of saline and levamisole co-treatment

The report is the same as in Chapter 6, Section 6.3.6.4 iv.

v. Figures 6.10 i and j: Liver sections A and B from the S+LMS group after 42 days of saline and levamisole co-treatment

The report is the same as in Chapter 6, Section 6.3.6.4 v.

vi. Figures 8.9 a and b: Liver sections A and B from the PAR+LMS group after 2 days of paracetamol and levamisole co-treatment

Representative photographs of rat livers after 2 days of daily paracetamol and levamisole co-treatment. The report: “In liver section A, vacuolar degeneration is mild, while minimal single cell necrosis could also be demonstrated in the liver parenchyma. Section B showed moderate degenerative changes and mild cytonecrosis.”

vii. Figures 8.9 c and d: Liver sections A and B from the PAR+LMS group after 7 days of paracetamol and levamisole co-treatment

Representative photographs of rat livers after 7 days of daily paracetamol and levamisole co-treatment. The report: “Minimal cytoplasmic swelling and single cell necrosis are present within liver sections A and B.”

viii. Figures 8.9 e and f: Liver sections A and B from the PAR+LMS group after 14 days of paracetamol and levamisole co-treatment

Representative photographs of rat livers after 14 days of daily paracetamol and levamisole co-treatment. The report: “Only minimal cellular swelling and vacuolar degeneration were seen in both liver sections. No cytonecrosis, centrilobular zonal necrosis, or hepatocyte mitosis were visible.”

ix. Figures 8.9 g and h: Liver sections A and B from the PAR+LMS group after 28 days of paracetamol and levamisole co-treatment

Representative photographs of rat livers after 28 days of daily paracetamol and levamisole co-treatment. The report: “Minimal granular vacuolar degeneration and cell swelling were observed in both liver sections, as well as minimal cytonecrosis in

section A. Furthermore, there was no sign of centrilobular zonal necrosis, while hepatocyte mitosis was seen in section B."

x. Figures 8.9 i and j: Liver sections A and B from the PAR+LMS group after 42 days of paracetamol and levamisole co-treatment
Representative photographs of rat livers after 42 days of daily paracetamol and levamisole co-treatment. The report: "Hepatocyte cytoplasmic granular vacuolar changes and single cell necrosis are minimal in liver sections A and B."

In view of the histopathology photographs (Figures 8.9 a – j), reports and tally tables (Tables 8.16 a and b), it was concluded that co-treatment with paracetamol and levamisole did not cause the same degree of liver injury as paracetamol alone.

(b) Liver histopathology photographs
Histopathology photographs of the S+LMS group (Figures 6.10 a – j) are presented in Chapter 6, Section 6.3.6.4 b. Figures 8.9 a – j are representative of randomly selected liver sections of the PAR+LMS group.

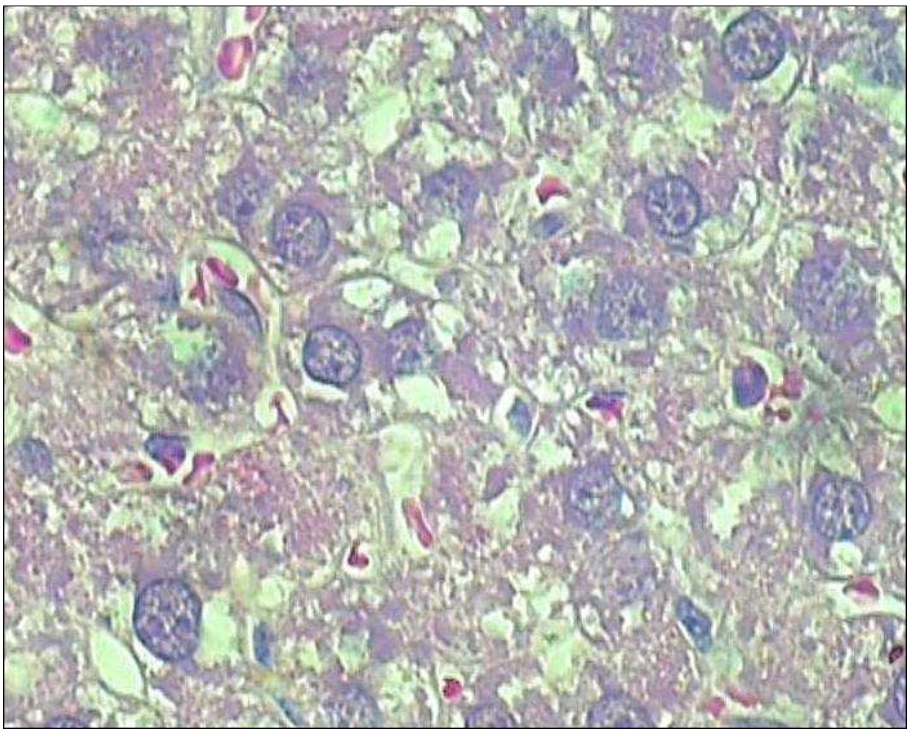

Figure 8.9 a): Liver section A from the PAR+LMS group after 2 days of treatment, showing mild vacuolar degeneration and minimal single cell necrosis

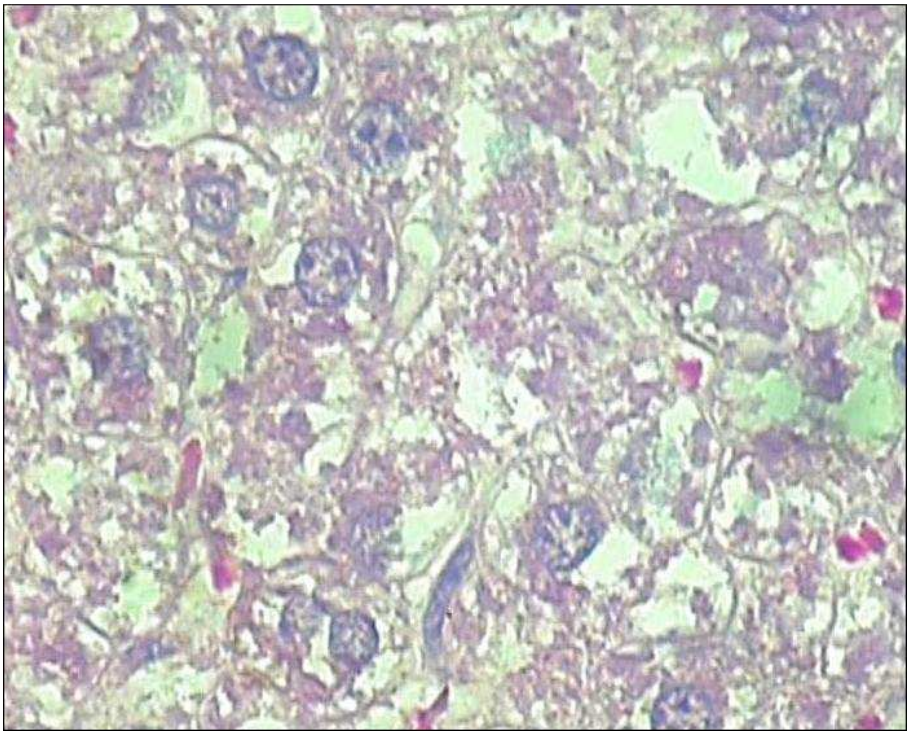

Figure 8.9 b): Liver section B from the PAR+LMS group after 2 days of treatment, showing moderate degenerative changes and mild cytonecrosis

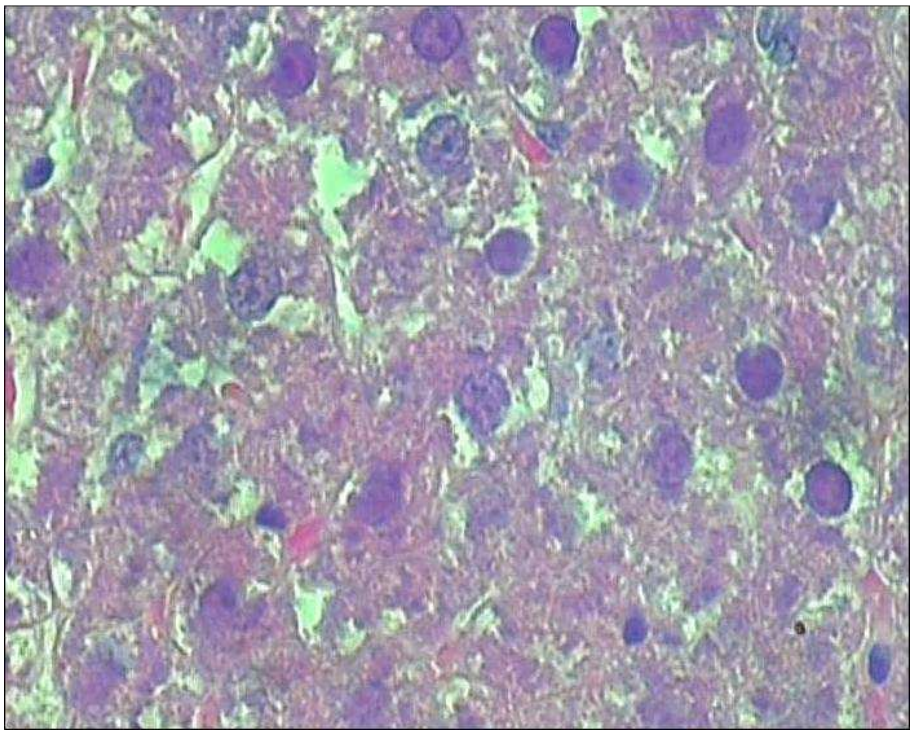

Figure 8.9 c): Liver section A from the PAR+LMS group after 7 days of treatment, showing minimal cytoplasmic swelling and single cell necrosis

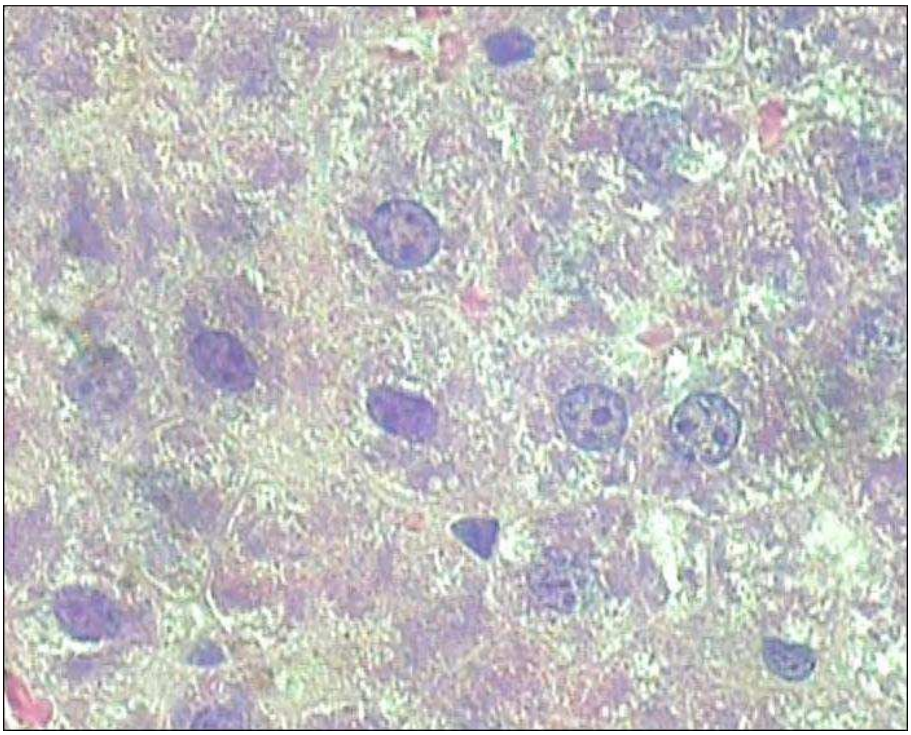

Figure 8.9 d): Liver section B from the PAR+LMS group after 7 days of treatment, showing minimal degeneration and single cell necrosis

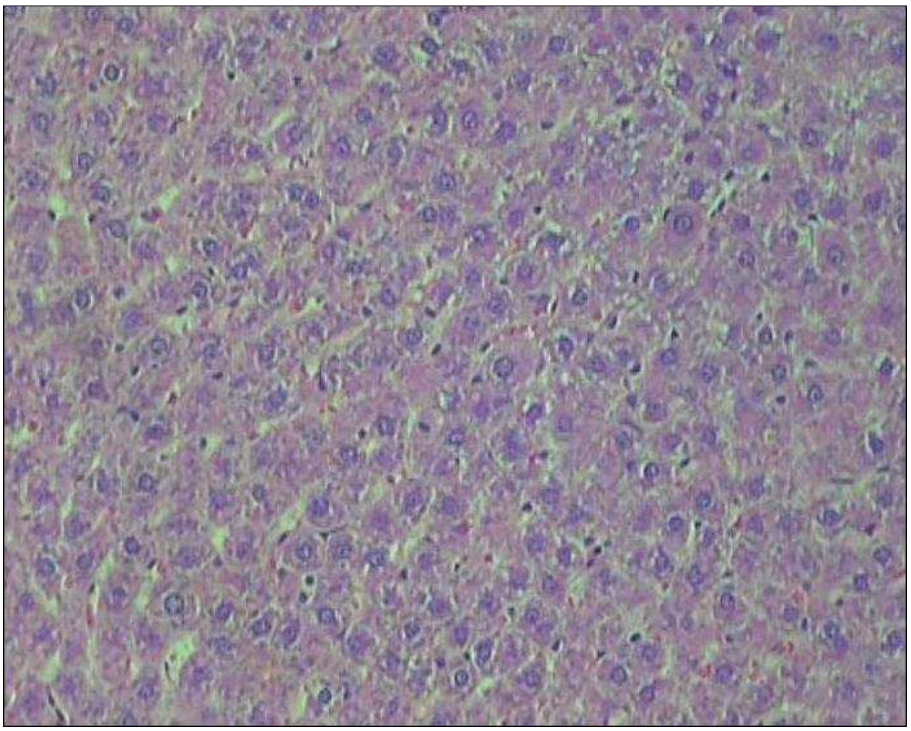

Figure 8.9 e): Liver section A from the PAR+LMS group after 14 days of treatment, showing hepatocytes with normal cords, and only minimal cellular swelling and vacuolar degeneration

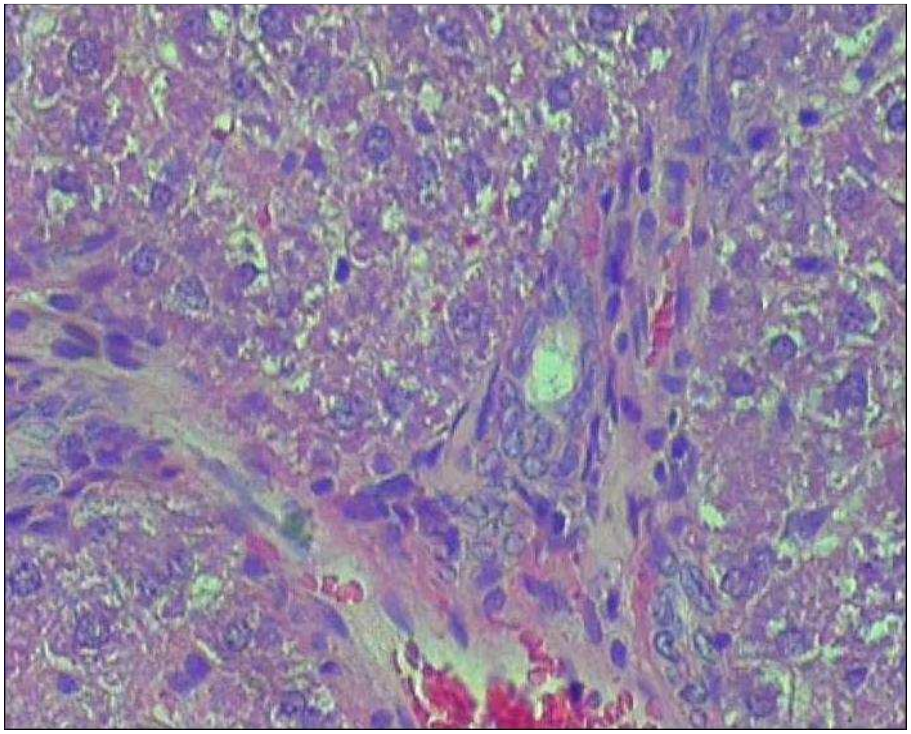

Figure 8.9 f): Liver section B from the PAR+LMS group after 14 days of treatment, showing some degeneration in the portal area

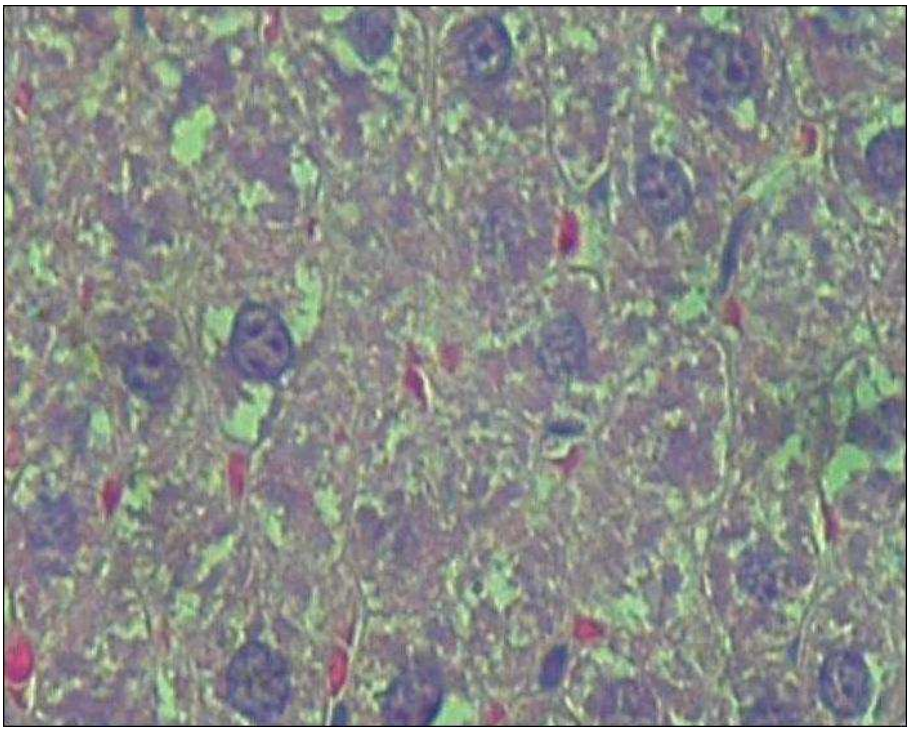

Figure 8.9 g): Liver section A from the PAR+LMS group after 28 days of treatment, showing minimal granular vacuolar degeneration, cell swelling and cytonecrosis

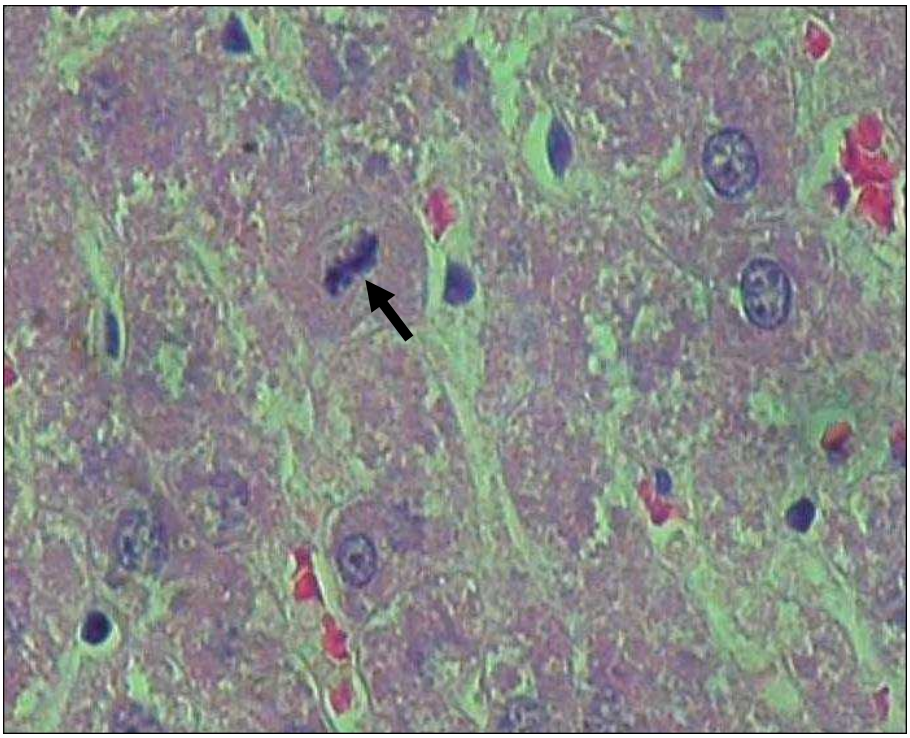

Figure 8.9 h): Liver section B from the PAR+LMS group after 28 days of treatment, showing minimal degeneration and cell swelling, with a mitotic figure

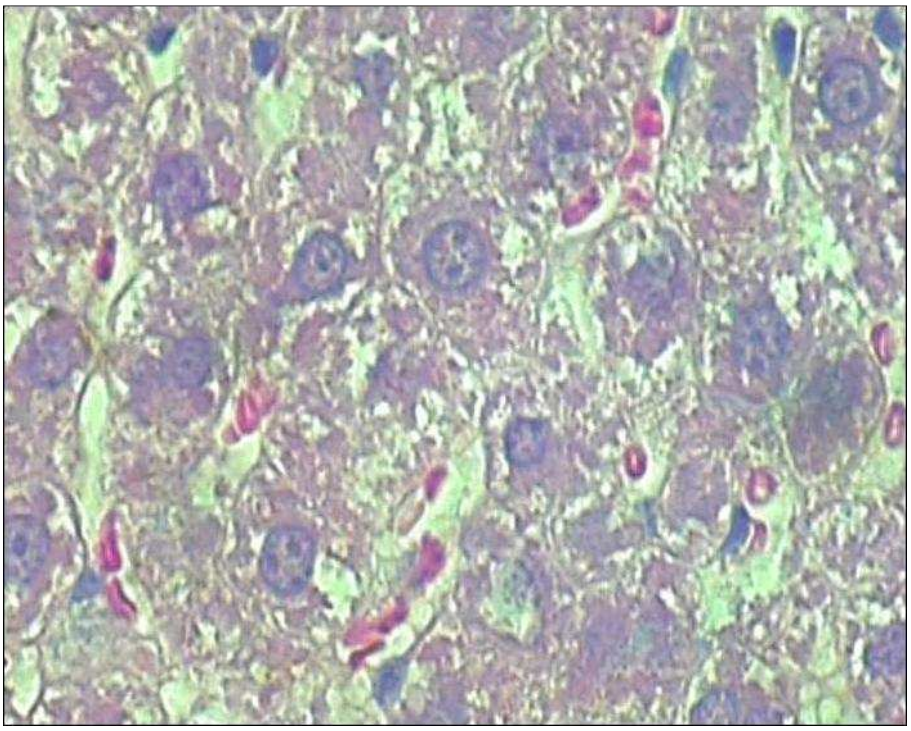

Figure 8.9 i): Liver section A from the PAR+LMS group after 42 days of treatment, showing minimal degeneration, cytoplasmic swelling and cytonecrosis

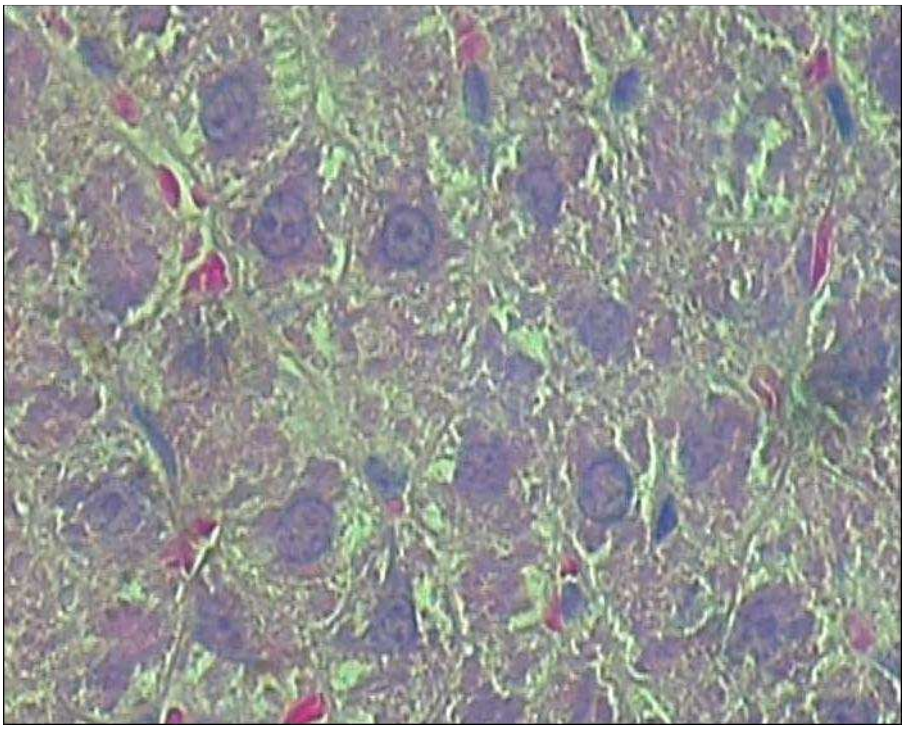

Figure 8.9 j): Liver section B from the PAR+LMS group after 42 days of treatment, showing minimal hepatocyte cytoplasmic granular vacuolar changes and single cell necrosis

Table 8.16 a): Tally of main pathology lesions (lesions score) in livers of untreated rats and the S+LMS group

Group	UnRx	S+LMS									
		2 Days		7 Days		14 Days		28 Days		42 Days	
(n = 2)	Fig.6.3a	Fig.6.10a	Fig.6.10b	Fig.6.10c	Fig.6.10d	Fig.6.10e	Fig.6.10f	Fig.6.10g	Fig.6.10h	Fig.6.10i	Fig.6.10j
Histopathological lesions											
Vacuolar degeneration	0	0	0	0	0	0	1+	1+	1+	1+	1+
Cell swelling	0	0	0	0	0	0	1+	1+	1+	1+	1+
Cytonecrosis	0	0	0	0	0	0	0	0	0	0	0
Centrilobular necrosis	0	0	0	0	0	0	0	0	0	0	0
Hepatocyte mitosis	0	0	0	1+	2+	0	0	0	1+	0	0
Average lesions score											
Vacuolar degeneration	0	0		0		0.5+		1+		1+	
Cell swelling	0	0		0		0.5+		1+		1+	
Cytonecrosis	0	0		0		0		0		0	
Centrilobular necrosis	0	0		0		0		0		0	
Hepatocyte mitosis	0	0		1.5+		0		0.5+		0	
Total lesion score	**0**	**0**		**1.5+**		**1+**		**2.5+**		**2+**	

UnRx = untreated; S = saline; LMS = levamisole

Table 8.16 b): Tally of main pathology lesions (lesions score) in livers of untreated rats and the PAR+LMS group

Group	UnRx	PAR+LMS									
		2 Days		7 Days		14 Days		28 Days		42 Days	
(n = 2)	Fig.8.2a	Fig.8.9a	Fig.8.9b	Fig.8.9c	Fig.8.9d	Fig.8.9e	Fig.8.9f	Fig.8.9g	Fig.8.9h	Fig.8.9i	Fig.8.9j
Histopathological lesions											
Vacuolar degeneration	0	2+	3+	1+	1+	1+	1+	1+	1+	1+	1+
Cell swelling	0	2+	3+	1+	1+	1+	1+	1+	1+	1+	1+
Cytonecrosis	0	1+	2+	1+	1+	0	0	1+	0	1+	1+
Centrilobular necrosis	0	0	0	0	0	0	0	0	0	0	0
Hepatocyte mitosis	0	0	0	0	0	0	0	1+	1+	0	0
Average lesions score											
Vacuolar degeneration	0	2.5+		1+		1+		1+		1+	
Cell swelling	0	2.5+		1+		1+		1+		1+	
Cytonecrosis	0	1.5+		1+		0		0.5+		1+	
Centrilobular necrosis	0	0		0		0		0		0	
Hepatocyte mitosis	0	0		0		0		1+		0	
Total lesion score	**0**	**6.5+**		**3+**		**2+**		**3.5+**		**3+**	

UnRx = untreated; PAR = paracetamol; LMS = levamisole

8.3.7 Paracetamol concentrations

Table 8.17 shows paracetamol concentrations of the PAR and PAR+LMS groups, while Figure 8.10 is a graphical illustration of the same. By day 42, paracetamol concentrations in the PAR+LMS group had declined ($p = 0.0278$). Overall, paracetamol levels in the PAR group were higher than in the PAR+LMS group, and most different on day 7 ($p = 0.0079$).

Table 8.17: Average (mean ± SD) paracetamol concentrations of the PAR and PAR+LMS groups

Group	PAR	PAR+LMS
	PAR concentration	PAR concentration
(n = 5)	(µg/ml)	(µg/ml)
2 Days	0.786 ± 0.22	0.879 ± 0.61
7 Days	0.721 ± 0.28	0.279 ± 0.14
14 Days	0.535 ± 0.12	0.379 ± 0.34
28 Days	0.637 ± 0.49	0.359 ± 0.49
42 Days	0.046 ± 0.10	0.000 ± 0.00

PAR = paracetamol; LMS = levamisole

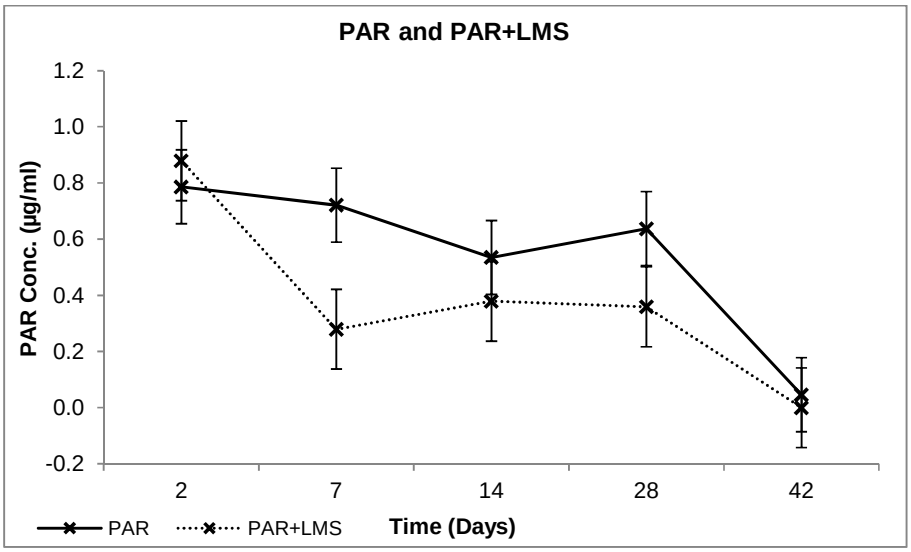

Figure 8.10: Paracetamol concentrations of the PAR and PAR+LMS groups over 42 days

8.3.8 Specific immunology tests

8.3.8.1 Direct observations

Table 8.18 shows changes in body weight of the S, S+LMS, PAR and PAR+LMS groups over the treatment period. All groups showed weight gain at all times, except for the S+LMS and PAR+LMS groups after 2 days (Refer to Appendix H-1 and H-4 for baseline weights).

Table 8.18: Average (mean ± SD) change in rat weights of the S, S+LMS, PAR and PAR+LMS groups

Group (n = 5)	S change in weight (g)	S+LMS change in weight (g)	PAR change in weight (g)	PAR+LMS change in weight (g)
2 Days	9.2±4	-1.4±1	9.7±6	-5.1±2
7 Days	35.6±8	19.4±10	34.6±4	29.4±3
14 Days	84.6±5	57.0±26	62.6±13	58.9±2
28 Days	107.8±10	96.1±13	103.5±14	105.4±30
42 Days	171.4±27	153.4±23	147.5±20	112.3±16

S = saline; LMS = levamisole; PAR = paracetamol

8.3.8.2 Cytokines

Table 8.19 shows IL-2 and IL-10 concentrations of the S, S+LMS, PAR and PAR+LMS groups, while Figures 8.11 a – b are graphical illustrations of the same. Throughout the treatment period IL-2 concentrations in the PAR+LMS groups were unchanged and always lower than in the PAR group (p = 0.0500). By day 42, IL-10 concentrations had decreased (p = 0.0500), but were significantly higher than in the PAR groups until day 14 (p = 0.0500).

Table 8.19: Average (mean ± SD) cytokine concentrations of the S, S+LMS, PAR and PAR+LMS groups

Group	Cytokine		Group	Cytokine	
	IL-2	IL-10		IL-2	IL-10
(n = 3)	(pg/ml)	(pg/ml)	(n = 3)	(pg/ml)	(pg/ml)
Untreated					
0 Days	65.46 ± 2.0	31.08 ± 1.2			
S			**PAR**		
2 Days	74.87 ± 6.5	29.96 ± 2.8	2 Days	66.66 ± 0.7	28.51 ± 2.4
7 Days	77.26 ± 5.8	34.58 ± 0.7	7 Days	60.66 ± 4.6	28.33 ± 5.6
14 Days	77.85 ± 6.6	35.69 ± 5.4	14 Days	58.01 ± 1.9	29.17 ± 2.3
28 Days	78.81 ± 4.6	32.46 ± 4.2	28 Days	59.83 ± 2.9	29.35 ± 2.4
42 Days	74.39 ± 5.7	32.03 ± 2.5	42 Days	65.40 ± 6.5	28.79 ± 3.1
S+LMS			**PAR+LMS**		
2 Days	62.89 ± 2.2	30.57 ± 0.9	2 Days	41.00 ± 9.6	38.43 ± 1.1
7 Days	62.89 ± 0.8	31.31 ± 2.0	7 Days	45.67 ± 16.6	38.52 ± 2.7
14 Days	62.61 ± 9.4	32.44 ± 6.5	14 Days	46.33 ± 8.1	40.11 ± 1.4
28 Days	65.82 ± 3.7	29.82 ± 1.7	28 Days	38.50 ± 8.8	34.69 ± 3.1
42 Days	69.45 ± 3.6	29.26 ± 3.1	42 Days	38.00 ± 5.7	30.94 ± 5.6

IL = interleukin; S = saline; LMS = levamisole; PAR = paracetamol

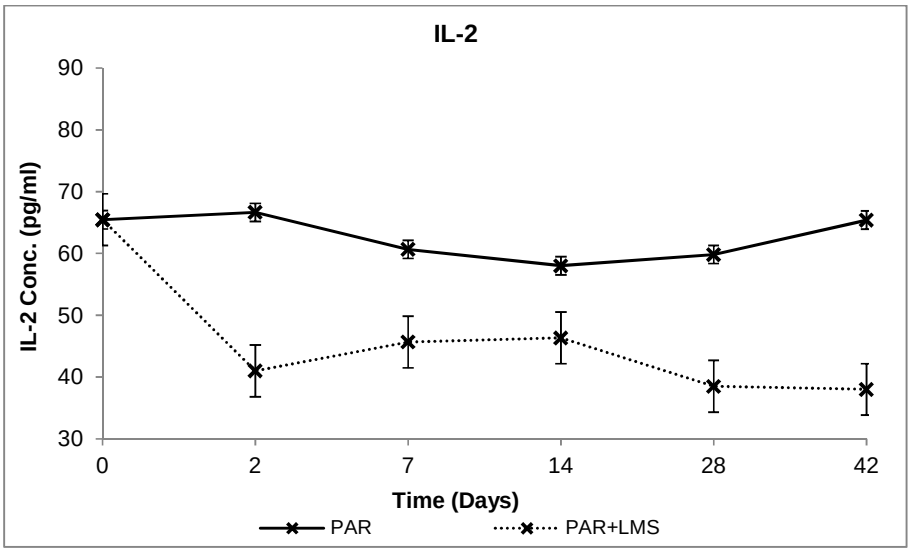

Figure 8.11 a): IL-2 concentrations of the PAR and PAR+LMS groups over 42 days

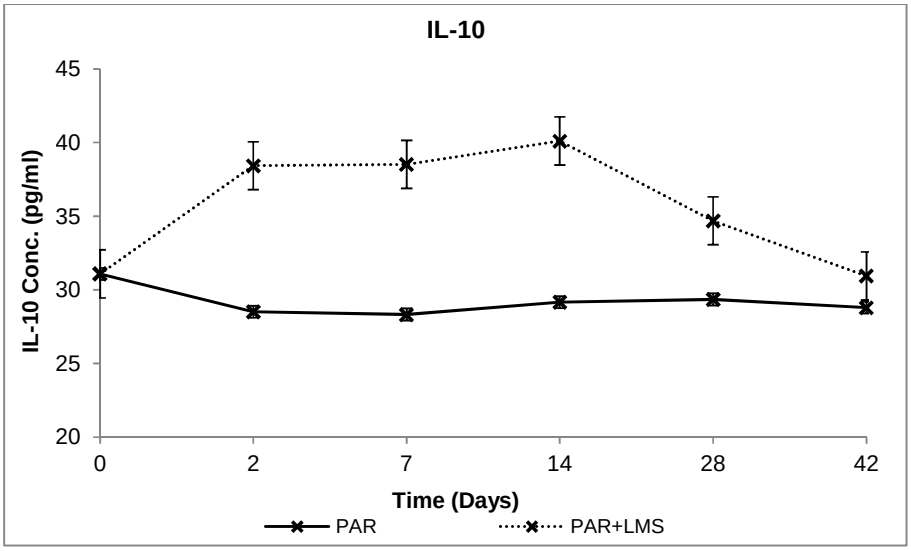

Figure 8.11 b): IL-10 concentrations of the PAR and PAR+LMS groups over 42 days

8.3.8.3 CD4 and CD8 counts

Table 8.20 shows CD4 and CD8 counts of the S, S+LMS, PAR and PAR+LMS groups, while Figures 8.12 a – b are graphical illustrations of the same. By day 42 in the PAR+LMS group, the CD4 count had become lower but this had no statistical significance, while the CD8 count had declined completely (p = 0.0500). The CD8 count was also lower than in the PAR group on day 42 (p = 0.0500).

Table 8.20: Average (mean ± SD) CD4 and CD8 counts of the S, S+LMS, PAR and PAR+LMS groups

Group	**Ly**	**T-Ly**		**Group**	**Ly**	**T-Ly**	
		CD4	**CD8**			**CD4**	**CD8**
(n = 3)	**(x10^9/l)**	**(x10^9/l)**	**(x10^9/l)**	**(n = 3)**	**(x10^9/l)**	**(x10^9/l)**	**(x10^9/l)**
Untreated							
0 Days	4.67±1.8	2.23±1.3	1.42±0.7				
S				**PAR**			
2 Days	5.18±0.7	2.27±0.3	1.35±0.2	2 Days	5.96±0.0	3.04±0.0	1.51±0.0
7 Days	4.07±2.0	1.72±0.8	1.07±0.5	7 Days	6.22±0.8	2.53±0.3	1.66±0.3
14 Days	4.21±0.7	1.69±0.2	1.17±0.2	14 Days	3.42±0.6	1.66±0.5	0.92±0.1
28 Days	6.15±0.8	2.45±0.2	1.58±0.3	28 Days	4.19±1.1	1.84±0.1	1.01±0.5
42 Days	3.23±0.3	1.47±0.1	0.79±0.2	42 Days	3.86±0.4	1.53±0.2	1.14±0.1
S+LMS				**PAR+LMS**			
2 Days	5.99±0.4	2.25±0.1	1.58±0.0	2 Days	5.46±1.4	2.38±0.7	1.32±0.2
7 Days	5.10±0.0	2.09±0.0	1.34±0.0	7 Days	5.30±1.6	2.25±0.8	1.24±0.2
14 Days	3.82±1.8	1.64±0.8	0.97±0.5	14 Days	6.13±0.4	2.60±0.3	1.56±0.1
28 Days	4.27±1.6	1.69±0.6	1.15±0.5	28 Days	4.67±1.3	1.91±0.6	1.23±0.2
42 Days	5.69±0.9	2.26±0.3	0.01±0.0	42 Days	3.90±2.0	1.68±0.7	0.00±0.0

Ly = lymphocytes; CD = cluster of differentiation; S = saline; LMS = levamisole; PAR = paracetamol

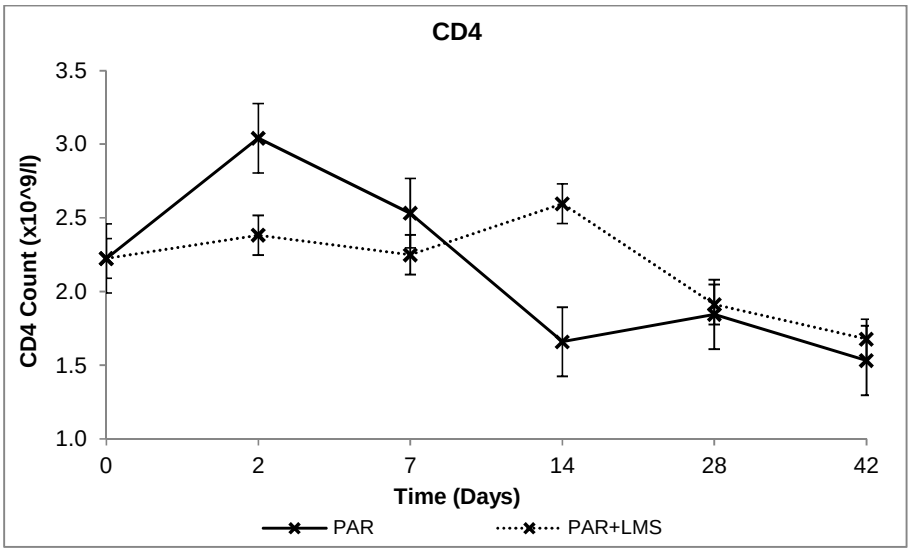

Figure 8.12 a): CD4 counts of the PAR and PAR+LMS groups over 42 days

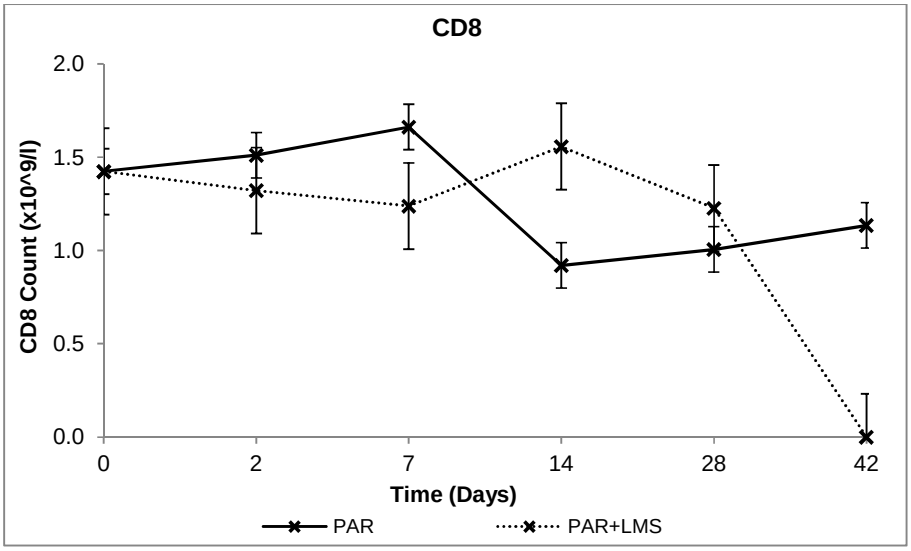

Figure 8.12 b): CD8 counts of the PAR and PAR+LMS groups over 42 days

8.3.8.4 Immunoglobulins

Table 8.21 shows concentrations of IgM and IgG of the S, S+LMS, PAR and PAR+LMS groups, while Figures 8.13 a – b are graphical illustrations of the same. By day 42 in the PAR+LMS group, IgM concentrations had increased ($p = 0.0500$). It was significantly lower than in the PAR group until day 14, but higher by day 42 ($p = 0.0500$). For IgG concentrations, it was elevated by day 42 ($p = 0.0500$), and significantly lower than in the PAR group on day 28 ($p = 0.0500$).

Table 8.21: Average (mean ± SD) immunoglobulin concentrations of the S, S+LMS, PAR and PAR+LMS groups

Group	**Immunoglobulin**		**Group**	**Immunoglobulin**	
	IgM	**IgG**		**IgM**	**IgG**
(n = 3)	**(mg/ml)**	**(mg/ml)**	**(n = 3)**	**(mg/ml)**	**(mg/ml)**
Untreated					
0 Days	0.109±0.02	14.434±1.10			
S			**PAR**		
2 Days	0.104±0.04	14.137±0.91	2 Days	0.123±0.02	11.745±0.27
7 Days	0.110±0.04	14.302±0.70	7 Days	0.088±0.01	11.837±2.16
14 Days	0.110±0.03	12.617±0.29	14 Days	0.091±0.02	12.919±1.86
28 Days	0.075±0.03	16.350±1.00	28 Days	0.074±0.01	13.199±2.46
42 Days	0.046±0.01	17.109±0.26	42 Days	0.057±0.01	13.135±0.62
S+LMS			**PAR+LMS**		
2 Days	0.040±0.00	9.486±0.95	2 Days	0.049±0.03	9.847±0.23
7 Days	0.045±0.02	12.487±1.21	7 Days	0.039±0.02	9.810±0.06
14 Days	0.059±0.02	11.961±1.72	14 Days	0.047±0.01	12.301±0.58
28 Days	0.053±0.02	12.095±1.15	28 Days	0.102±0.05	6.949±2.02
42 Days	0.046±0.01	13.013±1.30	42 Days	0.108±0.02	14.023±0.71

IgM = immunoglobulin M; IgG = immunoglobulin G; S = saline; PAR = paracetamol; LMS = levamisole

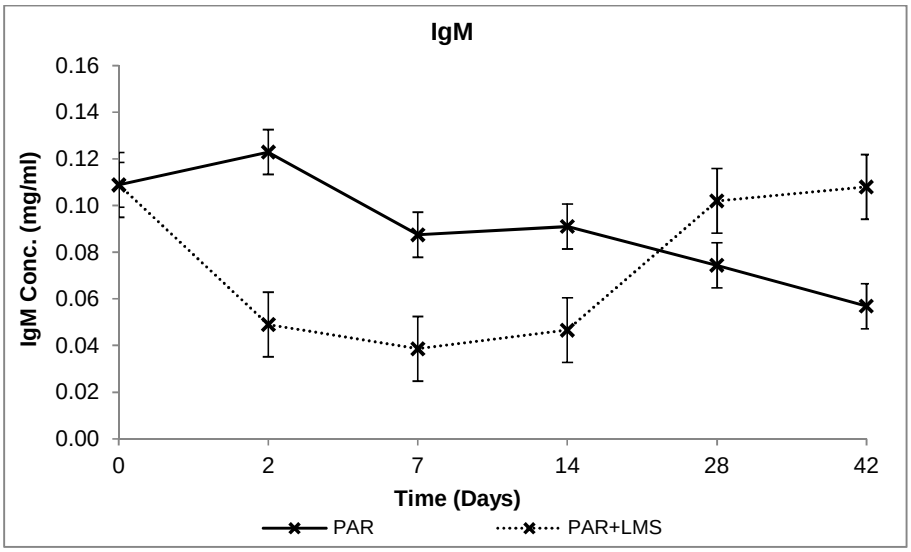

Figure 8.13 a): IgM concentrations of the PAR and PAR+LMS groups over 42 days

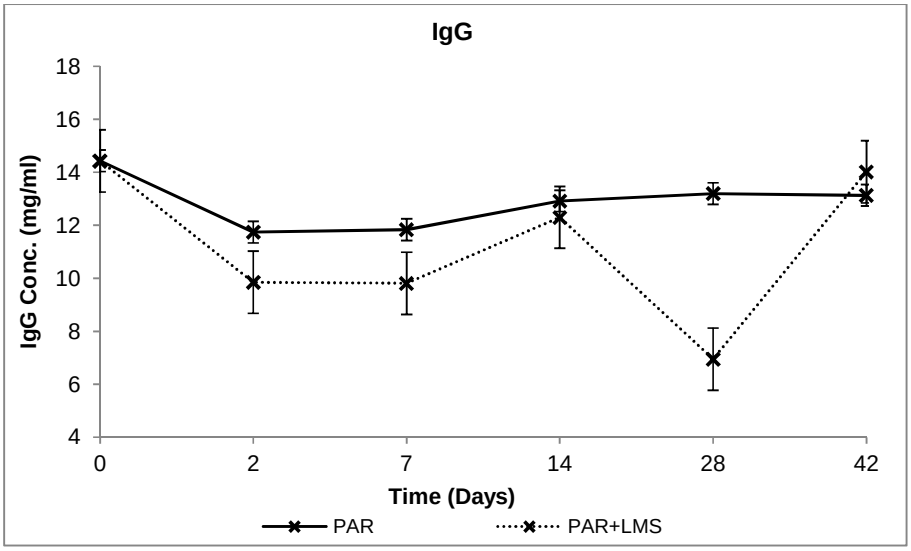

Figure 8.13 b): IgG concentrations of the PAR and PAR+LMS groups over 42 days

8.3.9 Main observations

- From the histopathology report, the liver injury was only minimal.
- Bay day 42 paracetamol concentrations were low.
- IL-2 concentrations were unchanged, while IL-10 was always higher than with paracetamol alone.
- IgM and IgG concentrations were mostly lower than with paracetamol alone.

C. Phase III: Co-treatment with a CYP450 inducer

8.3.10 Physiological observations (function tests)

8.3.10.1 Full blood count

Table 8.22 shows results of the full blood count of the S, S+CBZ, PAR and PAR+CBZ groups. By day 42 in the PAR+CBZ group, the red cell count, haemoglobin, haematocrit and MCHC were elevated ($p = 0.0500$), and were also higher than in the PAR group ($p = 0.0500$). The MCV and MCH had decreased ($p = 0.0500$). For white blood count parameters, the white cell count, neutrophils, lymphocytes and monocytes were always higher than in the PAR group by day 42 ($p = 0.0500$).

Table 8.22: Average (mean ± SD) full blood count and platelets results of the S, S+CBZ, PAR and PAR+CBZ groups

Group (n = 3)	RCC ($x10^{12}$/l)	Hb (g/dl)	Hct (l/l)	MCV (fl)	MCH (pg)	MCHC (g/dl)	Plt ($x10^9$/l)	WCC ($x10^9$/l)	Neu ($x10^9$/l)	Ly ($x10^9$/l)	Mo ($x10^9$/l)	Eos ($x10^9$/l)	Bas ($x10^9$/l)
Untreated													
0 Days	6.28±0.2	12.9±0.3	0.398±0.01	63.5±2.5	20.5±0.4	32.3±0.8	860±221.1	6.95±2.7	0.77±0.2	4.67±1.8	0.19±0.1	0.02±0.0	0.01±0.0
S													
2 Days	6.67±0.2	13.7±0.1	0.422±0.01	63.3±2.3	20.6±0.6	32.4±0.6	849±81.6	6.50±0.9	0.60±0.2	5.18±0.7	0.21±0.0	0.50±0.2	0.01±0.0
7 Days	7.53±0.9	15.3±1.7	0.451±0.04	60.1±2.5	20.3±0.2	33.9±1.2	1033±79.8	5.44±2.4	1.03±0.8	4.07±2.0	0.30±0.3	0.04±0.0	0.01±0.0
14 Days	6.77±0.6	13.9±1.1	0.417±0.03	61.8±2.8	20.5±0.6	33.2±0.6	721±196.4	5.22±1.2	0.63±0.5	4.21±0.7	0.18±0.1	0.18±0.1	0.05±0.0
28 Days	7.07±0.7	13.9±1.3	0.390±0.04	55.1±1.0	19.7±0.1	35.8±0.6	961±172.5	7.38±1.0	0.91±0.2	6.15±0.8	0.24±0.1	0.07±0.0	0.01±0.0
42 Days	6.93±0.8	13.4±1.8	0.374±0.05	53.9±1.0	19.3±0.4	35.8±0.2	839±166.0	3.93±0.3	0.54±0.1	3.23±0.3	0.11±0.0	0.04±0.0	0.01±0.0
S+CBZ													
2 Days	6.74±0.2	13.2±0.1	0.406±0.00	60.4±2.0	19.6±0.6	28.8±6.5	953±95.9	6.48±1.0	0.51±0.0	5.46±1.1	0.24±0.3	0.03±0.0	0.01±0.0
7 Days	6.62±0.4	13.1±0.6	0.414±0.02	62.5±1.3	19.8±0.4	31.7±0.2	779±187.7	7.32±2.4	0.59±0.2	6.20±2.1	0.27±0.1	0.27±0.3	0.01±0.0
14 Days	7.32±0.1	14.7±0.3	0.442±0.01	60.4±2.8	20.1±0.5	33.3±0.8	861±149.2	8.41±0.7	0.76±0.0	7.28±0.5	0.33±0.2	0.05±0.0	0.01±0.0
28 Days	7.27±0.9	14.5±1.7	0.424±0.05	58.4±0.8	19.9±0.3	34.1±0.9	637±79.9	5.48±1.3	0.84±0.3	4.32±1.0	0.23±0.3	0.08±0.0	0.00±0.0
42 Days	7.90±0.1	15.4±0.4	0.451±0.01	57.1±1.4	19.5±0.4	34.2±0.2	727±48.7	6.47±0.6	0.66±0.1	5.55±0.5	0.21±0.0	0.05±0.0	0.01±0.0
PAR													
2 Days	6.77±0.0	14.8±0.0	0.461±0.00	68.1±0.0	21.9±0.0	32.1±0.0	419±0.0	7.54±0.0	0.75±0. 0	5.96±0.0	0.37±0.0	0.46±0.0	0.01±0.0
7 Days	6.75±0.2	13.9±0.1	0.426±0.00	63.2±2.4	20.7±0.8	32.7±0.1	807±22.8	7.32±0.8	0.80±0.2	6.22±0.8	0.23±0.0	0.07±0.0	0.01±0.0
14 Days	6.66±0.2	13.8±0.3	0.411±0.01	61.7±0.5	20.7±0.1	33.6±0.4	834±43.0	4.19±0.4	0.55±0.0	3.42±0.6	0.09±0.1	0.13±0.2	0.00±0.0
28 Days	7.10±0.2	14.1±0.2	0.397±0.00	56.0±2.2	19.8±0.8	35.4±0.4	880±87.0	5.32±1.2	0.88±0.1	4.19±1.1	0.21±0.1	0.04±0.0	0.01±0.0
42 Days	7.33±0.3	14.5±0.3	0.401±0.01	54.8±1.8	19.8±0.5	36.2±0.3	961±85.8	4.91±0.3	0.80±0.2	3.86±0.4	0.20±0.0	0.05±0.0	0.01±0.0
PAR+CBZ													
2 Days	5.96±0.9	12.4±1.7	0.378±0.07	63.1±2.9	20.8±0.4	33.1±2.2	911±81.7	7.15±2.4	0.89±0.5	5.86±1.7	0.27±0.1	0.12±0.1	0.01±0.0
7 Days	6.56±0.2	13.4±0.4	0.427±0.01	65.1±0.3	20.4±0.1	31.4±0.3	821±96.1	6.65±1.3	0.68±0.1	5.66±1.4	0.26±0.1	0.04±0.0	0.01±0.0
14 Days	7.12±0.4	14.2±0.6	0.426±0.01	59.8±1.3	19.9±0.3	33.2±0.4	900±49.3	7.36±0.9	0.83±0.2	6.12±0.6	0.34±0.1	0.07±0.0	0.01±0.0
28 Days	7.55±0.2	15.6±0.4	0.452±0.01	59.8±0.3	20.7±0.4	34.5±0.7	770±64.8	5.63±0.8	0.81±0.2	4.63±0.8	0.14±0.1	0.04±0.0	0.01±0.0
42 Days	7.90±0.4	15.9±0.7	0.438±0.02	55.5±0.5	20.2±0.1	36.3±0.2	921±94.9	6.40±0.5	0.88±0.1	4.91±1.0	0.26±0.1	0.34±0.3	0.01±0.0

RCC = red cell count; Hb = haemoglobin; Hct = haematocrit; MCV = mean corpuscular volume; MCH = mean corpuscular haemoglobin; MCHC = mean corpuscular haemoglobin concentration; Plt = platelets; WCC = white cell count; Neu = neutrophils; Ly = lymphocytes; Mo = monocytes; Eos = eosinophils; Bas = basophils; S = saline; CBZ = carbamazepine; PAR = paracetamol

8.3.10.2 Renal function tests

Table 8.23 shows the changes of BUN and Cr of the S, S+CBZ, PAR and PAR+CBZ groups. In all groups BUN and Cr levels were normal. Here, Cr levels also spiked on day 28, but were still within the normal range (p = 0.0500).

Table 8.23: Average (mean ± SD) renal function test results of the S, S+CBZ, PAR and PAR+CBZ groups

Group	**RFT**		**Group**	**RFT**	
	BUN	**Cr**		**BUN**	**Cr**
(n = 3)	**(mmol/l)**	**(µmol/l)**	**(n = 3)**	**(mmol/l)**	**(µmol/l)**
Untreated					
0 Days	7.2 ± 1	37 ± 8			
S			**PAR**		
2 Days	7.3 ± 1	39 ± 2	2 Days	6.7 ± 1	36 ± 6
7 Days	8.1 ± 0	46 ± 7	7 Days	7.8 ± 1	37 ± 5
14 Days	7.5 ± 1	39 ± 3	14 Days	7.3 ± 1	36 ± 2
28 Days	10.6 ± 2	73 ± 17	28 Days	7.3 ± 1	73 ± 4
42 Days	5.8 ± 1	38 ± 9	42 Days	6.9 ± 1	24 ± 5
S+CBZ			**PAR+CBZ**		
2 Days	6.5 ± 0	36 ± 2	2 Days	6.3 ± 1	21 ± 6
7 Days	6.8 ± 1	33 ± 6	7 Days	6.7 ± 0	28 ± 10
14 Days	6.4 ± 1	36 ± 6	14 Days	10.8 ± 2	32 ± 14
28 Days	7.2 ± 1	64 ± 12	28 Days	9.1 ± 2	54 ± 4
42 Days	7.2 ± 1	8 ± 6	42 Days	7.3 ± 1	20 ± 4

RFT = renal function test; BUN = blood urea nitrogen; Cr = creatinine; S = saline; CBZ = carbamazepine; PAR = paracetamol

8.3.10.3 Liver function tests

Table 8.24 shows the changes of ALT, AST and ALP of the S, S+CBZ, PAR and PAR+CBZ groups. Over the 42 days, the results were similar in all groups.

Table 8.24: Average (mean ± SD) liver function test results of the S, S+CBZ, PAR and PAR+CBZ groups

Group	LFT			Group	LFT		
	ALT	AST	ALP		ALT	AST	ALP
(n = 3)	(U/l)	(U/l)	(U/l)	(n = 3)	(U/l)	(U/l)	(U/l)
Untreated							
0 Days	50±5	88±14	352±76				
S				**PAR**			
2 Days	46±2	90±7	400±7	2 Days	54±4	109±6	402±64
7 Days	49±10	103±25	304±13	7 Days	58±2	104±30	349±27
14 Days	58±4	127±37	508±37	14 Days	53±2	95±12	364±38
28 Days	47±2	115±44	216±19	28 Days	49±4	122±35	224±46
42 Days	46±6	76±28	109±76	42 Days	48±5	64±3	55±64
S+CBZ				**PAR+CBZ**			
2 Days	52±4	90±12	332±18	2 Days	51±8	126±14	358±73
7 Days	43±6	86±8	341±28	7 Days	57±2	125±29	417±63
14 Days	47±2	85±2	356±1	14 Days	50±8	112±24	277±24
28 Days	52±5	97±7	152±78	28 Days	45±18	86±31	120±97
42 Days	45±2	91±20	104±74	42 Days	46±7	83±22	211±1

LFT = liver function test; ALT = alanine aminotransferase; AST = aspartate aminotransferase; ALP = alkaline phosphatase; S = saline; CBZ = carbamazepine; PAR = paracetamol

8.3.10.4 Liver histopathology

(a) Liver histopathology reports

Liver sections for histopathology (Figures 6.15 a – j and 8.14 a – j) were randomly selected and the main histopathology lesions are summarised in the tally tables (Tables 8.25 a and b). The following report is a summary of the features of the lesion:

i. Figures 6.15 a and b: Liver sections A and B from the S+CBZ group after 2 days of saline and carbamazepine co-treatment

The report is the same as in Chapter 6, Section 6.3.10.4 i.

ii. Figures 6.15 c and d: Liver sections A and B from the S+CBZ group after 7 days of saline and carbamazepine co-treatment

The report is the same as in Chapter 6, Section 6.3.10.4 ii.

iii. Figures 6.15 e and f: Liver sections A and B from the S+CBZ group after 14 days of saline and carbamazepine co-treatment

The report is the same as in Chapter 6, Section 6.3.10.4 iii.

iv. Figures 6.15 g and h: Liver sections A and B from the S+CBZ group after 28 days of saline and carbamazepine co-treatment

The report is the same as in Chapter 6, Section 6.3.10.4 iv.

v. Figures 6.15 i and j: Liver sections A and B from the S+CBZ group after 42 days of saline and carbamazepine co-treatment

The report is the same as in Chapter 6, Section 6.3.10.4 v.

vi. Figures 8.14 a and b: Liver sections A and B from the PAR+CBZ group after 2 days of paracetamol and carbamazepine co-treatment

Representative photographs of rat livers, after 2 days of daily paracetamol and carbamazepine co-treatment. The report: “The degeneration, cytonecrosis and hepatocyte mitosis are minimal in the liver parenchyma of section A. Morphological findings in liver section B are similar to those in liver section A.”

vii. Figures 8.14 c and d: Liver sections A and B from the PAR+CBZ group after 7 days of paracetamol and carbamazepine co-treatment

Representative photographs of rat livers, after 7 days of daily paracetamol and carbamazepine co-treatment. The report: “In liver section A, the vacuolar changes and single cell necrosis are graded as minimal, while minimal mitoses could be demonstrated as well. Also, incidental mild portal lymphocytic cuffing is detected in this liver. The degeneration as well as the cytonecrosis are graded as minimal in liver section B.”

viii. Figures 8.14 e and f: Liver sections A and B from the PAR+CBZ group after 14 days of paracetamol and carbamazepine co-treatment

Representative photographs of rat livers, after 14 days of daily paracetamol and carbamazepine co-treatment. The report: “Only minimal granular vacuolar degeneration and cell swelling were present in liver section A. No further lesions were observed.”

ix. Figures 8.14 g and h: Liver sections A and B from the PAR+CBZ group after 28 days of paracetamol and carbamazepine co-treatment

Representative photographs of rat livers, after 28 days of daily paracetamol and carbamazepine co-treatment. The report: “Liver pathology had completely regenerated by day 28, as no lesions were observed and all cells and cords appeared morphologically normal.”

x. Figures 8.14 i and j: Liver sections A and B from the PAR+CBZ group after 42 days of paracetamol and carbamazepine co-treatment

Representative photographs of rat livers, after 42 days of daily paracetamol and carbamazepine co-treatment. The report: “Mild vacuolar degeneration could be demonstrated within the parenchymal cells, while single cell necrosis appears minimal in liver sections A and B.”

In view of the histopathology photographs (Figures 6.15 a – j and 8.14 a – j), reports and tally tables (Tables 8.25 a and b), it appeared that co-treatment with paracetamol and carbamazepine did not cause the same degree of liver injury as with paracetamol alone.

(b) Liver histopathology photographs

Histopathology photographs of the S+CBZ group (Figures 6.15 a – j) are presented in Chapter 6, Section 6.3.10.4 b. Figures 8.14 a – j are representative of randomly selected liver sections of the PAR+CBZ group.

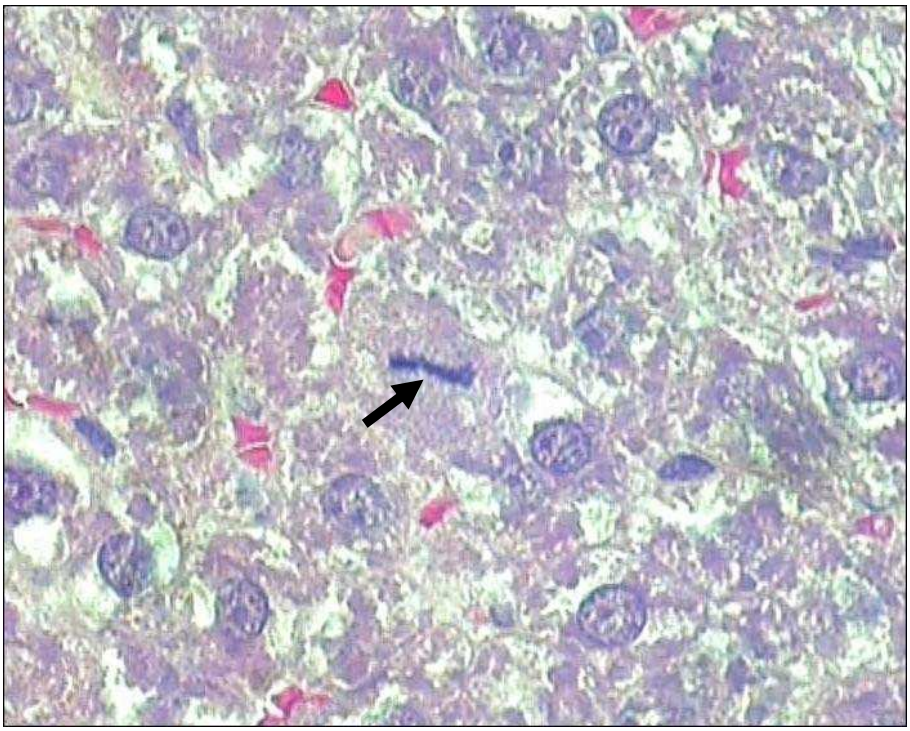

Figure 8.14 a): Liver section A from the PAR+CBZ group after 2 days of treatment, showing minimal degeneration, cytonecrosis and hepatocyte mitosis in the liver parenchyma

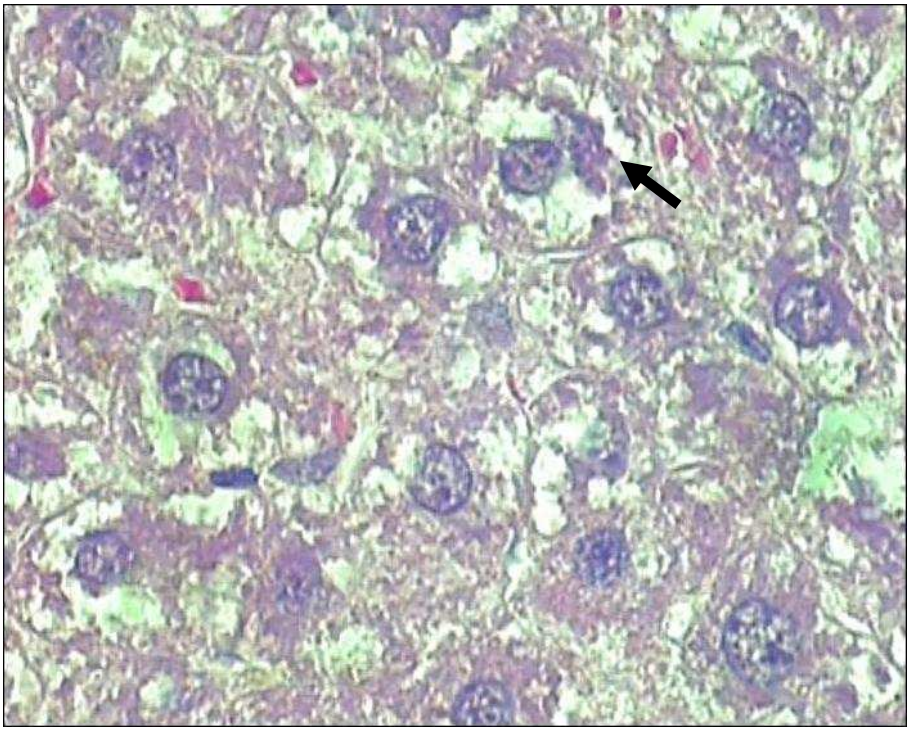

Figure 8.14 b): Liver section B from the PAR+CBZ group after 2 days of treatment, showing similar degeneration, cytonecrosis and hepatocyte mitosis

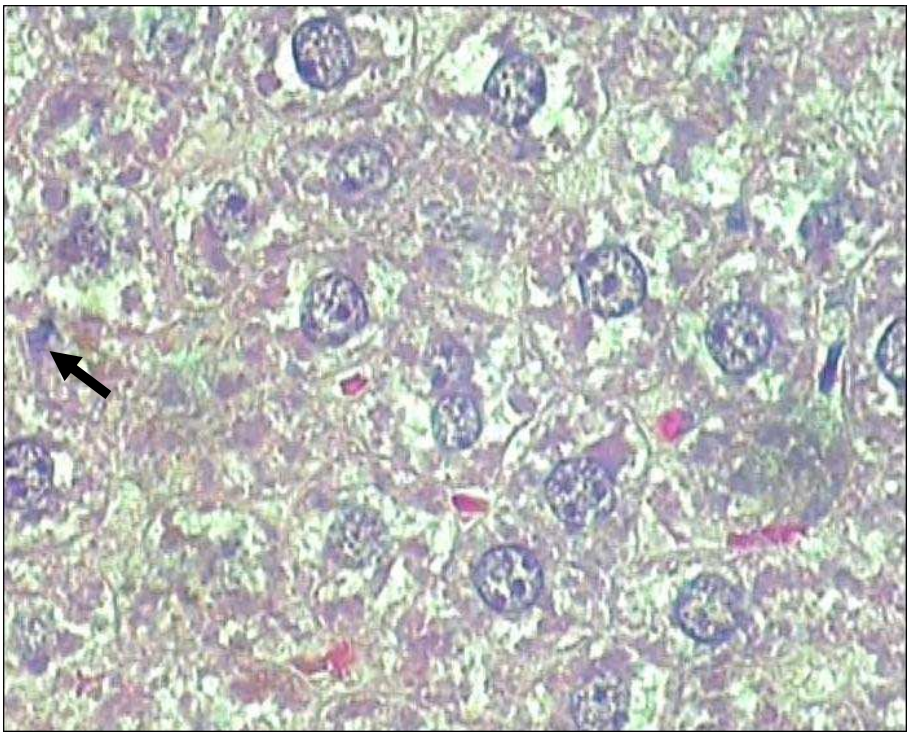

Figure 8.14 c): Liver section A from the PAR+CBZ group after 7 days of treatment, showing minimal vacuolar changes and single cell necrosis, and minimal mitoses

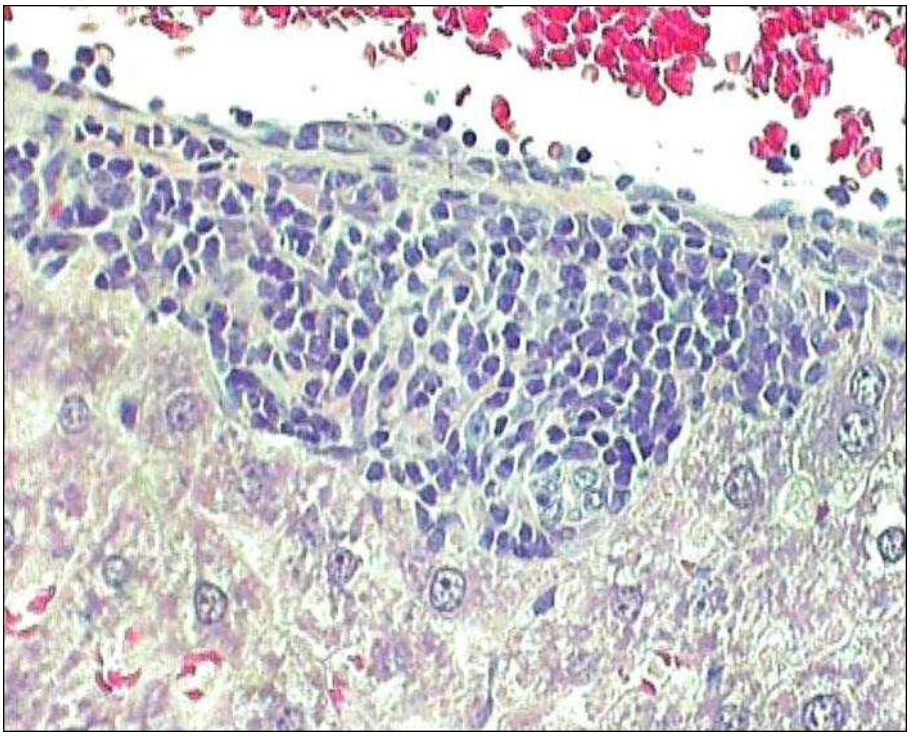

Figure 8.14 d): Liver section B from the PAR+CBZ group after 7 days of treatment, showing incidental mild portal lymphocytic cuffing

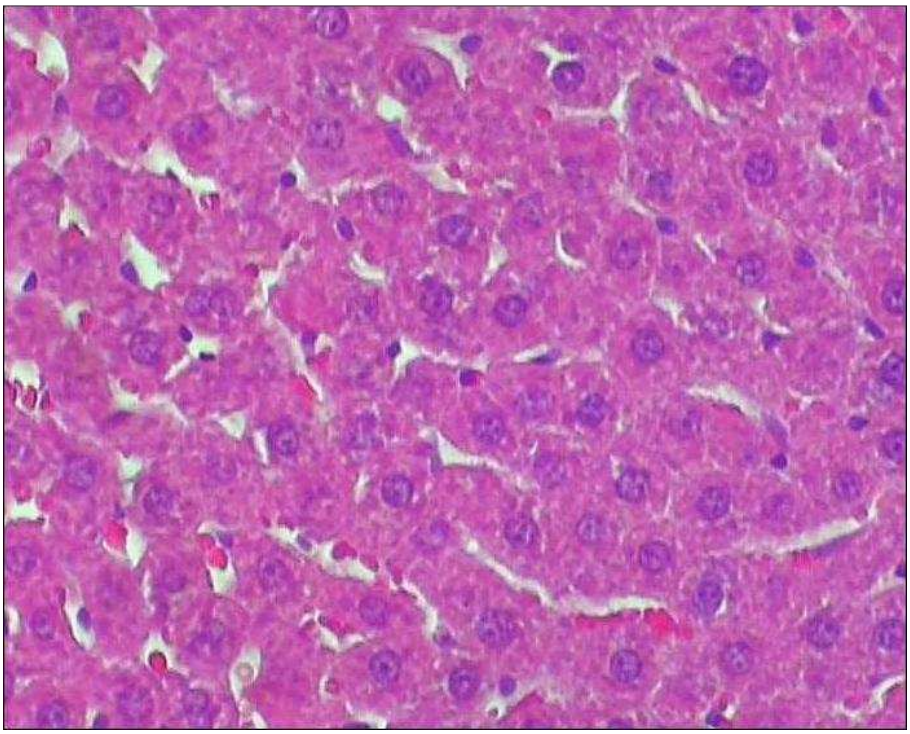

Figure 8.14 e): Liver section A from the PAR+CBZ group after 14 days of treatment, showing hepatic cords, sinusoids, and minimal vacuolar degeneration

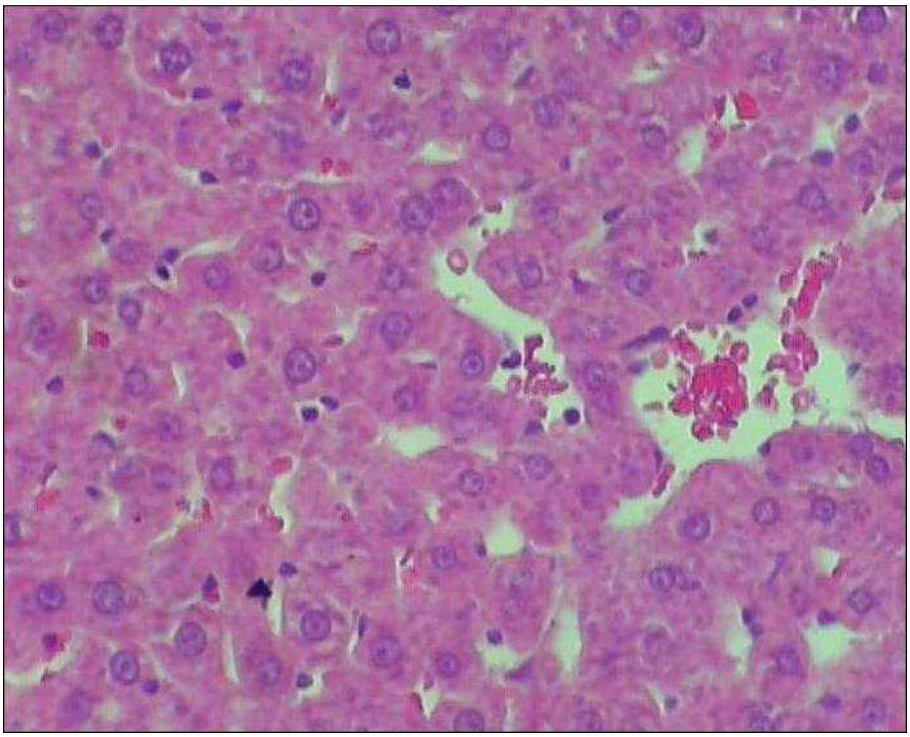

Figure 8.14 f): Liver section B from the PAR+CBZ group after 14 days of treatment, showing morphologically normal centrilobular hepatocytes and central vein area

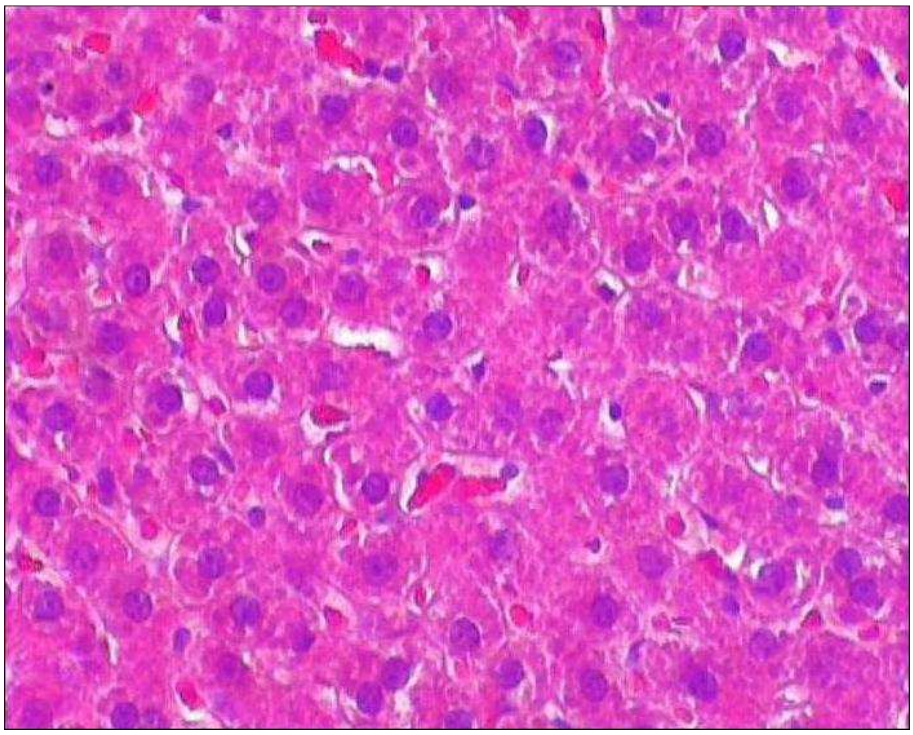

Figure 8.14 g): Liver section A from the PAR+CBZ group after 28 days of treatment, showing normal hepatic parenchyma

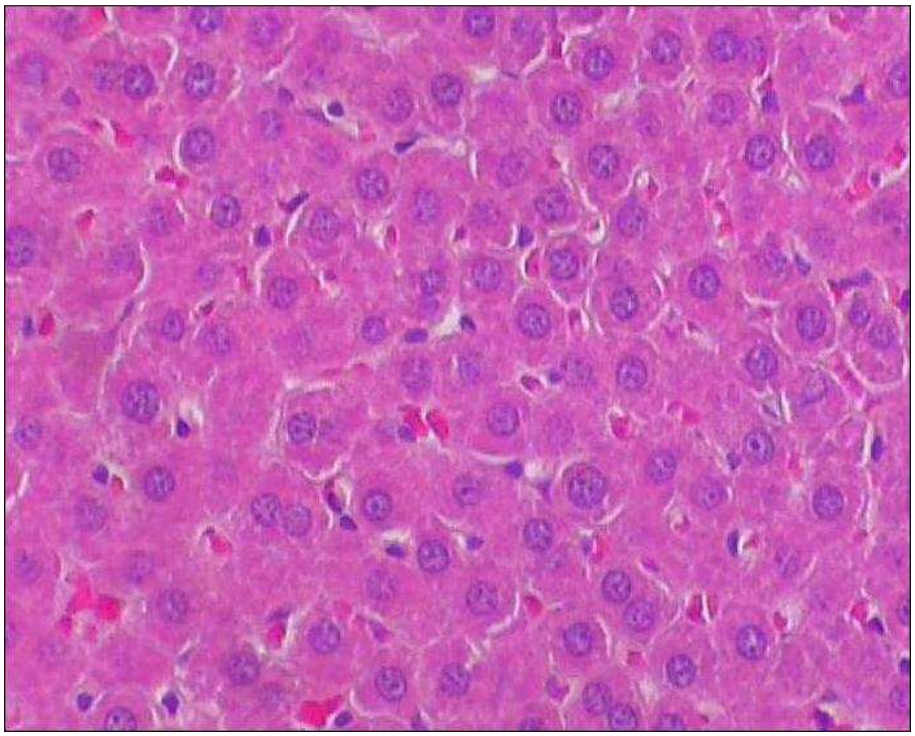

Figure 8.14 h): Liver section B from the PAR+CBZ group after 28 days of treatment, showing morphologically normal hepatic cords

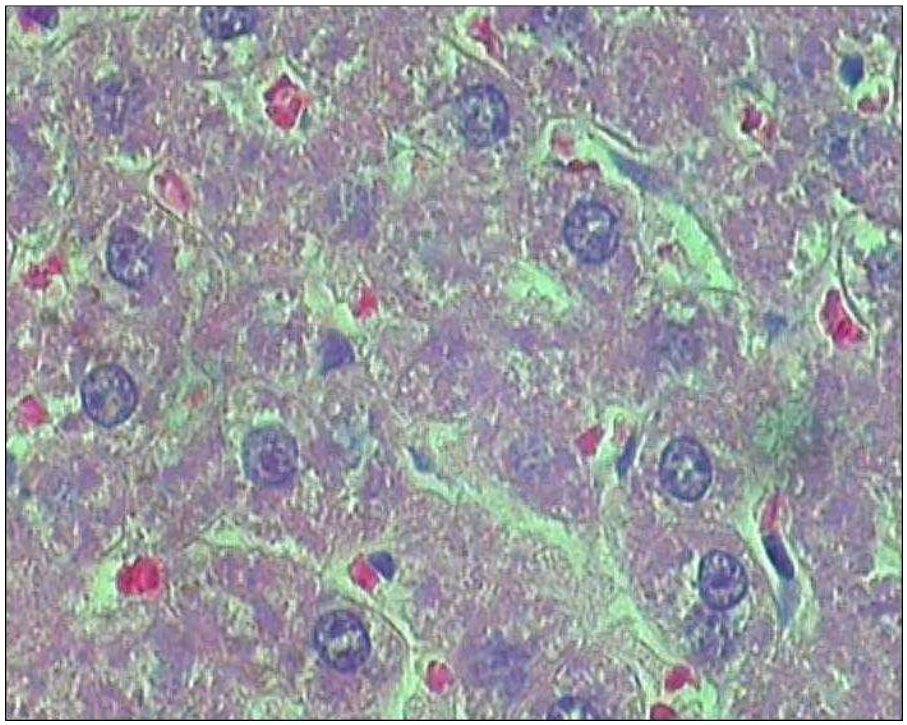

Figure 8.14 i): Liver section A from the PAR+CBZ group after 42 days of treatment, showing mild vacuolar degeneration and minimal single cell necrosis

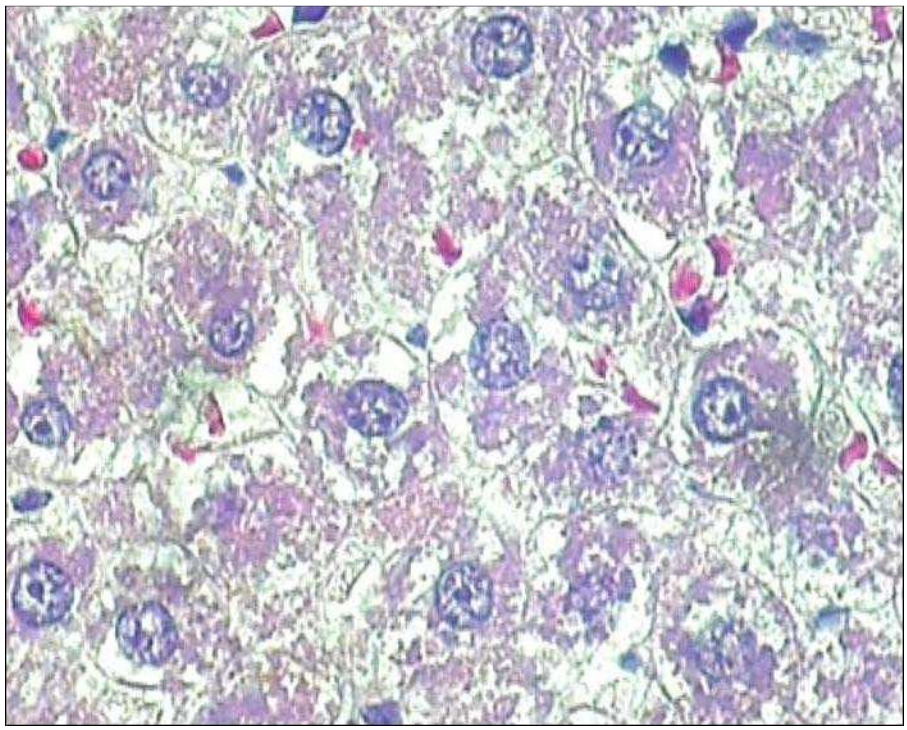

Figure 8.14 j): Liver section B from the PAR+CBZ group after 42 days of treatment, showing mild vacuolar changes and minimal single cell necrosis

Table 8.25 a): Tally of main pathology lesions (lesions score) in livers of untreated rats and the S+CBZ group

Group	UnRx	S+CBZ									
		2 Days		7 Days		14 Days		28 Days		42 Days	
(n = 2)	Fig.6.3a	Fig.6.15a	Fig.6.15b	Fig.6.15c	Fig.6.15d	Fig.6.15e	Fig.6.15f	Fig.6.15g	Fig.6.15h	Fig.6.15i	Fig.6.15j
Histopathological lesions											
Vacuolar degeneration	0	0	1+	1+	1+	2+	2+	1+	1+	1+	1+
Cell swelling	0	0	1+	1+	1+	2+	2+	1+	1+	1+	1+
Cytonecrosis	0	0	0	0	0	2+	2+	0	1+	1+	1+
Centrilobular necrosis	0	0	0	0	0	0	0	0	0	0	0
Hepatocyte mitosis	0	0	0	0	0	0	0	0	0	0	0
Average lesions score											
Vacuolar degeneration	0	0.5+		1+		2+		1+		1+	
Cell swelling	0	0.5+		1+		2+		1+		1+	
Cytonecrosis	0	0		0		2+		0.5+		1+	
Centrilobular necrosis	0	0		0		0		0		0	
Hepatocyte mitosis	0	0		0		0		0		0	
Total lesion score	**0**	**1+**		**2+**		**6+**		**2.5+**		**3+**	

UnRx = untreated; S = saline; CBZ = carbamazepine

Table 8.25 b): Tally of main pathology lesions (lesions score) in livers of untreated rats and the PAR+CBZ group

Group	UnRx	PAR+CBZ									
		2 Days		7 Days		14 Days		28 Days		42 Days	
(n = 2)	Fig.8.2a	Fig.8.14a	Fig.8.14b	Fig.8.14c	Fig.8.14d	Fig.8.14e	Fig.8.14f	Fig.8.14g	Fig.8.14h	Fig.8.14i	Fig.8.14j
Histopathological lesions											
Vacuolar degeneration	0	1+	1+	1+	1+	1+	0	0	0	2+	2+
Cell swelling	0	1+	1+	1+	1+	1+	0	0	0	2+	2+
Cytonecrosis	0	1+	1+	1+	1+	0	0	0	0	1+	1+
Centrilobular necrosis	0	0	0	0	0	0	0	0	0	0	0
Hepatocyte mitosis	0	1+	1+	1+	0	0	0	0	0	0	0
Average lesions score											
Vacuolar degeneration	0	1+		1+		0.5+		0		2+	
Cell swelling	0	1+		1+		0.5+		0		2+	
Cytonecrosis	0	1+		1+		0		0		1+	
Centrilobular necrosis	0	0		0		0		0		0	
Hepatocyte mitosis	0	1+		0.5+		0		0		0	
Total lesion score	**0**	**4+**		**3.5+**		**1+**		**0**		**5+**	

UnRx = untreated; PAR = paracetamol; CBZ = carbamazepine

8.3.11 Paracetamol concentrations

Table 8.26 shows paracetamol concentrations of the PAR and PAR+CBZ groups, while Figure 8.15 is a graphical illustration of the same. For the PAR+CBZ group, paracetamol levels had declined by day 42 ($p = 0.0140$), and were lower than in the PAR group until day 28 ($p = 0.0079$).

Table 8.26: Average (mean ± SD) paracetamol concentrations of the PAR and PAR+CBZ groups

Group	PAR	PAR+CBZ
	PAR concentration	PAR concentration
(n = 5)	(µg/ml)	(µg/ml)
2 Days	0.786 ± 0.22	0.303 ± 0.19
7 Days	0.721 ± 0.28	0.123 ± 0.10
14 Days	0.535 ± 0.12	0.157 ± 0.09
28 Days	0.637 ± 0.49	0.193 ± 0.48
42 Days	0.046 ± 0.10	0.061 ± 0.08

PAR = paracetamol; CBZ = carbamazepine

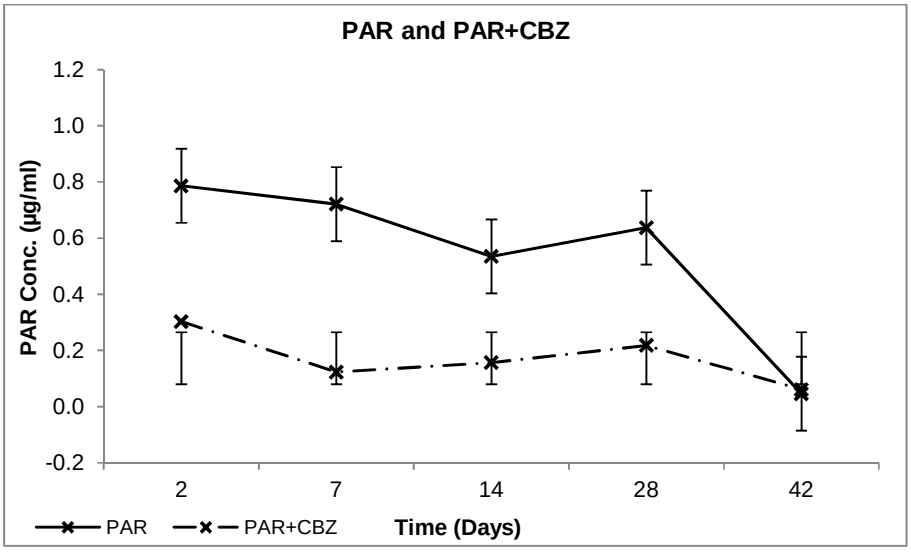

Figure 8.15: Paracetamol concentrations of the PAR and PAR+CBZ groups over 42 days

8.3.12 Specific immunology tests

8.3.12.1 Direct observations

Table 8.27 shows changes in body weight of the S, S+CBZ, PAR and PAR+CBZ groups over the treatment period. All groups showed weight gain at all times, except for the S+CBZ group after 2 days (Refer to Appendix H-1 and H-4 for baseline weights).

Table 8.27: Average (mean ± SD) change in rat weights of the S, S+CBZ, PAR and PAR+CBZ groups

Group (n = 5)	S change in weight (g)	S+CBZ change in weight (g)	PAR change in weight (g)	PAR+CBZ change in weight (g)
2 Days	9.2±4	-3.9±2	9.7±6	4.1±1
7 Days	35.6±8	34.0±4	34.6±4	27.0±4
14 Days	84.6±5	66.2±4	62.6±13	45.4±2
28 Days	107.8±10	97.3±30	103.5±14	97.3±17
42 Days	171.4±27	142.5±182	147.5±20	130.1±23

S = saline; CBZ = carbamazepine; PAR = paracetamol

8.3.12.2 Cytokines

Table 8.28 shows IL-2 and IL-10 concentrations of the S, S+CBZ, PAR and PAR+CBZ groups, while Figures 8.16 a – b are graphical illustrations of the same. In the PAR+CBZ group, IL-2 concentrations showed some fluctuations, but were elevated by day 42 ($p = 0.0500$). With the exception of day 28, IL-2 was always higher than in the PAR group ($p = 0.0500$). Although IL-10 had increased by day 42, this was not statistically significant. However, it was always higher than in the PAR group ($p = 0.0500$).

Table 8.28: Average (mean ± SD) cytokine concentrations of the S, S+CBZ, PAR and PAR+CBZ groups

Group	Cytokine		Group	Cytokine	
	IL-2	IL-10		IL-2	IL-10
(n = 3)	(pg/ml)	(pg/ml)	(n = 3)	(pg/ml)	(pg/ml)
Untreated					
0 Days	65.46 ± 2.0	31.08 ± 1.2			
S			**PAR**		
2 Days	74.87 ± 6.5	29.96 ± 2.8	2 Days	66.66 ± 0.7	28.51 ± 2.4
7 Days	77.26 ± 5.8	34.58 ± 0.7	7 Days	60.66 ± 4.6	28.33 ± 5.6
14 Days	77.85 ± 6.6	35.69 ± 5.4	14 Days	58.01 ± 1.9	29.17 ± 2.3
28 Days	78.81 ± 4.6	32.46 ± 4.2	28 Days	59.83 ± 2.9	29.35 ± 2.4
42 Days	74.39 ± 5.7	32.03 ± 2.5	42 Days	65.40 ± 6.5	28.79 ± 3.1
S+CBZ			**PAR+CBZ**		
2 Days	52.33 ± 14.6	37.61 ± 2.3	2 Days	90.00 ± 22.6	37.63 ± 7.8
7 Days	112.50 ± 7.8	40.11 ± 4.7	7 Days	103.33 ± 29.1	43.10 ± 4.1
14 Days	120.83 ± 12.8	40.11 ± 3.1	14 Days	225.50 ± 17.7	38.98 ± 6.3
28 Days	117.75 ± 23.7	40.67 ± 6.0	28 Days	57.00 ± 8.5	39.53 ± 2.6
42 Days	92.50 ± 46.0	36.00 ± 4.9	42 Days	191.00 ± 75.3	40.69 ± 3.3

IL = interleukin; S = saline; CBZ = carbamazepine; PAR = paracetamol

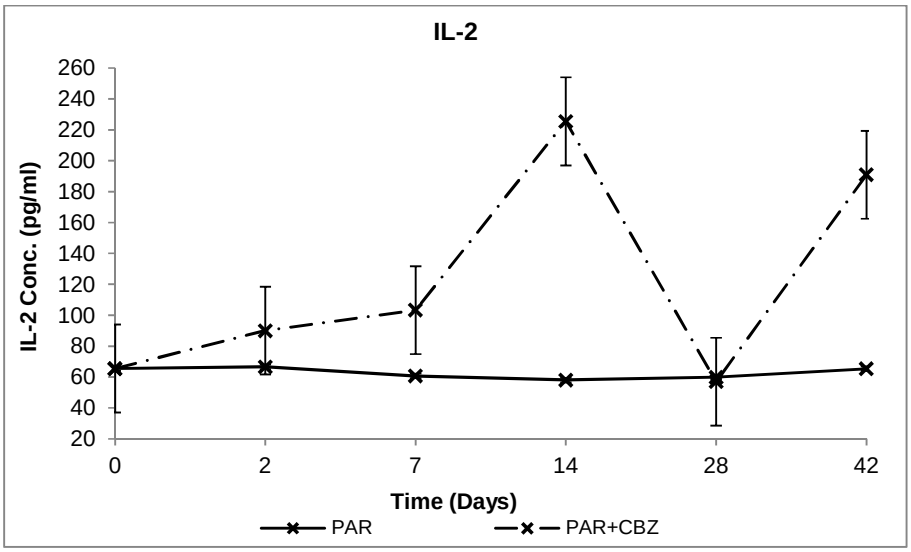

Figure 8.16 a): IL-2 concentrations of the PAR and PAR+CBZ groups over 42 days

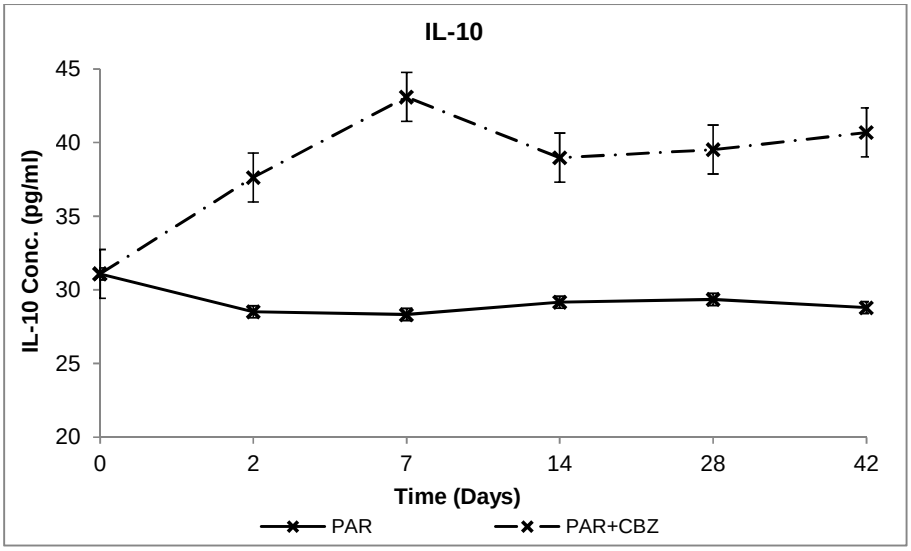

Figure 8.16 b): IL-10 concentrations of the PAR and PAR+CBZ groups over 42 days

8.3.12.3 CD4 and CD8 counts

Table 8.29 shows CD4 and CD8 counts of the S, S+CBZ, PAR and PAR+CBZ groups, while Figures 8.17 a – b are graphical illustrations of the same. For the PAR+CBZ group, the CD4 and CD8 counts were normal and unchanged over the treatment period.

Table 8.29: Average (mean ± SD) CD4 and CD8 counts of the S, S+CBZ, PAR and PAR+CBZ groups

Group	**Ly**	**T-Ly**		**Group**	**Ly**	**T-Ly**	
		CD4	**CD8**			**CD4**	**CD8**
(n = 3)	**(x10^9/l)**	**(x10^9/l)**	**(x10^9/l)**	**(n = 3)**	**(x10^9/l)**	**(x10^9/l)**	**(x10^9/l)**
Untreated							
0 Days	4.67±1.8	2.23±1.3	1.42±0.7				
S				**PAR**			
2 Days	5.18±0.7	2.27±0.3	1.35±0.2	2 Days	5.96±0.0	3.04±0.0	1.51±0.0
7 Days	4.07±2.0	1.72±0.8	1.07±0.5	7 Days	6.22±0.8	2.53±0.3	1.66±0.3
14 Days	4.21±0.7	1.69±0.2	1.17±0.2	14 Days	3.42±0.6	1.66±0.5	0.92±0.1
28 Days	6.15±0.8	2.45±0.2	1.58±0.3	28 Days	4.19±1.1	1.84±0.1	1.01±0.5
42 Days	3.23±0.3	1.47±0.1	0.79±0.2	42 Days	3.86±0.4	1.53±0.2	1.14±0.1
S+CBZ				**PAR+CBZ**			
2 Days	5.46±1.1	1.96±0.3	1.39±0.1	2 Days	5.86±1.7	2.30±0.5	1.37±0.6
7 Days	6.20±2.1	2.33±0.8	1.41±0.4	7 Days	5.66±1.4	2.46±0.5	1.38±0.4
14 Days	7.28±0.5	2.70±0.2	1.59±0.2	14 Days	6.12±0.6	1.91±0.2	1.25±0.2
28 Days	4.32±1.0	1.79±0.3	0.00±0.0	28 Days	4.63±0.8	1.94±0.1	1.13±0.3
42 Days	5.55±0.5	1.73±0.2	1.54±0.2	42 Days	4.91±1.0	1.87±0.2	1.28±0.2

Ly = lymphocytes; CD = cluster of differentiation; S = saline; CBZ = carbamazepine; PAR = paracetamol

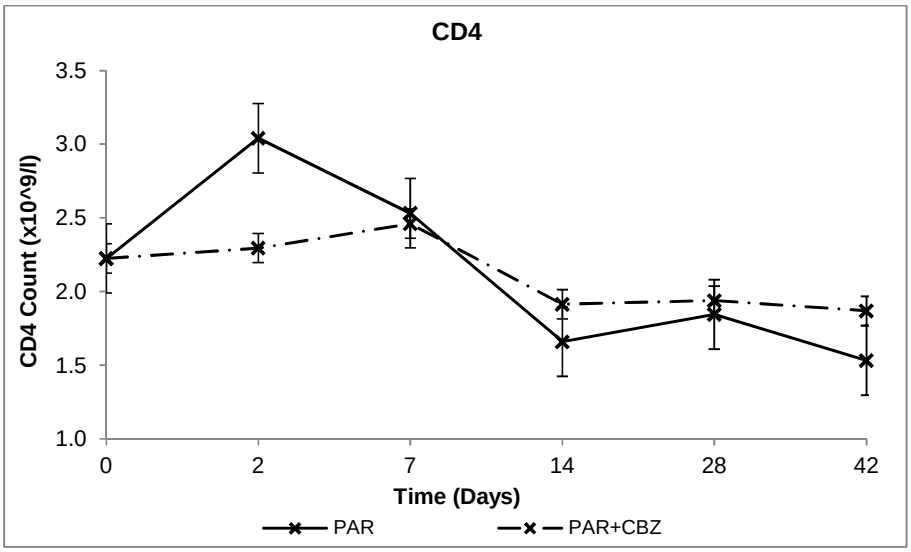

Figure 8.17 a): CD4 counts of the PAR and PAR+CBZ groups over 42 days

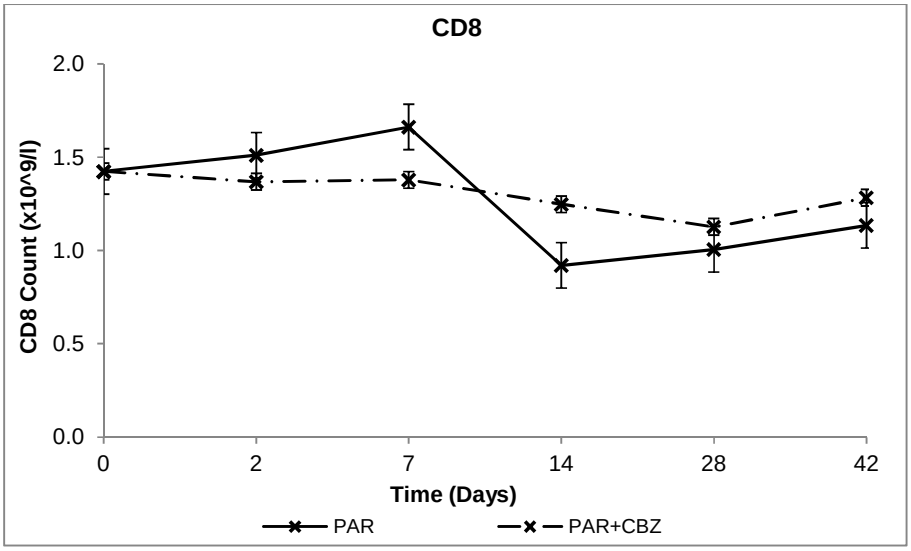

Figure 8.17 b): CD8 counts of the PAR and PAR+CBZ groups over 42 days

8.3.12.4 Immunoglobulins

Table 8.30 shows concentrations of IgM and IgG of the S, S+CBZ, PAR and PAR+CBZ groups, while Figures 8.18 a – b are graphical illustrations of the same. By day 42, IgM levels were higher in the PAR+CBZ group compared to the PAR group (p = 0.0500). For IgG, it was always lower than in the PAR group (p = 0.0500).

Table 8.30: Average (mean ± SD) immunoglobulin concentrations of the S, S+CBZ, PAR and PAR+CBZ groups

Group	**Immunoglobulin**		**Group**	**Immunoglobulin**	
	IgM	**IgG**		**IgM**	**IgG**
(n = 3)	**(mg/ml)**	**(mg/ml)**	**(n = 3)**	**(mg/ml)**	**(mg/ml)**
Untreated					
0 Days	0.109±0.02	14.434±1.10			
S			**PAR**		
2 Days	0.104±0.04	14.137±0.91	2 Days	0.123±0.02	11.745±0.27
7 Days	0.110±0.04	14.302±0.70	7 Days	0.088±0.01	11.837±2.16
14 Days	0.110±0.03	12.617±0.29	14 Days	0.091±0.02	12.919±1.86
28 Days	0.075±0.03	16.350±1.00	28 Days	0.074±0.01	13.199±2.46
42 Days	0.046±0.01	17.109±0.26	42 Days	0.057±0.01	13.135±0.62
S+CBZ			**PAR+CBZ**		
2 Days	0.107±0.01	9.823±1.19	2 Days	0.117±0.03	8.875±0.40
7 Days	0.108±0.00	8.483±0.63	7 Days	0.105±0.01	8.059±0.28
14 Days	0.083±0.01	9.751±2.10	14 Days	0.097±0.00	8.096±1.00
28 Days	0.084±0.01	11.525±2.12	28 Days	0.086±0.01	8.690±1.20
42 Days	0.071±0.01	12.140±1.83	42 Days	0.116±0.03	9.134±0.83

IgM = immunoglobulin M; IgG = immunoglobulin G; S = saline; CBZ = carbamazepine; PAR = paracetamol

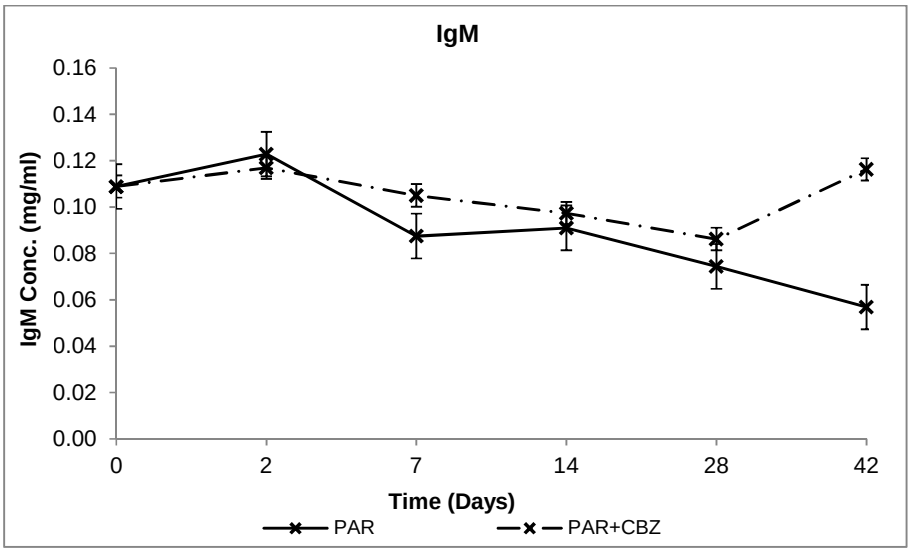

Figure 8.18 a): IgM concentrations of the PAR and PAR+CBZ groups over 42 days

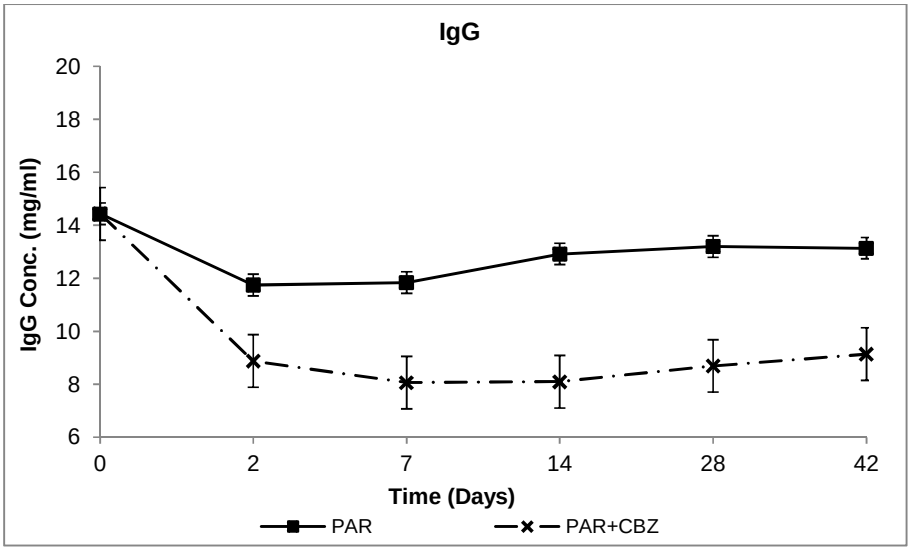

Figure 8.18 b): IgG concentrations of the PAR and PAR+CBZ groups over 42 days

8.3.13 Activity of rat CYP1A2, CYP2E1 and CYP3A2 *in vivo*

8.3.13.1 Protein concentrations

The results of BSA calibration samples are the same as shown in Section 8.3.4.1. Table 8.31 shows microsomal protein concentrations of untreated rats, and the PAR and PAR+CBZ groups.

Table 8.31: Average (mean ± SD) microsomal protein concentrations of the untreated, PAR and PAR+CBZ groups

Selected liver (n = 3)	Prot. conc. (mg/ml)	Abs. (nm)	Selected liver (n = 3)	Prot. conc. (mg/ml)	Abs. (nm)
Untreated					
Rat 1	49.40 ± 6.8	0.13 ± 0.0			
Rat 2	55.02 ± 1.1	0.15 ± 0.0			
Rat 4	51.00 ± 5.1	0.14 ± 0.0			
PAR-2D			**PAR+CBZ-2D**		
Rat 1	20.05 ± 1.3	0.04 ± 0.0	Rat 2	31.50 ± 1.3	0.06 ± 0.0
Rat 2	44.32 ± 3.2	0.09 ± 0.0	Rat 3	23.94 ± 1.0	0.05 ± 0.0
Rat 4	28.98 ± 2.3	0.06 ± 0.0	Rat 5	47.76 ± 2.9	0.10 ± 0.0
PAR-7D			**PAR+CBZ-7D**		
Rat 1	39.70 ± 4.0	0.07 ± 0.0	Rat 3	45.62 ± 0.3	0.09 ± 0.0
Rat 2	29.54 ± 3.7	0.05 ± 0.0	Rat 4	55.55 ± 3.0	0.11 ± 0.0
Rat 5	30.24 ± 4.7	0.05 ± 0.0	Rat 5	26.46 ± 2.7	0.05 ± 0.0
PAR-14D			**PAR+CBZ-14D**		
Rat 1	33.47 ± 9.4	0.07 ± 0.0	Rat 2	29.45 ± 3.0	0.07 ± 0.0
Rat 3	36.06 ± 3.7	0.08 ± 0.0	Rat 3	37.48 ± 2.3	0.08 ± 0.0
Rat 4	28.98 ± 5.0	0.07 ± 0.0	Rat 4	33.94 ± 1.3	0.08 ± 0.0

Prot. conc. = protein concentration; Abs. = absorption; PAR = paracetamol; CBZ = carbamazepine; D = days

8.3.13.2 CYP1A2, CYP2E1 and CYP3A2 activity in vivo

Table 8.32 shows CYP1A2, CYP2E1 and CYP3A2 activity after 2, 7 and 14 days of paracetamol alone (PAR) and concomitant paracetamol and carbamazepine (PAR+CBZ) treatment, while Figures 8.19 a – c are graphical illustrations of the same. The combination of paracetamol and carbamazepine increased CYP1A2 activity on all occasions ($p = 0.0286$), and there was no statistical difference from treatment with paracetamol alone. For CYP2E1, the activity was increased on all occasions, but was only different from the normal on day 7 ($p = 0.0119$). Although CYP2E1 activity was mostly lower after paracetamol and carbamazepine co-administration, it was not statistically different form paracetamol alone. CYP3A2

activity was not affected by paracetamol and carbamazepine co-treatment, and this was similar to paracetamol alone, and carried no statistical significance.

Table 8.32: Average (mean ± SD) CYP1A2, CYP2E1 and CYP3A2 activity

Group (n = 3)	CYP1A2 (pmol/min*mg)	CYP2E1 (nmol/min*mg)	CYP3A2 (pmol/min*mg)
Untreated			
0 Days	4.40 ± 0.8	0.77 ± 0.1	84.63 ± 6.9
PAR			
2 Days	7.08 ± 1.1	2.02 ± 0.3	83.60 ± 6.5
7 Days	9.72 ± 0.6	1.23 ± 0.1	68.40 ± 7.5
14 Days	12.63 ± 1.4	0.74 ± 0.0	72.23 ± 5.2
PAR+CBZ			
2 Days	7.98 ± 0.2	1.35 ± 0.2	72.71 ± 0.4
7 Days	9.36 ± 1.6	1.13 ± 0.2	80.29 ± 9.8
14 Days	11.79 ± 1.7	0.83 ± 0.2	68.48 ± 7.5

RR = reaction rate; PAR = paracetamol; CBZ = carbamazepine

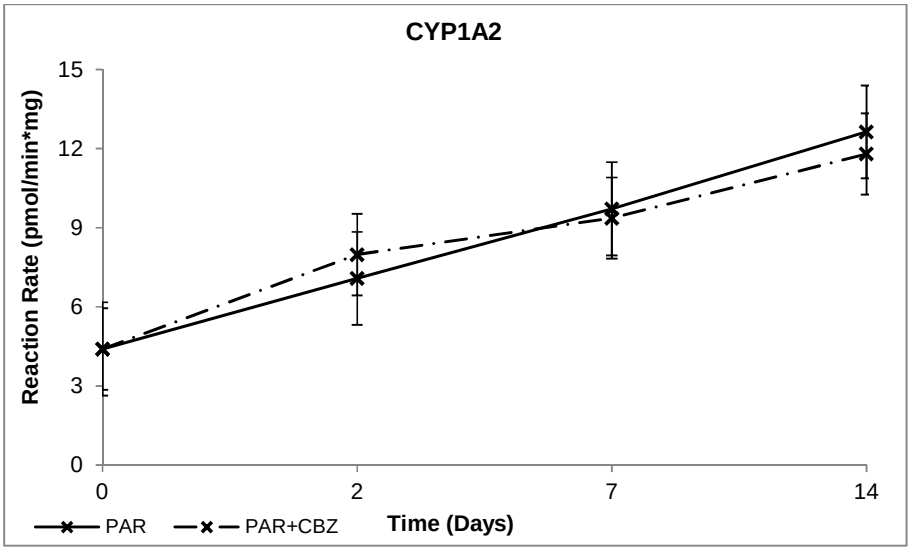

Figure 8.19 a): CYP1A2 activity after paracetamol alone, and carbamazepine co-treatment

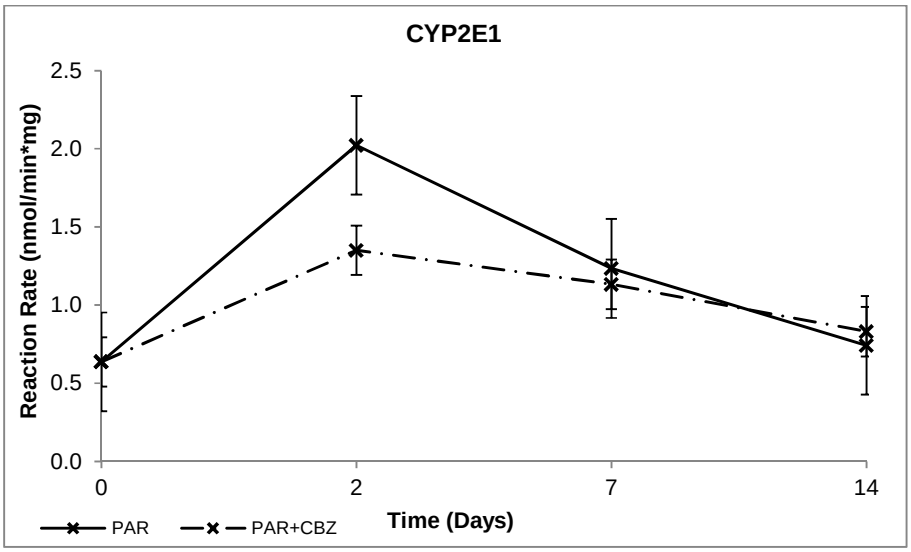

Figure 8.19 b): CYP2E1 activity after paracetamol alone, and carbamazepine co-treatment

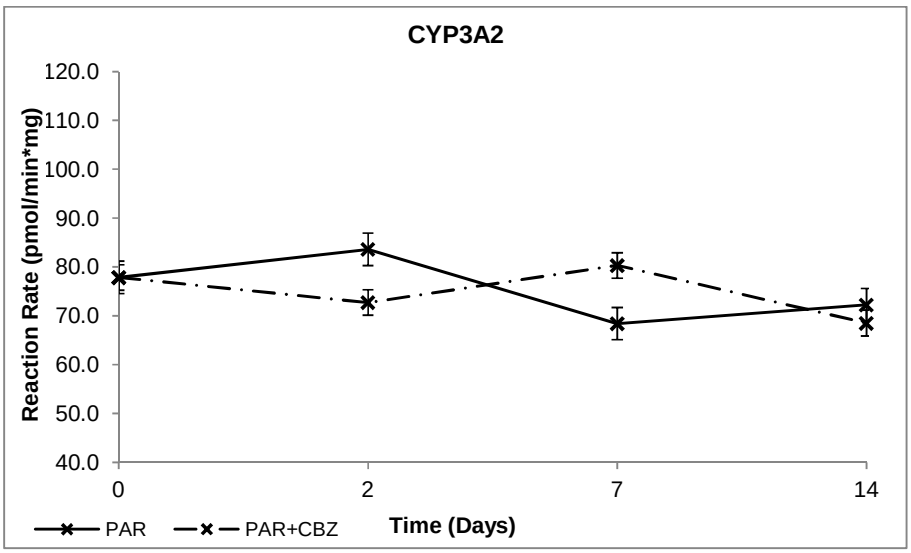

Figure 8.19 c): CYP3A2 activity after paracetamol alone, and carbamazepine co-treatment

8.3.14 Main observations

- Co-treatment with carbamazepine caused only minimal liver injury.
- Paracetamol was metabolised even more extensively in combination with carbamazepine.
- IL-2 and IL-10 were always higher than with paracetamol alone.
- Co-treatment with carbamazepine did not influence the production of effector cells of the immune system, and was similar to paracetamol alone.
- Co-administration with carbamazepine did not provoke a humoral immune response. IgG levels were also always lower than with paracetamol alone.
- CYP1A2 and CYP2E1 activity was increased.

8.4 SUMMARY OF THE RESULTS

The immune system and CYP450 enzymes are implicated in the mechanism of paracetamol-induced hepatotoxicity. As such, this study aimed at indicating the changes in physiological and early pathological processes, as monitored by the relevant biomarkers during daily paracetamol administration at normal doses in animals that did not develop clinical hepatotoxicity. The changes of the different parameters occurred within their normal concentration ranges. Specifically, the liver injury was subclinical (*i.e.*, normal liver function tests) and the concentrations for paracetamol and chemokines were within their normal ranges. Therefore, changes or responses between the different groups need not be statistically significant in order to make sense.

These early changes were presumed to be part of premonitory symptoms of the major pathological process, *i.e.*, during normal dosing the body responds to drug insults by chemokines or enzyme activity, but within the respective normal range. It was envisaged that comprehending these early changes may be used to determine the mechanism of paracetamol-induced hepatotoxicity, as well as strategies to prevent the liver injury.

Table 8.33 is a summary of the changes or responses in the test groups relative to those in the control or PAR groups, while Table 8.34 shows the relation between lesions scores and paracetamol concentrations, and Figure 8.20 is a graphical illustration of the same. Treatment with paracetamol alone caused subclinical liver injury within the first 14 days, followed by recovery by day 28 (Table 8.34; Figure 8.20). The injury was not associated with an immune response, as all immune parameters were mostly lower than in the control. It was, however, associated with low paracetamol concentrations and increased CYP1A2 and CYP2E1 activity (Table 8.33; PAR alone). The subsequent recovery is because at a therapeutic paracetamol dose, as used here, NAPQI is rapidly detoxified by glutathione to non-toxic metabolites (Rowden *et al*., 2005).

Co-treatment with an immune stimulant, levamisole, caused less liver injury, and was associated with low paracetamol concentrations and increased IgM and IL-10 (Th2

response; Table 8.33; PAR+LMS). Here, the strong anti-inflammatory actions along with NAPQI detoxification most probably prevented the development of liver injury. Treatment with levamisole alone did not cause liver injury.

Co-treatment with carbamazepine, an enzyme inducer, caused minimal to mild injury (Table 8.34; Figure 8.20). Here, CYP1A2 activity was increased, paracetamol concentrations were low, and IL-2 increased up to day 42 (Th1 response) versus an increase in IL-10 from day 2 to day 28 (Th2 response; Table 8.33; PAR+CBZ). Treatment with carbamazepine alone caused mild liver injury up to day 14. Therefore, the strong Th1 response was counteracted by a strong Th2 response and resulted in minimal liver injury.

Table 8.33: Description of the changes or responses in the test group relative to those in the control or PAR group

PAR alone

	Period 1 0 – 14 days of treatment	**Period 2 14 – 42 days of treatment**
	PAR vs. S	**PAR vs. S**
Liver injury (score)	Increased, 8+ (d14)	Decreased, 2+ (d28)
PAR conc. (µg/ml)	Peak at d7 (0.8 µg/ml)	Lower than phase 1
CYP1A2(pmol/min*mg)	Increased CYP1A2 activity by d2 and continued so	

PAR+LMS

	Period 1 0 – 14 days of treatment	**Period 2 14 – 42 days of treatment**
	PAR+LMS vs. PAR alone	**PAR+LMS vs. PAR alone**
Liver injury (score)	Mild, 2+ (d14)	Mild, 3+ (d42)
PAR conc. (µg/ml)	Peak at d2 (0.9 µg/ml)	Lower
IL-10 conc. (pg/ml)	Moderate increase, peak at d14	Higher than PAR alone group

PAR+CBZ

	Period 1 0 – 14 days of treatment	**Period 2 14 – 42 days of treatment**
	PAR+CBZ vs. PAR alone	**PAR+CBZ vs. PAR alone**
Liver injury (score)	Minimal, 1+ (d14)	Mild, 5+ (d42)
PAR conc. (µg/ml)	Peak at d2 (0.3 µg/ml)	Lower
IL-2 conc. (pg/ml)	Sharp increase, peak at d14	Sharp increase from d28 to d42
IL-10 conc. (pg/ml)	Sharp increase, peak at d7	Higher than in PAR alone group
CYP1A2(pmol/min*mg)	Similar to PAR alone group	

PAR = paracetamol; LMS = levamisole; CBZ = carbamazepine; d = day

Table 8.34: The relation between paracetamol concentrations and histopathological lesions in the PAR, PAR+LMS and PAR+CBZ groups

Group	Lesions score (score)	PAR concentration (µg/ml)
Untreated		
0 Days	0+	0.000
PAR		
2 Days	8.5+	0.786
7 Days	5+	0.721
14 Days	8.5+	0.535
28 Days	2+	0.637
42 Days	4+	0.046
PAR+LMS		
2 Days	6.5+	0.879
7 Days	3+	0.279
14 Days	2+	0.379
28 Days	3.5+	0.359
42 Days	3+	0.000
PAR+CBZ		
2 Days	4+	0.303
7 Days	3.5+	0.123
14 Days	1+	0.157
28 Days	0+	0.193
42 Days	5+	0.061

PAR = paracetamol; LMS = levamisole; CBZ = carbamazepine

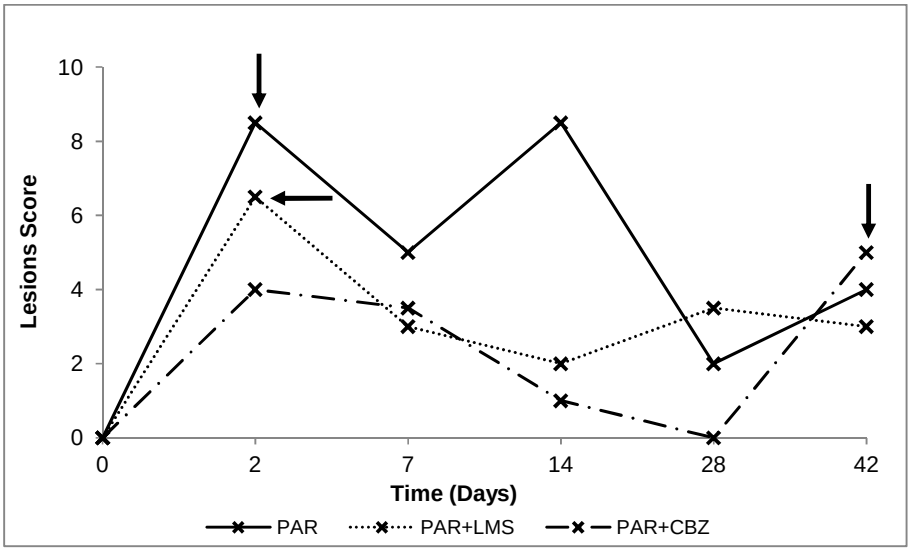

Figure 8.20: Lesions score chart of the PAR, PAR+LMS and PAR+CBZ groups over 42 days

Only in the CYP450 induction phase was there immune activation of the Th1 and Th2 responses. This may imply that the immune system is involved in paracetamol-induced liver injury on a cytokine signalling level, but this may not occur at normal paracetamol doses.

In conclusion, paracetamol did not exhibit a distinct pattern of immune response by which to associate it with the liver injury, most probably because the concentrations were too low for generation of toxic metabolites.

CHAPTER 9

COMPARISON OF THE MECHANISMS INVOLVED IN SUBCLINICAL ISONIAZID, NEVIRAPINE AND PARACETAMOL-INDUCED LIVER INJURY

In general, isoniazid and nevirapine led to stimulation of the immune system that was characterised by an initial pro-inflammatory (Th1) response over the first 14 days, followed by an anti-inflammatory (Th2) response over the next 14 days. The Th2 response was associated with a subclinical liver injury, the severity of which peaked at 14 days, while the Th2 response was associated with healing of the liver injury and this was complete by day 42 of treatment.

The above observation is indicative of an adaptive immune response proposed earlier by Holt and Ju (2006). It was postulated that upon interaction with cellular structure or proteins, the hepatotoxic drug forms antigenic protein adducts which stimulate the pro-inflammatory immune response, and this attempts to kill hepatocytes expressing the antigenic adducts, hence the liver injury. This is then countered by the anti-inflammatory immune response (Th2) that inhibits the production of Th1 cytokines, and presumably mediates the recognition of the adducts as part of the self via the HLA system. It was further postulated that failure of the Th2 counter response may be responsible for unhindered progression of the destructive Th1 response and progression to clinical hepatotoxicity. In effect, a rat model for the initial signals of isoniazid and nevirapine induced liver injury has been established in animals that did not develop clinical hepatotoxicity. This model can then be used to study factors that can lead to progression to clinical hepatotoxicity, one of which is the drug concentration.

Regarding the drug concentration, the severity of isoniazid liver injury was associated with higher isoniazid concentrations, while severity for nevirapine liver injury was associated with low plasma concentrations of nevirapine. For isoniazid, it implies that the parent drug is responsible for the liver injury, while for nevirapine it is most probably a secondary product, metabolite.

Co-treatment with the immune stimulant, levamisole, led to delayed onset of the severe liver injury for both isoniazid and nevirapine, *i.e.*, 7 and 14 days, respectively, while co-treatment with carbamazepine led to reduced livery injury with increasing severity for both drugs. For isoniazid, carbamazepine led to severe liver injury at day 42. The fact the liver injury still occurred in these groups implies that the liver injury and subsequent immune responses are vital for the immune adaptation to the hepatotoxic drug.

On the other hand, although isoniazid and nevirapine therapy were associated with increased activity of CYP2E1 and CYP3A2, respectively, these were not closely associated with changes in the liver injury or immune response.

Paracetamol therapy was not closely associated with the changes in the immune parameters and CYP450 activity, *i.e.*, there was no clear pattern in the two. This was most probably because, at normal doses used here, only a small amount of the toxic metabolite was formed, and this was quenched by the glutathione antioxidant mechanism. Nevertheless, the model will enable further studies at higher concentrations as indicated for the other two drugs.

In conclusion, the pattern of immune response to prolonged treatment with isoniazid and nevirapine shows that the immune system is involved in the two drug-induced liver injuries, most probably as a protective mechanism against further drug toxicity.

CHAPTER 10

CONCLUSIONS AND FUTURE STUDIES

The objectives of this study were achieved as follows:

- A method for the simultaneous determination of isoniazid, nevirapine in plasma by high performance liquid chromatography was successfully developed.
  - It was used to measure isoniazid, nevirapine and paracetamol plasma concentrations in rats treated with 20 mg/kg isoniazid, 200 mg/kg nevirapine or 500 mg/kg paracetamol for 2, 7, 14, 28 and 42 days.

- The immune response to prolonged administration of isoniazid, nevirapine and paracetamol revealed that:
  - the immune system was involved in the subclinical liver injury caused by isoniazid and nevirapine, most likely to counteract development of further drug toxicity;
  - paracetamol did not exhibit a distinct pattern of immune response by which to associate it with the liver injury.

- The cytochrome P450 response to prolonged administration of isoniazid, nevirapine and paracetamol showed that:
  - although isoniazid and nevirapine therapy were associated with increased activity of CYP2E1 and CYP3A2, respectively, these were not closely associated with changes in the liver injury or immune response;
  - paracetamol therapy was not closely associated with the changes in CYP450 activity.

Implications:

- In order to prevent and/or treat drug-induced liver injury, the following strategies should be considered:
  - therapeutic drug monitoring, as the liver injury was associated with high isoniazid concentrations, but low nevirapine concentrations;
  - manipulation of the immune response, since early anti-inflammatory activity might be able to prevent drug-induced liver injury; and

- manipulation of the CYP450 response in order to control the formation of harmful metabolites.

Future studies:

- Regarding the immune response, it is necessary to search for drugs or substances alike which can stimulate a Th2 immune response, without causing further drug toxicity or interactions.
- CYP450 quantification by SDS-PAGE and Western Blot analysis during the Th1 and Th2 immune response.

CHAPTER 11

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APPENDICES

APPENDIX A: HPLC CALIBRATIONS OF ISONIAZID, NEVIRAPINE, AND PARACETAMOL OVER 5 DAYS

Appendix A-1: Isoniazid calibration, day 1

Table A-1: Isoniazid calibration data, day 1

INH Conc. (µg/ml)	Ratio
1	0.051
2	0.070
4	0.187
6	0.226
10	0.330

INH = isoniazid; Conc. = concentration

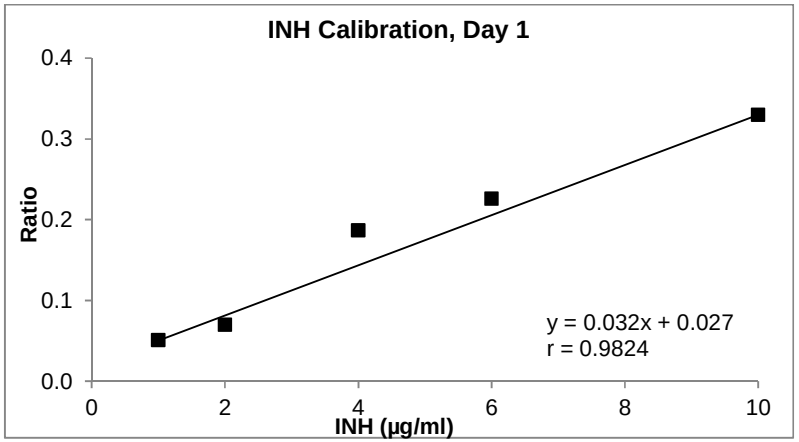

Figure A-1: Isoniazid calibration curve, day 1

Appendix A-2: Isoniazid calibration, day 2

Table A-2: Isoniazid calibration data, day 2

INH Conc. (µg/ml)	Ratio
1	0.044
2	0.062
4	0.127
6	0.148
10	0.248

INH = isoniazid; Conc. = concentration

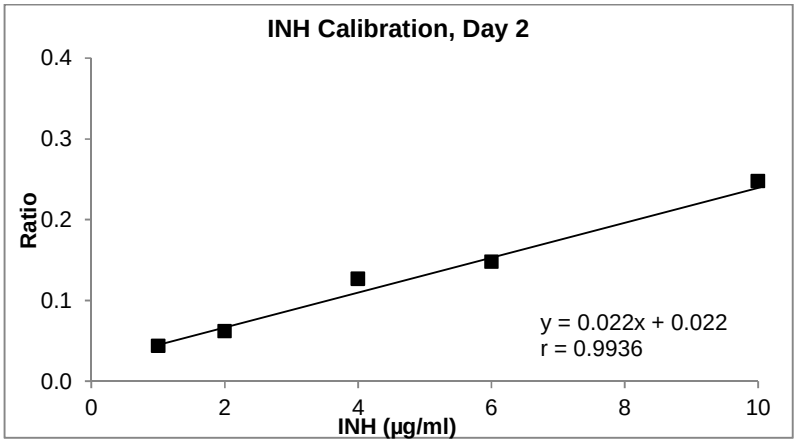

Figure A-2: Isoniazid calibration curve, day 2

Appendix A-3: Isoniazid calibration, day 3

Table A-3: Isoniazid calibration data, day 3

INH Conc. (µg/ml)	Ratio
1	0.066
2	0.100
4	0.156
6	0.203
10	0.334

INH = isoniazid; Conc. = concentration

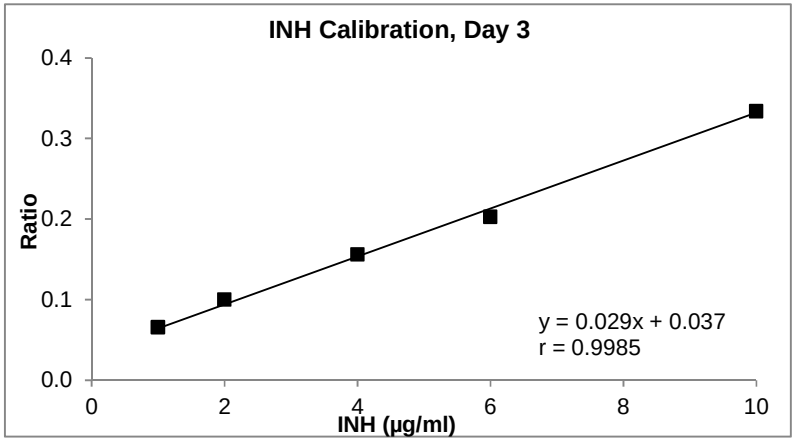

Figure A-3: Isoniazid calibration curve, day 3

Appendix A-4: Isoniazid calibration, day 4

Table A-4: Isoniazid calibration data, day 4

INH Conc. (µg/ml)	Ratio
1	0.041
2	0.078
4	0.157
6	0.250
10	0.355

INH = isoniazid; Conc. = concentration

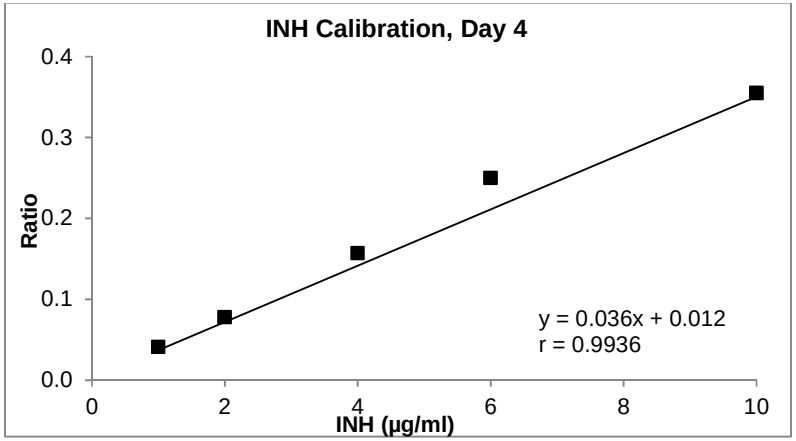

Figure A-4: Isoniazid calibration curve, day 4

Appendix A-5: Isoniazid calibration, day 5

Table A-5: Isoniazid calibration data, day 5

INH Conc. (µg/ml)	Ratio
1	0.048
2	0.082
4	0.136
6	0.204
10	0.302

INH = isoniazid; Conc. = concentration

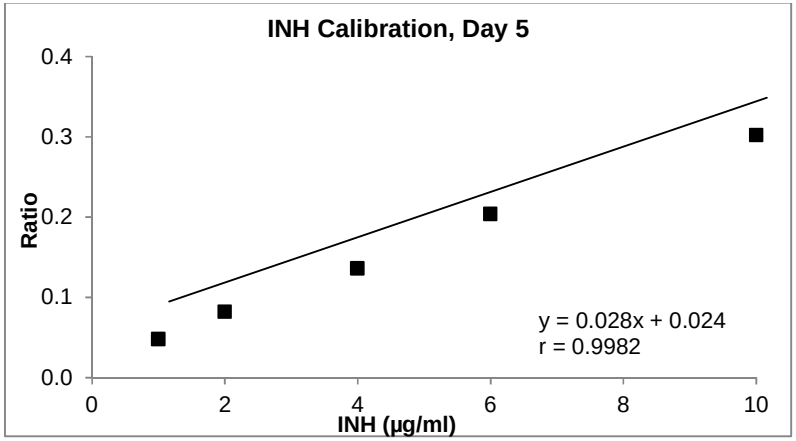

Figure A-5: Isoniazid calibration curve, day 5

Appendix A-6: Nevirapine calibration, day 1

Table A-6: Nevirapine calibration data, day 1

NVP Conc. (µg/ml)	Ratio
1	0.170
3	0.254
5	0.324
8	0.420
10	0.484

NVP = nevirapine; Conc. = concentration

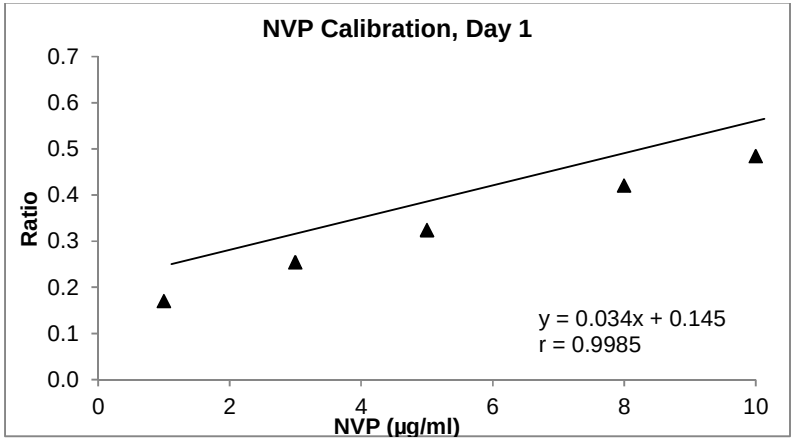

Figure A-6: Nevirapine calibration curve, day 1

Appendix A-7: Nevirapine calibration, day 2

Table A-7: Nevirapine calibration data, day 2

NVP Conc. (µg/ml)	Ratio
1	0.142
3	0.262
5	0.326
8	0.465
10	0.524

NVP = nevirapine; Conc. = concentration

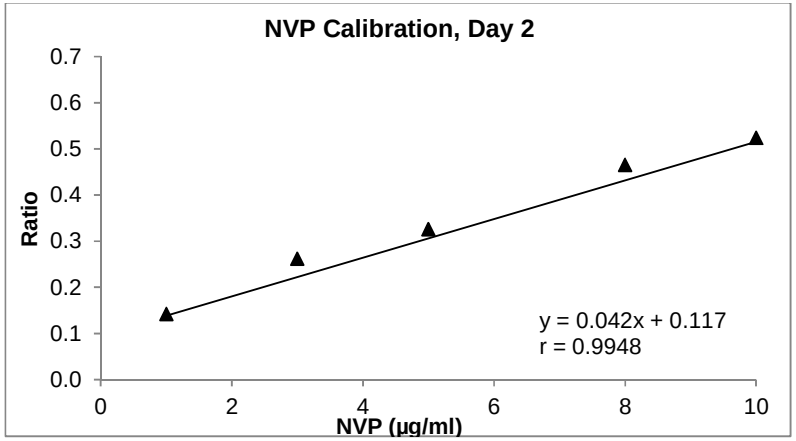

Figure A-7: Nevirapine calibration curve, day 2

Appendix A-8: Nevirapine calibration, day 3

Table A-8: Nevirapine calibration data, day 3

NVP Conc. (µg/ml)	Ratio
1	0.164
3	0.257
5	0.366
8	0.523
10	0.626

NVP = nevirapine; Conc. = concentration

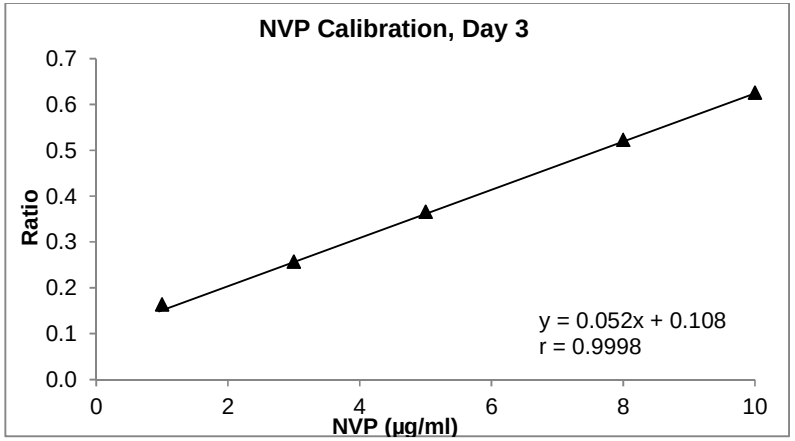

Figure A-8: Nevirapine calibration curve, day 3

Appendix A-9: Nevirapine calibration, day 4

Table A-9: Nevirapine calibration data, day 4

NVP Conc. (µg/ml)	Ratio
1	0.177
3	0.282
5	0.340
8	0.393
10	0.465

NVP = nevirapine; Conc. = concentration

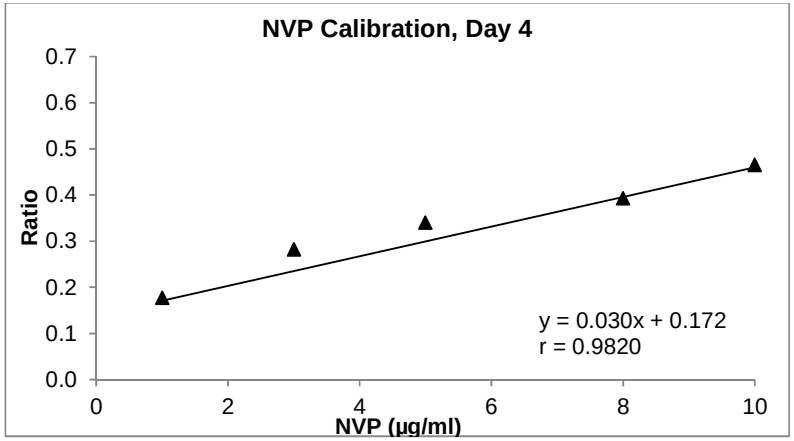

Figure A-9: Nevirapine calibration curve, day 4

Appendix A-10: Nevirapine calibration, day 5

Table A-10: Nevirapine calibration data, day 5

NVP Conc. (µg/ml)	Ratio
1	0.144
3	0.292
5	0.361
8	0.539
10	0.668

NVP = nevirapine; Conc. = concentration

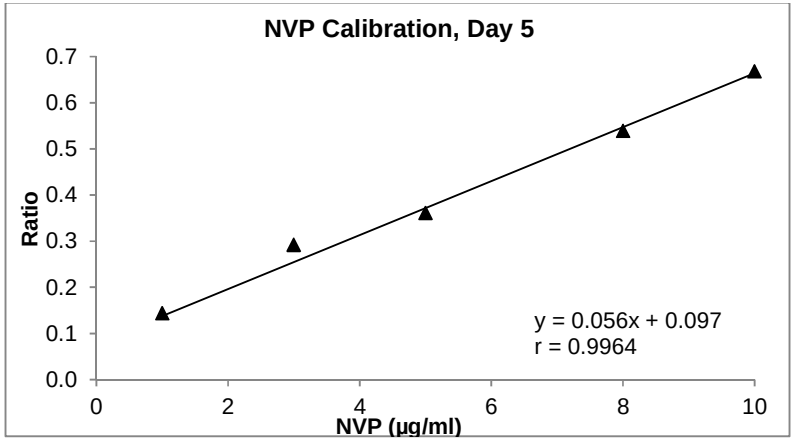

Figure A-10: Nevirapine calibration curve, day 5

Appendix A-11: Paracetamol calibration, day 1

Table A-11: Paracetamol calibration data, day 1

PAR Conc. (µg/ml)	Ratio
1	0.167
5	0.539
10	1.139
15	1.338
20	2.034

PAR = paracetamol; Conc. = concentration

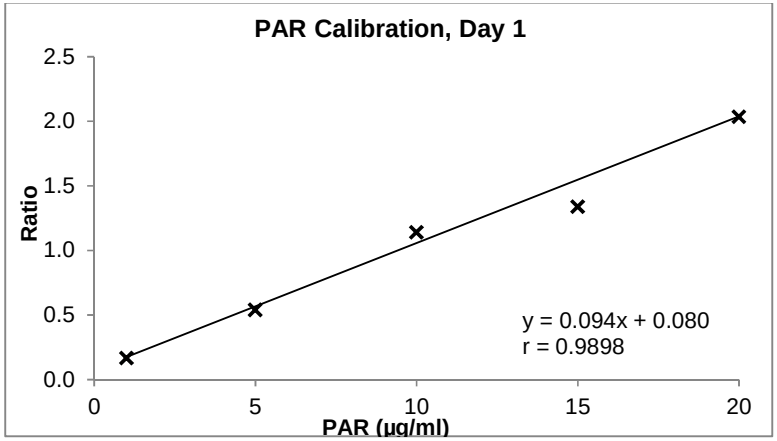

Figure A-11: Paracetamol calibration curve, day 1

Appendix A-12: Paracetamol calibration, day 2

Table A-12: Paracetamol calibration data, day 2

PAR Conc. (µg/ml)	Ratio
1	0.165
5	0.532
10	0.909
15	1.411
20	1.846

PAR = paracetamol; Conc. = concentration

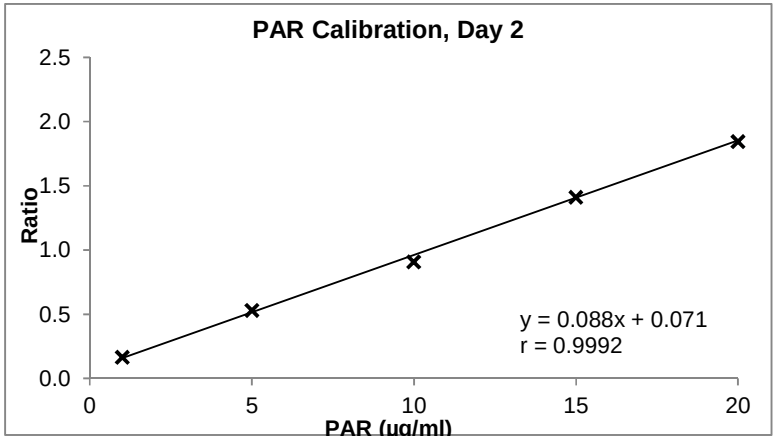

Figure A-12: Paracetamol calibration curve, day 2

Appendix A-13: Paracetamol calibration, day 3

Table A-13: Paracetamol calibration data, day 3

PAR Conc. (µg/ml)	Ratio
1	0.163
5	0.542
10	1.039
15	1.573
20	2.012

PAR = paracetamol; Conc. = concentration

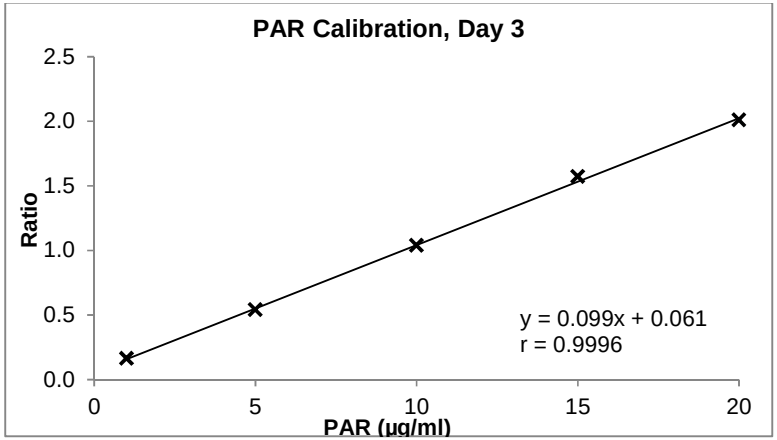

Figure A-13: Paracetamol calibration curve, day 3

Appendix A-14: Paracetamol calibration, day 4

Table A-14: Paracetamol calibration data, day 4

PAR Conc. (µg/ml)	Ratio
1	0.151
5	0.660
10	1.207
15	1.688
20	2.283

PAR = paracetamol; Conc. = concentration

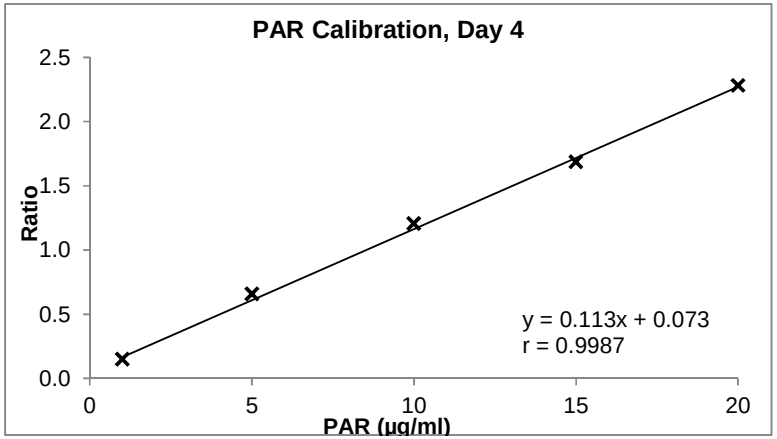

Figure A-14: Paracetamol calibration curve, day 4

Appendix A-15: Paracetamol calibration, day 5

Table A-15: Paracetamol calibration data, day 5

PAR Conc. (µg/ml)	Ratio
1	0.124
5	0.529
10	1.000
15	1.575
20	1.842

PAR = paracetamol; Conc. = concentration

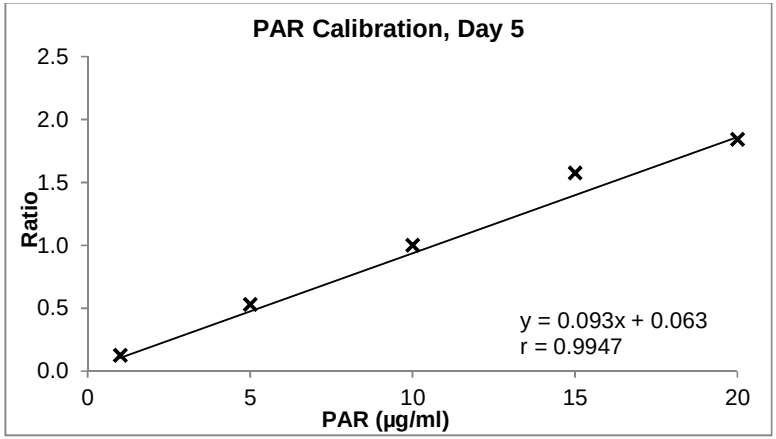

Figure A-15: Paracetamol calibration curve, day 5

APPENDIX B: ACCURACY DETERMINATION OF ISONIAZID, NEVIRAPINE AND PARACETAMOL

Appendix B-1: Accuracy of isoniazid

Table B-1: Accuracy data of isoniazid

Conc. Prep. (µg/ml)	Conc. 1 (µg/ml)	Conc. 2 (µg/ml)	Conc. 3 (µg/ml)	Conc. 4 (µg/ml)	Conc. 5 (µg/ml)	Mean	SD	Acc. (%)	CV (%)
1	1.015	1.089	0.999	0.991	0.991	**1.017**	0.04	**101.7**	4.1
4	3.918	3.870	3.676	3.662	4.400	**3.905**	0.30	**97.6**	7.7
10	9.773	10.183	10.286	10.509	9.849	**10.120**	0.31	**101.2**	3.0

Conc. = concentration; SD = standard deviation; Acc = accuracy; CV = coefficient of variation

Appendix B-2: Accuracy of nevirapine

Table B-2: Accuracy data of nevirapine

Conc. Prep. (µg/ml)	Conc. 1 (µg/ml)	Conc. 2 (µg/ml)	Conc. 3 (µg/ml)	Conc. 4 (µg/ml)	Conc. 5 (µg/ml)	Mean	SD	Acc. (%)	CV (%)
1	1.015	1.015	0.807	1.099	0.773	**0.942**	0.14	**94.2**	15.2
5	4.800	5.262	4.681	4.661	4.632	**4.807**	0.26	**96.1**	5.5
10	10.030	9.781	9.846	9.960	10.592	**10.042**	0.32	**100.4**	3.2

Conc. = concentration; SD = standard deviation; Acc = accuracy; CV = coefficient of variation

Appendix B-3: Accuracy of paracetamol

Table B-3: Accuracy data of paracetamol

Conc. Prep. (µg/ml)	Conc. 1 (µg/ml)	Conc. 2 (µg/ml)	Conc. 3 (µg/ml)	Conc. 4 (µg/ml)	Conc. 5 (µg/ml)	Mean	SD	Acc. (%)	CV (%)
1	1.036	0.938	1.088	1.062	0.823	**0.989**	0.11	**98.9**	11.0
10	9.047	9.437	9.198	10.853	9.897	**9.686**	0.73	**96.9**	7.5
20	20.822	17.803	19.998	20.034	20.051	**19.742**	1.14	**98.7**	5.8

Conc. = concentration; SD = standard deviation; Acc = accuracy; CV = coefficient of variation

APPENDIX C: STABILITY DETERMINATION OF ISONIAZID, NEVIRAPINE AND PARACETAMOL

Appendix C-1: Short-term stability of INH (4 µg/ml) at ambient temperature

Table C-1: INH (4 µg/ml) stability data at ambient temperature at 8, 12 and 24 hours

Time	Prep. Conc. (µg/ml)	Conc. 1 (µg/ml)	Conc. 2 (µg/ml)	Mean	SD	Stab. (%)	CV (%)
0 H	4	5.156	4.061	**4.609**	0.77	**115**	16.8
8 H	4	2.853	2.038	**2.446**	0.58	**61**	23.6
12 H	4	2.010	2.234	**2.122**	0.16	**53**	7.5
24 H	4	2.150	2.543	**2.347**	0.28	**59**	11.8

Conc. = concentration; SD = standard deviation; Stab. = stability; CV = coefficient of variation; H = hours

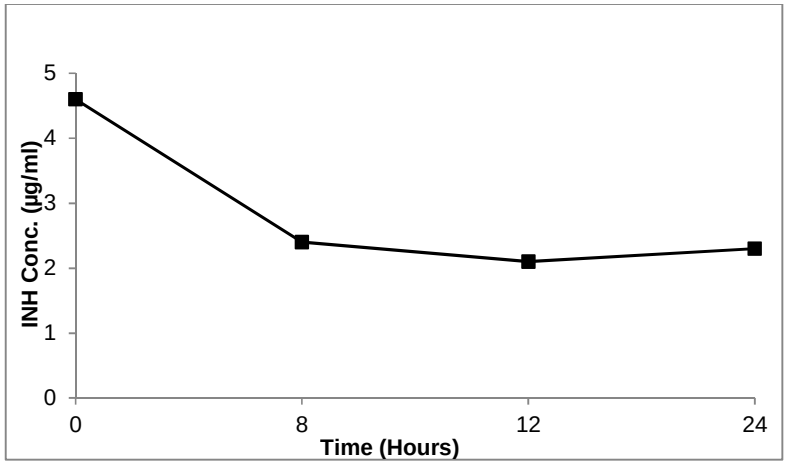

Figure C-1: Plot of stability of INH (4 µg/ml) at ambient temperature

Appendix C-2: Short-term stability of INH (10 µg/ml) at ambient temperature

Table C-2: INH (10 µg/ml) stability data at ambient temperature at 8, 12 and 24 hours

Time	Prep. Conc. (µg/ml)	Conc. 1 (µg/ml)	Conc. 2 (µg/ml)	Mean	SD	Stab. (%)	CV (%)
0 H	10	8.149	9.576	**8.863**	1.01	**89**	11.4
8 H	10	7.880	7.045	**7.463**	0.59	**75**	7.9
12 H	10	7.341	8.257	**7.799**	0.65	**78**	8.3
24 H	10	10.303	8.526	**9.415**	1.26	**94**	13.3

Conc. = concentration; SD = standard deviation; Stab. = stability; CV = coefficient of variation; H = hours

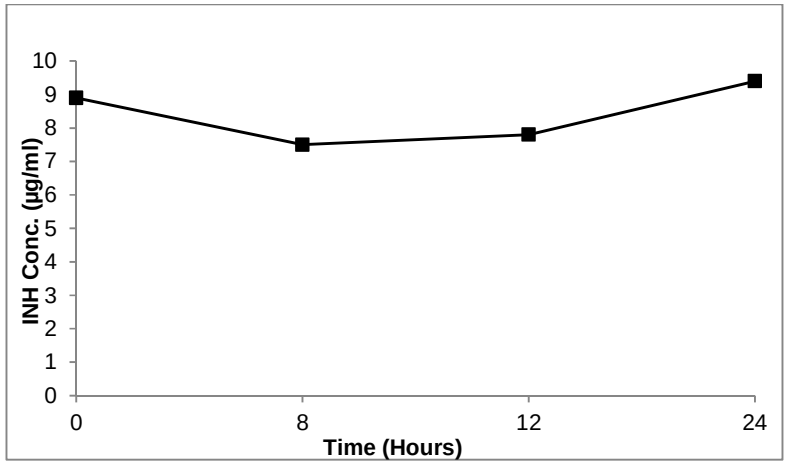

Figure C-2: Plot of stability of INH (10 µg/ml) at ambient temperature

Appendix C-3: Short-term stability of INH (4 µg/ml) at 4ºC

Table C-3: INH (4 µg/ml) stability data at 4ºC at 8, 12 and 24 hours

Time	Prep. Conc. (µg/ml)	Conc. 1 (µg/ml)	Conc. 2 (µg/ml)	Mean	SD	Stab. (%)	CV (%)
8 H	4	2.600	3.049	**2.825**	0.32	**71**	11.2
12 H	4	2.208	1.500	**1.854**	0.50	**46**	27.0
24 H	4	2.385	2.061	**2.223**	0.23	**56**	10.3

Conc. = concentration; SD = standard deviation; Stab. = stability; CV = coefficient of variation; H = hours

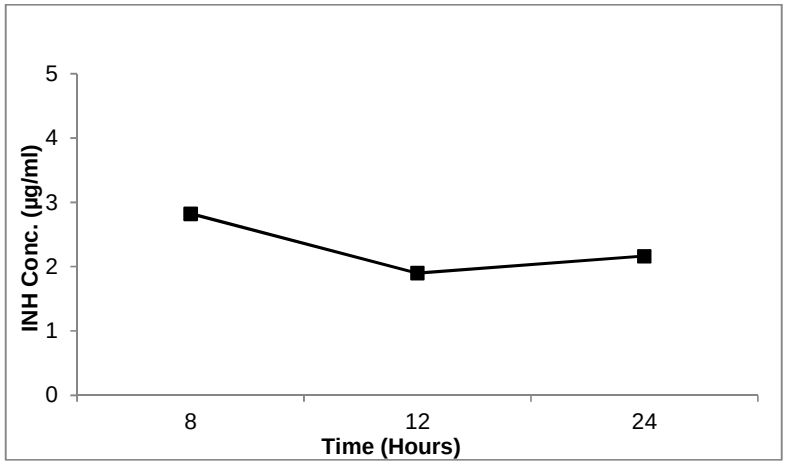

Figure C-3: Plot of stability of INH (4 µg/ml) at 4ºC

Appendix C-4: Short-term stability of INH (10 µg/ml) at 4ºC

Table C-4: INH (10 µg/ml) stability data at 4ºC at 8, 12 and 24 hours

Time	Prep. Conc. (µg/ml)	Conc. 1 (µg/ml)	Conc. 2 (µg/ml)	Mean	SD	Stab. (%)	CV (%)
8 H	10	8.688	4.918	**6.803**	2.67	**68**	39.2
12 H	10	9.765	6.722	**8.244**	2.15	**82**	26.1
24 H	10	10.169	5.995	**8.082**	2.95	**81**	36.5

Conc. = concentration; SD = standard deviation; Stab. = stability; CV = coefficient of variation; H = hours

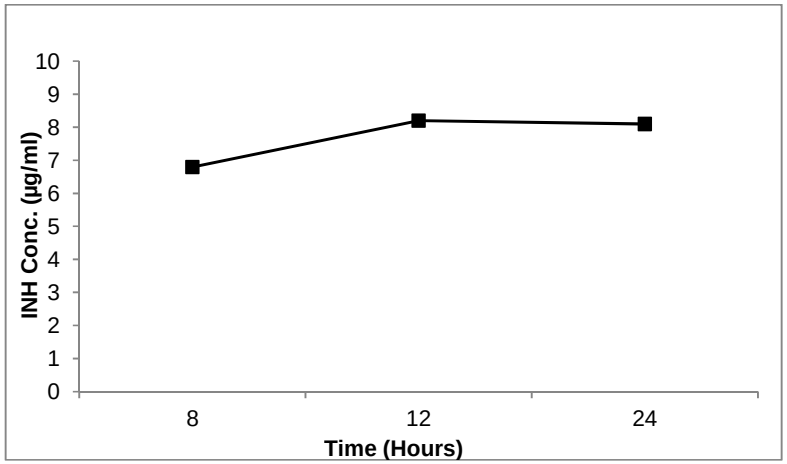

Figure C-4: Plot of stability of INH (10 µg/ml) at 4ºC

Appendix C-5: Short-term stability of INH (4 µg/ml) at -20ºC

Table C-5: INH (4 µg/ml) stability data at -20ºC at 8, 12 and 24 hours

Time	Prep. Conc. (µg/ml)	Conc. 1 (µg/ml)	Conc. 2 (µg/ml)	Mean	SD	Stab. (%)	CV (%)
8 H	4	2.002	1.845	**1.924**	0.11	**48**	5.8
12 H	4	1.251	1.363	**1.307**	0.08	**33**	6.1
24 H	4	3.358	2.263	**2.811**	0.77	**70**	27.6

Conc. = concentration; SD = standard deviation; Stab. = stability; CV = coefficient of variation; H = hours

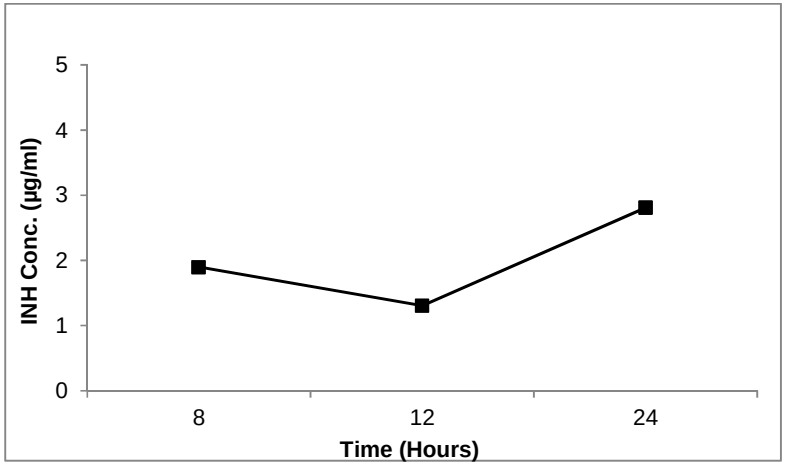

Figure C-5: Plot of short-term stability of INH (4 µg/ml) at -20ºC

Appendix C-6: Short-term stability of INH (10 µg/ml) at -20ºC

Table C-6: INH (10 µg/ml) stability data at -20ºC at 8, 12 and 24 hours

Time	Prep. Conc. (µg/ml)	Conc. 1 (µg/ml)	Conc. 2 (µg/ml)	Mean	SD	Stab. (%)	CV (%)
8 H	10	7.853	7.314	**7.584**	0.38	**76**	5.0
12 H	10	8.203	8.661	**8.432**	0.32	**84**	3.8
24 H	10	9.522	9.630	**9.576**	0.08	**96**	0.8

Conc. = concentration; SD = standard deviation; Stab. = stability; CV = coefficient of variation; H = hours

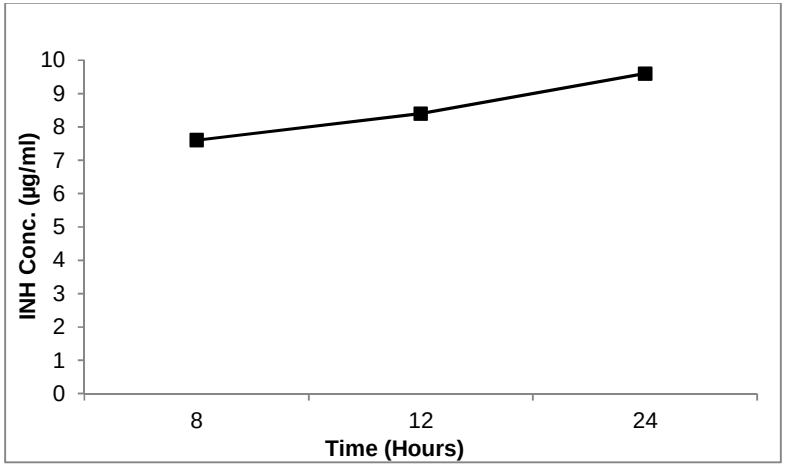

Figure C-6: Plot of short-term stability of INH (10 µg/ml) at -20ºC

Appendix C-7: Long-term Stability of INH (4 µg/ml) at -20ºC

Table C-7: INH (4 µg/ml) stability data at 7, 30 and 60 days

Time	Prep. Conc. (µg/ml)	Conc. 1 (µg/ml)	Conc. 2 (µg/ml)	Mean	SD	Stab. (%)	CV (%)
7 D	4	2.465	1.653	**2.059**	0.57	**52**	27.9
30 D	4	0.311	0.258	**0.285**	0.04	**7**	13.2
60 D	4	0.942	0.796	**0.869**	0.10	**22**	11.9

Conc. = concentration; SD = standard deviation; Stab. = stability; CV = coefficient of variation; D = days

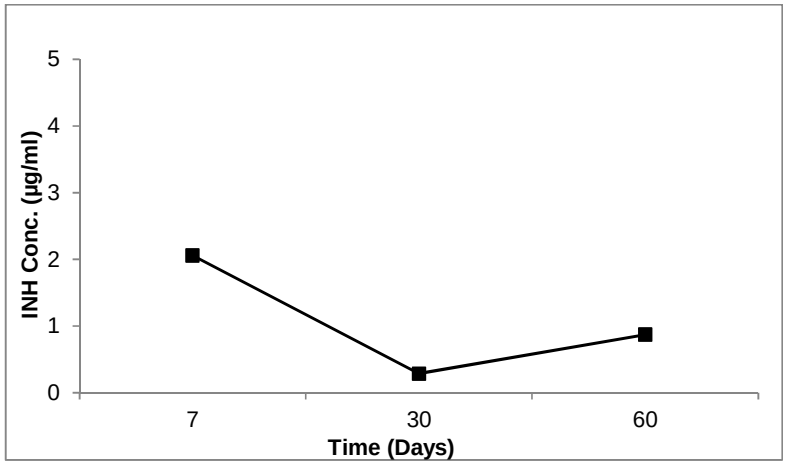

Figure C-7: Plot of long-term stability of INH (4 µg/ml) at -20ºC

Appendix C-8: Long-term Stability of INH (10 µg/ml) at -20ºC

Table C-8: INH (10 µg/ml) stability data at -20ºC at 7, 30 and 60 days

Time	Prep. Conc. (µg/ml)	Conc. 1 (µg/ml)	Conc. 2 (µg/ml)	Mean	SD	Stab. (%)	CV (%)
7 D	10	2.563	3.227	**2.895**	0.47	**29**	16.2
30 D	10	2.750	2.263	**2.507**	0.34	**25**	13.7
60 D	10	4.600	5.131	**4.866**	0.38	**49**	7.7

Conc. = concentration; SD = standard deviation; Stab. = stability; CV = coefficient of variation; D = days

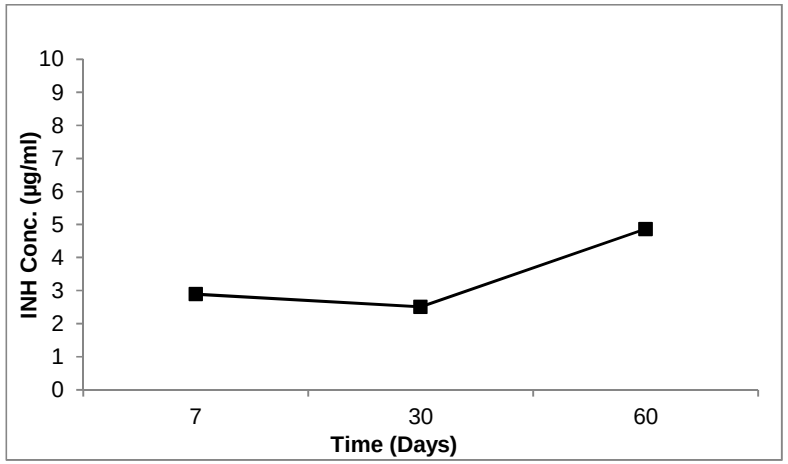

Figure C-8: Plot of long-term stability of INH (10 µg/ml) at -20ºC

Appendix C-9: Short-term stability of NVP (5 µg/ml) at ambient temperature

Table C-9: NVP (5 µg/ml) stability data at ambient temperature at 8, 12 and 24 hours

Time	Prep. Conc. (µg/ml)	Conc. 1 (µg/ml)	Conc. 2 (µg/ml)	Mean	SD	Stab. (%)	CV (%)
0 H	5	4.791	4.791	**4.791**	0.00	**96**	0.0
8 H	5	2.737	2.373	**2.555**	0.26	**51**	10.1
12 H	5	4.169	2.984	**3.577**	0.84	**72**	23.4
24 H	5	2.930	3.013	**2.958**	0.08	**59**	2.6

Conc. = concentration; SD = standard deviation; Stab. = stability; CV = coefficient of variation; H = hours

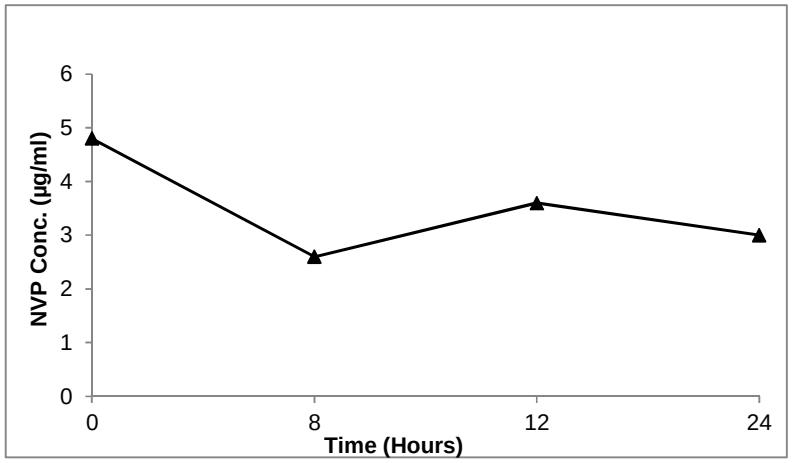

Figure C-9: Plot of stability of NVP (5 µg/ml) at ambient temperature

Appendix C-10: Short-term stability of NVP (10 µg/ml) at ambient temperature

Table C-10: NVP (10 µg/ml) stability data at ambient temperature at 8, 12 and 24 hours

Time	Prep. Conc. (µg/ml)	Conc. 1 (µg/ml)	Conc. 2 (µg/ml)	Mean	SD	Stab. (%)	CV (%)
0 H	10	9.991	9.266	**9.629**	0.51	**96**	5.3
8 H	10	8.608	9.201	**8.905**	0.42	**89**	4.7
12 H	10	10.561	10.210	**10.386**	0.25	**104**	2.4
24 H	10	10.824	9.223	**10.024**	1.13	**100**	11.3

Conc. = concentration; SD = standard deviation; Stab. = stability; CV = coefficient of variation; H = hours

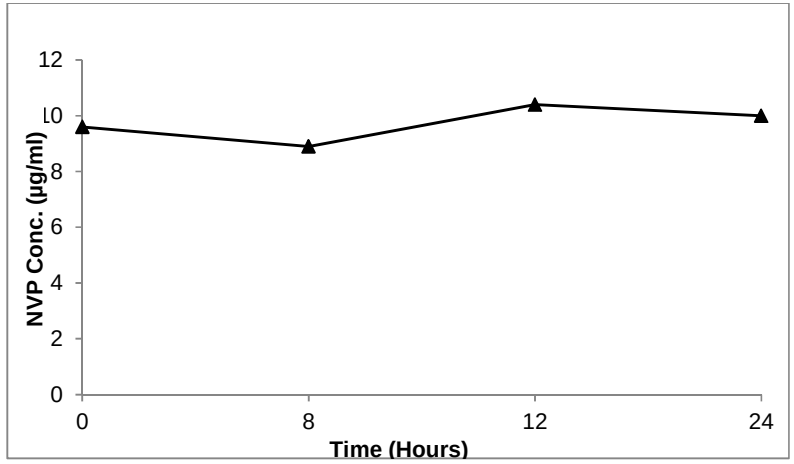

Figure C-10: Plot of stability of NVP (10 µg/ml) at ambient temperature

Appendix C-11: Short-term stability of NVP (5 µg/ml) at 4ºC

Table C-11: NVP (5 µg/ml) stability data at 4ºC at 8, 12 and 24 hours

Time	Prep. Conc. (µg/ml)	Conc. 1 (µg/ml)	Conc. 2 (µg/ml)	Mean	SD	Stab. (%)	CV (%)
8 H	5	6.745	4.143	**5.444**	1.84	**109**	33.8
12 H	5	4.625	4.592	**4.609**	0.02	**92**	0.5
24 H	5	2.572	4.581	**3.577**	1.42	**72**	39.7

Conc. = concentration; SD = standard deviation; Stab. = stability; CV = coefficient of variation; H = hours

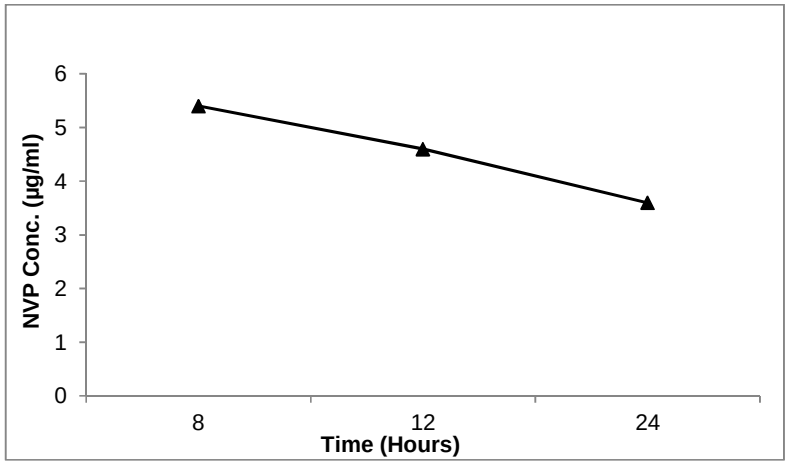

Figure C-11: Plot of stability of NVP (5 µg/ml) at 4ºC

Appendix C-12: Short-term stability of NVP (10 µg/ml) at 4ºC

Table C-12: NVP (10 µg/ml) stability data at 4ºC at 8, 12 and 24 hours

Time	Prep. Conc. (µg/ml)	Conc. 1 (µg/ml)	Conc. 2 (µg/ml)	Mean	SD	Stab. (%)	CV (%)
8 H	10	8.937	8.433	**8.685**	0.36	**87**	4.1
12 H	10	10.034	10.364	**10.199**	0.23	**102**	2.3
24 H	10	9.596	9.683	**9.640**	0.06	**96**	0.6

Conc. = concentration; SD = standard deviation; Stab. = stability; CV = coefficient of variation; H = hours

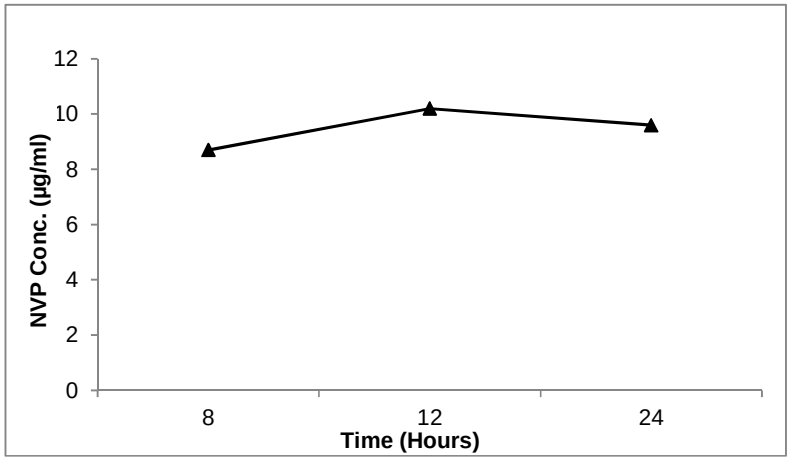

Figure C-12: Plot of stability of NVP (10 µg/ml) at 4ºC

Appendix C-13: Short-term stability of NVP (5 µg/ml) at -20ºC

Table C-13: NVP (5 µg/ml) stability data at -20ºC at 8, 12 and 24 hours

Time	Prep. Conc. (µg/ml)	Conc. 1 (µg/ml)	Conc. 2 (µg/ml)	Mean	SD	Stab. (%)	CV (%)
8 H	5	3.422	7.079	**5.251**	2.59	**105**	49.3
12 H	5	4.448	3.079	**3.764**	0.97	**75**	25.7
24 H	5	3.988	4.942	**4.465**	0.68	**89**	15.1

Conc. = concentration; SD = standard deviation; Stab. = stability; CV = coefficient of variation; H = hours

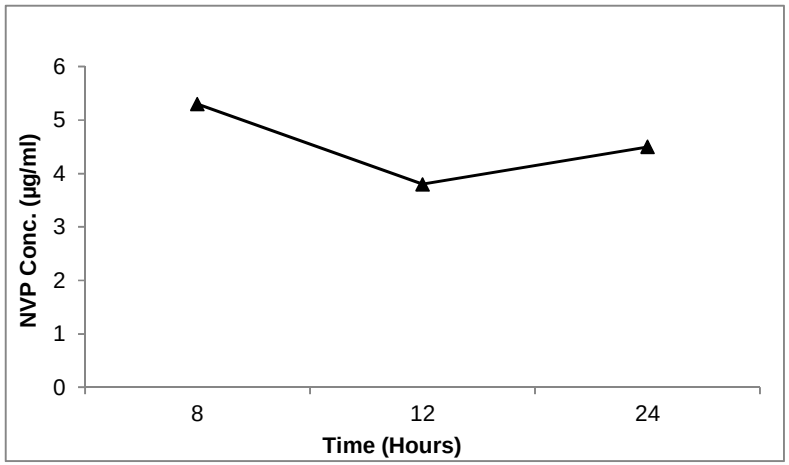

Figure C-13: Plot of short-term stability of NVP (5 µg/ml) at -20ºC

Appendix C-14: Short-term stability of NVP (10 µg/ml) at -20ºC

Table C-14: NVP (10 µg/ml) stability data at -20ºC at 8, 12 and 24 hours

Time	Prep. Conc. (µg/ml)	Conc. 1 (µg/ml)	Conc. 2 (µg/ml)	Mean	SD	Stab. (%)	CV (%)
8 H	10	8.762	8.740	**8.751**	0.02	**88**	0.2
12 H	10	11.000	11.263	**11.132**	0.19	**111**	1.7
24 H	10	10.122	9.969	**9.946**	0.11	**101**	1.1

Conc. = concentration; SD = standard deviation; Stab. = stability; CV = coefficient of variation; H = hours

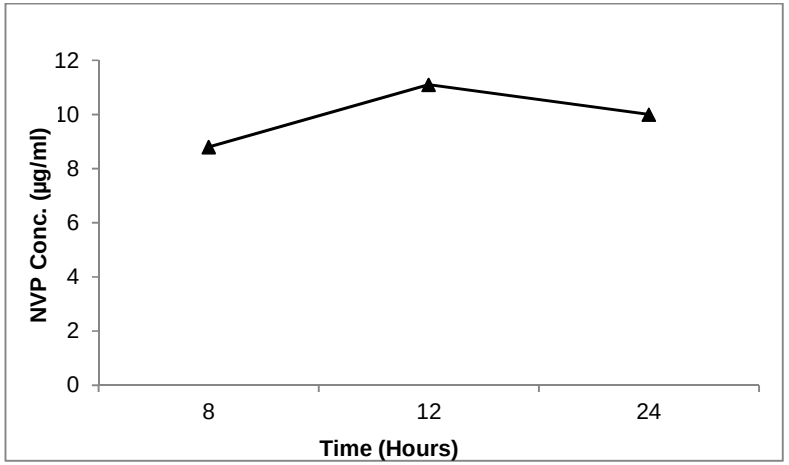

Figure C-14: Plot of short-term stability of NVP (10 µg/ml) at -20ºC

Appendix C-15: Long-term stability of NVP (5 µg/ml) at -20ºC

Table C-15: NVP (5 µg/ml) stability data at -20ºC at 7, 30 and 60 days

Time	Prep. Conc. (µg/ml)	Conc. 1 (µg/ml)	Conc. 2 (µg/ml)	Mean	SD	Stab. (%)	CV (%)
7 D	5	4.411	4.424	**4.418**	0.01	**88**	0.2
30 D	5	2.250	2.243	**2.247**	0.01	**45**	0.2
60 D	5	3.958	3.670	**3.814**	0.20	**76**	5.3

Conc. = concentration; SD = standard deviation; Stab. = stability; CV = coefficient of variation; D = days

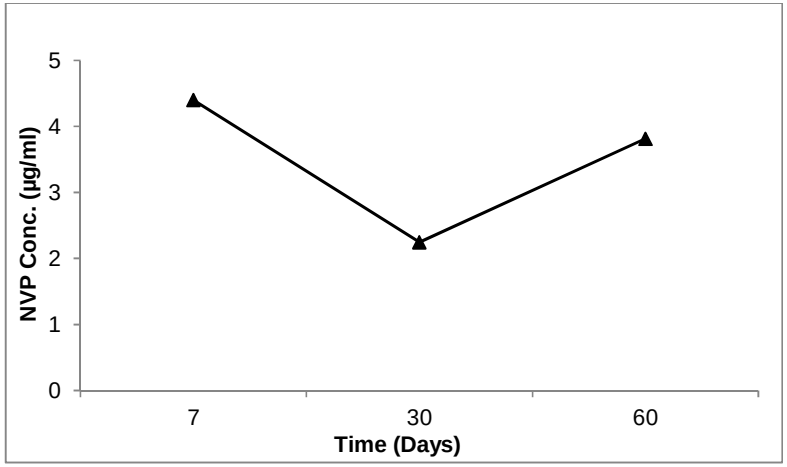

Figure C-15: Plot of long-term stability of NVP (5 µg/ml) at -20ºC

Appendix C-16: Long-term stability of NVP (10 µg/ml) at -20ºC

Table C-16: NVP (10 µg/ml) stability data at -20ºC at 7, 30 and 60 days

Time	Prep. Conc. (µg/ml)	Conc. 1 (µg/ml)	Conc. 2 (µg/ml)	Mean	SD	Stab. (%)	CV (%)
7 D	10	7.283	8.917	**8.100**	1.16	**81**	14.3
30 D	10	9.228	9.491	**9.360**	0.19	**94**	2.0
60 D	10	7.283	7.998	**7.641**	0.51	**76**	6.6

Conc. = concentration; SD = standard deviation; Stab. = stability; CV = coefficient of variation; D = days

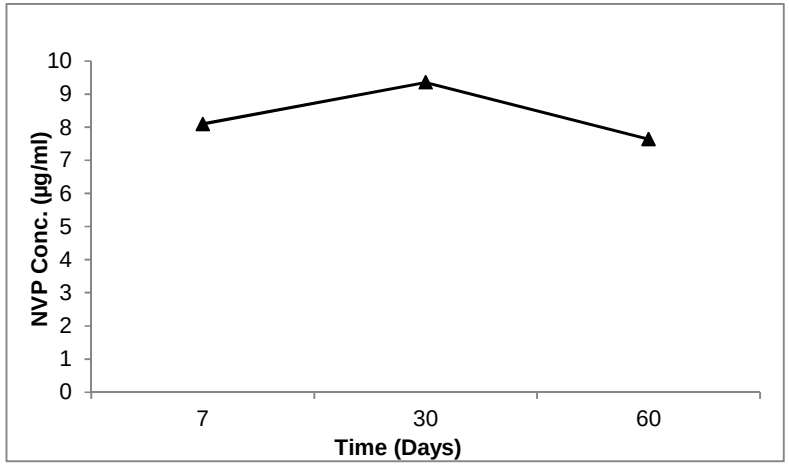

Figure C-16: Plot of long-term stability of NVP (10 µg/ml) at -20ºC

Appendix C-17: Short-term stability of PAR (10 µg/ml) at ambient temperature

Table C-17: PAR (10 µg/ml) stability data at ambient temperature at 8, 12 and 24 hours

Time	Prep. Conc. (µg/ml)	Conc. 1 (µg/ml)	Conc. 2 (µg/ml)	Mean	SD	Stab. (%)	CV (%)
0 H	10	10.050	11.112	**10.581**	0.75	**106**	7.1
8 H	10	9.932	10.082	**10.007**	0.11	**100**	1.1
12 H	10	10.232	10.232	**10.232**	0.00	**102**	0.0
24 H	10	9.943	10.436	**10.190**	0.35	**102**	3.4

Conc. = concentration; SD = standard deviation; Stab. = stability; CV = coefficient of variation; H = hours

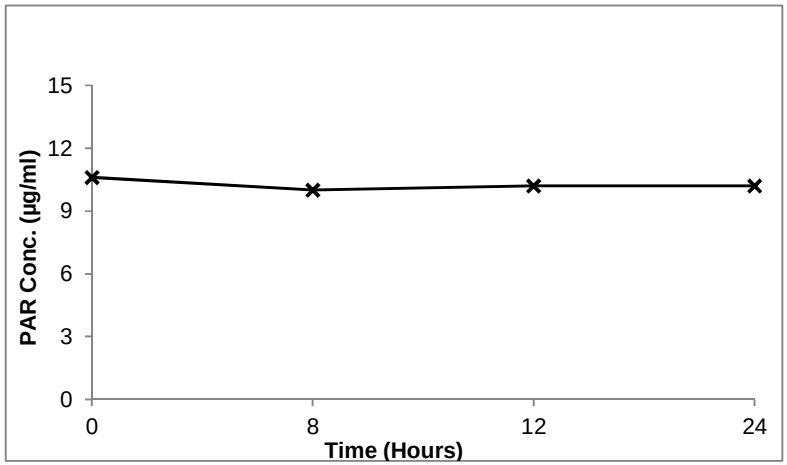

Figure C-17: Plot of stability of PAR (10 µg/ml) at ambient temperature

Appendix C-18: Short-term stability of PAR (20 µg/ml) at ambient temperature

Table C-18: PAR (20 µg/ml) stability data at ambient temperature at 8, 12 and 24 hours

Time	Prep. Conc. (µg/ml)	Conc. 1 (µg/ml)	Conc. 2 (µg/ml)	Mean	SD	Stab. (%)	CV (%)
0 H	20	19.752	20.092	**19.925**	0.24	**100**	1.2
8 H	20	17.238	17.213	**17.226**	0.02	**86**	0.1
12 H	20	18.751	18.889	**18.820**	0.10	**94**	0.5
24 H	20	18.695	17.186	**17.941**	1.07	**90**	5.9

Conc. = concentration; SD = standard deviation; Stab. = stability; CV = coefficient of variation; H = hours

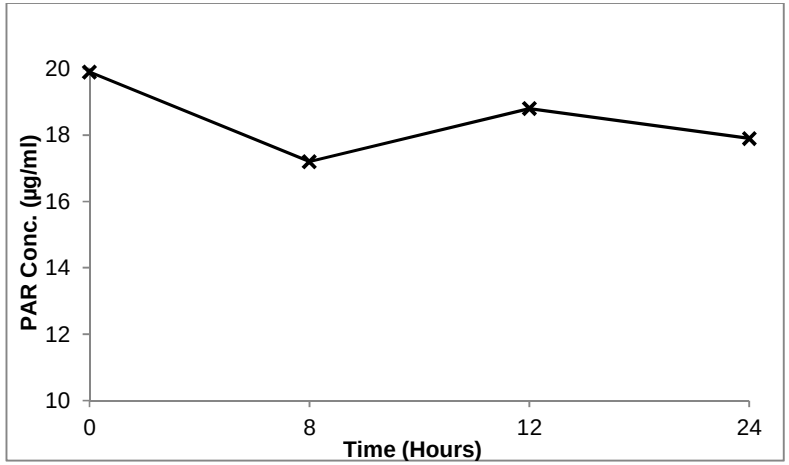

Figure C-18: Plot of stability of PAR (20 µg/ml) at ambient temperature

Appendix C-19: Short-term stability of PAR (10 µg/ml) at 4ºC

Table C-19: PAR (10 µg/ml) stability data at 4ºC at 8, 12 and 24 hours

Time	Prep. Conc. (µg/ml)	Conc. 1 (µg/ml)	Conc. 2 (µg/ml)	Mean	SD	Stab. (%)	CV (%)
8 H	10	10.114	10.157	**10.136**	0.03	**101**	0.3
12 H	10	9.965	9.934	**9.950**	0.02	**100**	0.2
24 H	10	11.562	12.066	**11.814**	0.36	**118**	3.0

Conc. = concentration; SD = standard deviation; Stab. = stability; CV = coefficient of variation; H = hours

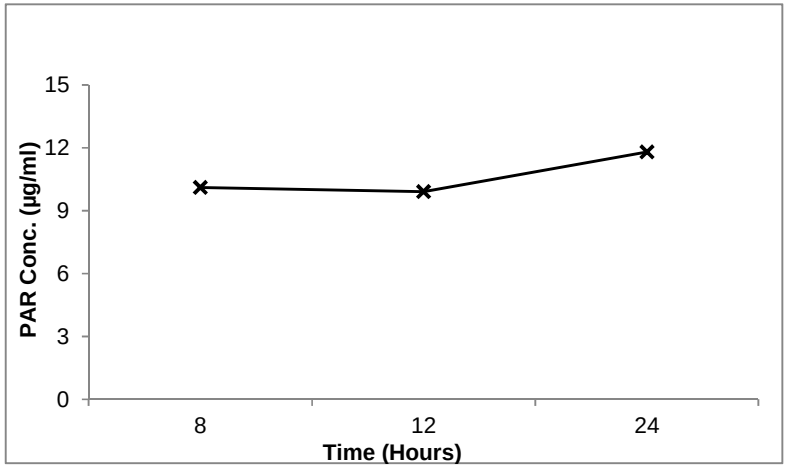

Figure C-19: Plot of stability of PAR (10 µg/ml) at 4ºC

Appendix C-20: Short-term stability of PAR (20 µg/ml) at 4ºC

Table C-20: PAR (20 µg/ml) stability data at 4ºC at 8, 12 and 24 hours

Time	Prep. Conc. (µg/ml)	Conc. 1 (µg/ml)	Conc. 2 (µg/ml)	Mean	SD	Stab. (%)	CV (%)
8 H	20	16.958	14.417	**15.688**	1.80	**78**	11.5
12 H	20	19.754	18.894	**19.324**	0.61	**97**	3.1
24 H	20	17.614	17.900	**17.757**	0.20	**89**	1.1

Conc. = concentration; SD = standard deviation; Stab. = stability; CV = coefficient of variation; H = hours

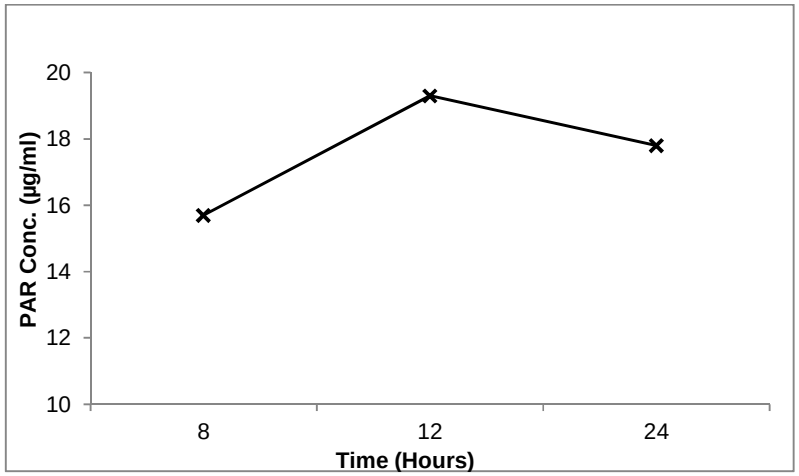

Figure C-20: Plot of stability of PAR (20 µg/ml) at 4ºC

Appendix C-21: Short-term stability of PAR (10 µg/ml) at -20ºC

Table C-21: PAR (10 µg/ml) stability data at -20ºC at 8, 12 and 24 hours

Time	Prep. Conc. (µg/ml)	Conc. 1 (µg/ml)	Conc. 2 (µg/ml)	Mean	SD	Stab. (%)	CV (%)
8 H	10	10.093	10.490	**10.292**	0.28	**103**	2.7
12 H	10	11.294	11.037	**11.166**	0.18	**112**	1.6
24 H	10	11.734	10.061	**10.898**	1.18	**109**	10.9

Conc. = concentration; SD = standard deviation; Stab. = stability; CV = coefficient of variation; H = hours

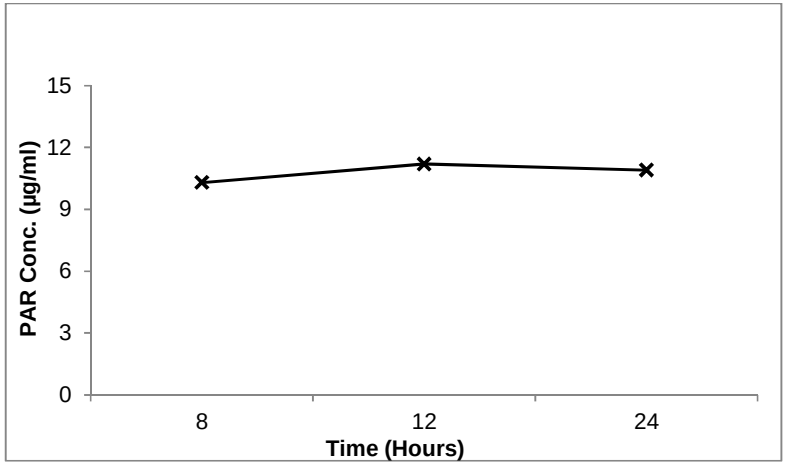

Figure C-21: Plot of short-term stability of PAR (10 µg/ml) at -20ºC

Appendix C-22: Short-term stability of PAR (20 µg/ml) at -20ºC

Table C-22: PAR (20 µg/ml) stability data at -20ºC at 8, 12 and 24 hours

Time	Prep. Conc. (µg/ml)	Conc. 1 (µg/ml)	Conc. 2 (µg/ml)	Mean	SD	Stab. (%)	CV (%)
8 H	20	16.425	15.834	**16.130**	0.42	**81**	2.6
12 H	20	19.304	19.085	**19.195**	0.16	**96**	1.0
24 H	20	18.513	18.246	**18.380**	0.19	**92**	1.0

Conc. = concentration; SD = standard deviation; Stab. = stability; CV = coefficient of variation; H = hours

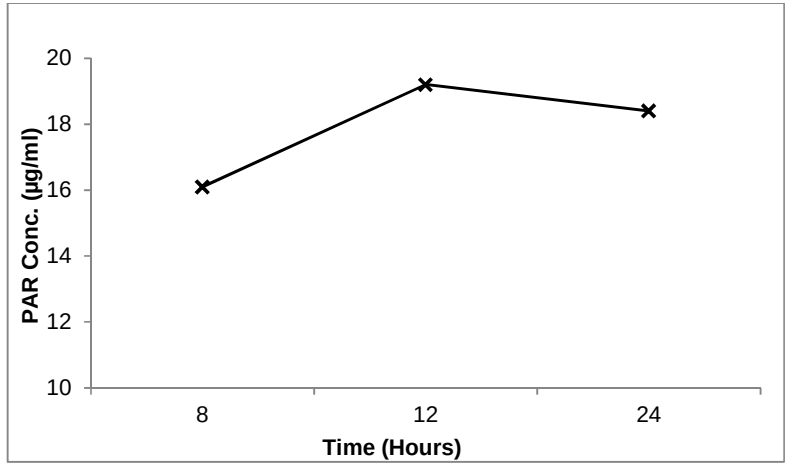

Figure C-22: Plot of long-term stability of PAR (20 µg/ml) at -20ºC

Appendix C-23: Long-term stability of PAR (10 µg/ml) at -20ºC

Table C-23: PAR (10µg/ml) stability data at -20ºC at 7, 30 and 60 days

Time	Prep. Conc. (µg/ml)	Conc. 1 (µg/ml)	Conc. 2 (µg/ml)	Mean	SD	Stab. (%)	CV (%)
7 D	10	9.070	11.559	**10.315**	1.76	**103**	17.1
30 D	10	12.341	12.180	**12.261**	0.11	**123**	0.9
60 D	10	7.132	6.013	**6.573**	0.79	**66**	12.0

Conc. = concentration; SD = standard deviation; Stab. = stability; CV = coefficient of variation; D = days

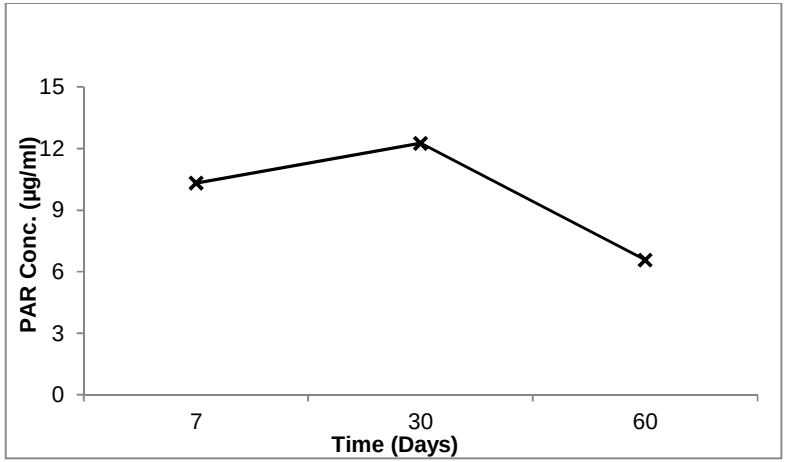

Figure C-23: Plot of long-term stability of PAR (10 µg/ml) at -20ºC

Appendix C-24: Long-term stability of PAR (20 µg/ml) at -20ºC

Table C-24: PAR (20µg/ml) stability data at -20ºC at 7, 30 and 60 days

Time	Prep. Conc. (µg/ml)	Conc. 1 (µg/ml)	Conc. 2 (µg/ml)	Mean	SD	Stab. (%)	CV (%)
7 D	20	17.820	18.063	**17.942**	0.17	**90**	1.0
30 D	20	11.603	16.038	**13.821**	3.14	**69**	22.7
60 D	20	8.226	8.863	**8.545**	0.45	**43**	5.3

Conc. = concentration; SD = standard deviation; Stab. = stability; CV = coefficient of variation; D = days

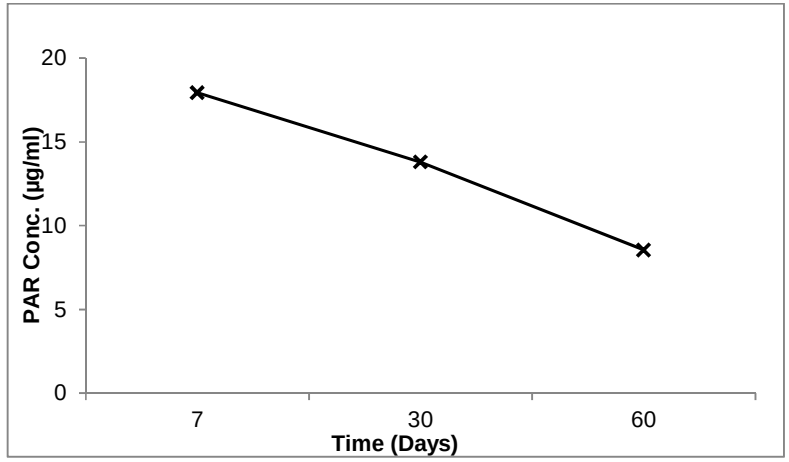

Figure C-24: Plot of long-term stability of PAR (20 µg/ml) at -20ºC

APPENDIX D: FULL BLOOD COUNT RESULTS

Appendix D-1: Full blood count results of the S, S+LMS and S+CBZ groups

Table D-1a: Full blood count results of the S group after 0, 2 and 7 days

Group (n = 3)	Rat 1	Rat 2	Rat 3	Mean	SD
Untreated					
WCC (x10^9/l)	4.16	9.55	7.15	**6.95**	2.7
RCC (x10^{12}/l)	6.42	5.99	6.42	**6.28**	0.2
Hb (g/dl)	13.1	12.5	13.0	**12.9**	0.3
Hct (l/l)	0.406	0.396	0.393	**0.398**	0.01
MCV (fl)	63.2	66.1	61.2	**63.5**	2.5
MCH (pg)	20.4	20.9	20.2	**20.5**	0.4
MCHC (g/dl)	32.3	31.6	33.1	**32.3**	0.8
Plt (x10^9/l)	934	611	1034	**860**	221.1
Neu (x10^9/l)	0.65	-*	0.89	**0.77**	0.2
Ly (x10^9/l)	3.37	-*	5.97	**4.67**	1.8
Mo (x10^9/l)	0.11	-*	0.27	**0.19**	0.1
Eos (x10^9/l)	0.02	-*	0.02	**0.02**	0.0
Bas (x10^9/l)	0.01	-*	0.00	**0.01**	0.0
S-2D (2 Days)					
WCC (x10^9/l)	5.64	6.50	7.37	**6.50**	0.9
RCC (x10^{12}/l)	6.71	6.81	6.50	**6.67**	0.2
Hb (g/dl)	13.8	13.6	13.7	**13.7**	0.1
Hct (l/l)	0.418	0.420	0.429	**0.422**	0.01
MCV (fl)	62.3	61.7	66.0	**63.3**	2.3
MCH (pg)	20.6	20.0	21.1	**20.6**	0.6
MCHC (g/dl)	33.0	32.4	31.9	**32.4**	0.6
Plt (x10^9/l)	932	845	769	**849**	81.6
Neu (x10^9/l)	0.44	0.57	0.80	**0.60**	0.2
Ly (x10^9/l)	4.60	4.93	6.01	**5.18**	0.7
Mo (x10^9/l)	0.17	0.22	0.24	**0.21**	0.0
Eos (x10^9/l)	0.43	0.77	0.31	**0.50**	0.2
Bas (x10^9/l)	0.00	0.01	0.01	**0.01**	0.0
S-7D (7 Days)					
WCC (x10^9/l)	7.90	3.11	5.30	**5.44**	2.4
RCC (x10^{12}/l)	7.03	6.96	8.59	**7.53**	0.9
Hb (g/dl)	14.4	14.2	17.3	**15.3**	1.7
Hct (l/l)	0.430	0.431	0.492	**0.451**	0.04
MCV (fl)	61.2	61.9	57.3	**60.1**	2.5
MCH (pg)	20.5	20.4	20.1	**20.3**	0.2
MCHC (g/dl)	33.5	32.9	35.2	**33.9**	1.2
Plt (x10^9/l)	1120	1017	963	**1033**	79.8
Neu (x10^9/l)	0.91	0.28	1.89	**1.03**	0.8
Ly (x10^9/l)	6.31	2.72	3.18	**4.07**	2.0
Mo (x10^9/l)	0.61	0.09	0.19	**0.30**	0.3
Eos (x10^9/l)	0.06	0.02	0.03	**0.04**	0.0
Bas (x10^9/l)	0.01	0.00	0.01	**0.01**	0.0

SD = standard deviation; S = saline; D = days; WCC = white cell count; RCC = red cell count; Hb = haemoglobin; Hct = haematocrit; MCV = mean corpuscular volume; MCH = mean corpuscular haemoglobin; MCHC = mean corpuscular haemoglobin concentration; Plt = platelets; Neu = neutrophils; Ly = lymphocytes; Mo = monocytes; Eos = eosinophils; Bas = basophils; * = differential count failed

Table D-1b: Full blood count results of the S group after 14, 28 and 42 days

Group (n = 3)	Rat 1	Rat 2	Rat 3	Mean	SD
S-14D (14 Days)					
WCC (x10^9/l)	6.57	4.63	4.45	**5.22**	1.2
RCC (x10^{12}/l)	6.04	7.22	7.04	**6.77**	0.6
Hb (g/dl)	12.6	14.3	14.7	**13.9**	1.1
Hct (l/l)	0.387	0.423	0.441	**0.417**	0.03
MCV (fl)	64.1	58.6	62.6	**61.8**	2.8
MCH (pg)	20.9	19.8	20.9	**20.5**	0.6
MCHC (g/dl)	32.6	33.8	33.3	**33.2**	0.6
Plt (x10^9/l)	539	694	929	**721**	196.4
Neu (x10^9/l)	1.16	0.40	0.34	**0.63**	0.5
Ly (x10^9/l)	5.00	3.83	3.79	**4.21**	0.7
Mo (x10^9/l)	0.32	0.13	0.09	**0.18**	0.1
Eos (x10^9/l)	0.01	0.26	0.27	**0.18**	0.1
Bas (x10^9/l)	0.08	0.01	0.05	**0.05**	0.0
S-28D (28 Days)					
WCC (x10^9/l)	8.54	6.91	6.68	**7.38**	1.0
RCC (x10^{12}/l)	7.74	7.08	6.39	**7.07**	0.7
Hb (g/dl)	15.2	14.0	12.6	**13.9**	1.3
Hct (l/l)	0.424	0.398	0.347	**0.390**	0.04
MCV (fl)	54.8	56.2	54.3	**55.1**	1.0
MCH (pg)	19.6	19.8	19.7	**19.7**	0.1
MCHC (g/dl)	35.8	35.2	36.3	**35.8**	0.6
Plt (x10^9/l)	1135	957	790	**961**	172.5
Neu (x10^9/l)	1.10	0.74	0.88	**0.91**	0.2
Ly (x10^9/l)	7.08	5.96	5.42	**6.15**	0.8
Mo (x10^9/l)	0.26	0.16	0.31	**0.24**	0.1
Eos (x10^9/l)	0.09	0.04	0.08	**0.07**	0.0
Bas (x10^9/l)	0.01	0.01	0.00	**0.01**	0.0
S-42D (42 Days)					
WCC (x10^9/l)	4.29	3.67	3.82	**3.93**	0.3
RCC (x10^{12}/l)	7.49	6.02	7.27	**6.93**	0.8
Hb (g/dl)	14.7	11.3	14.2	**13.4**	1.8
Hct (l/l)	0.409	0.318	0.395	**0.374**	0.05
MCV (fl)	54.6	52.8	54.3	**53.9**	1.0
MCH (pg)	19.6	18.8	19.5	**19.3**	0.4
MCHC (g/dl)	35.9	35.5	35.9	**35.8**	0.2
Plt (x10^9/l)	1004	672	841	**839**	166.0
Neu (x10^9/l)	0.62	0.56	0.44	**0.54**	0.1
Ly (x10^9/l)	3.54	2.90	3.26	**3.23**	0.3
Mo (x10^9/l)	0.08	0.16	0.08	**0.11**	0.0
Eos (x10^9/l)	0.04	0.04	0.04	**0.04**	0.0
Bas (x10^9/l)	0.01	0.01	0.00	**0.01**	0.0

SD = standard deviation; S = saline; D = days; WCC = white cell count; RCC = red cell count; Hb = haemoglobin; Hct = haematocrit; MCV = mean corpuscular volume; MCH = mean corpuscular haemoglobin; MCHC = mean corpuscular haemoglobin concentration; Plt = platelets; Neu = neutrophils; Ly = lymphocytes; Mo = monocytes; Eos = eosinophils; Bas = basophils

Table D-2a: Full blood count results of the S+LMS group after 2, 7 and 14 days

Group (n = 3)	Rat 1	Rat 2	Rat 3	Mean	SD
S+LMS-2D (2 Days)					
WCC (x10^9/l)	7.19	-*	7.46	**7.33**	0.2
RCC (x10^{12}/l)	6.21	-*	6.84	**6.53**	0.4
Hb (g/dl)	12.8	-*	13.6	**13.2**	0.6
Hct (l/l)	0.389	-*	0.426	**0.408**	0.03
MCV (fl)	62.6	-*	62.3	**62.5**	0.2
MCH (pg)	20.6	-*	19.9	**20.3**	0.5
MCHC (g/dl)	32.9	-*	31.9	**32.4**	0.7
Plt (x10^9/l)	586	-*	924	**755**	239.0
Neu (x10^9/l)	0.68	-*	0.63	**0.66**	0.0
Ly (x10^9/l)	5.73	-*	6.24	**5.99**	0.4
Mo (x10^9/l)	0.34	-*	0.35	**0.35**	0.0
Eos (x10^9/l)	0.44	-*	0.24	**0.34**	0.1
Bas (x10^9/l)	0.01	-*	0.00	**0.01**	0.0
S+LMS-7D (7 Days)					
WCC (x10^9/l)	-*	-*	6.75	**6.75**	0.0
RCC (x10^{12}/l)	-*	-*	6.91	**6.91**	0.0
Hb (g/dl)	-*	-*	13.4	**13.4**	0.0
Hct (l/l)	-*	-*	0.408	**0.408**	0.00
MCV (fl)	-*	-*	59.0	**59.0**	0.0
MCH (pg)	-*	-*	19.4	**19.4**	0.0
MCHC (g/dl)	-*	-*	32.8	**32.8**	0.0
Plt (x10^9/l)	-*	-*	850	**850**	0.0
Neu (x10^9/l)	-*	-*	0.93	**0.93**	0.0
Ly (x10^9/l)	-*	-*	5.10	**5.10**	0.0
Mo (x10^9/l)	-*	-*	0.38	**0.38**	0.0
Eos (x10^9/l)	-*	-*	0.33	**0.33**	0.0
Bas (x10^9/l)	-*	-*	0.01	**0.01**	0.0
S+LMS-14D (14 Days)					
WCC (x10^9/l)	5.38	2.70	5.86	**4.65**	1.7
RCC (x10^{12}/l)	6.84	6.68	7.46	**6.99**	0.4
Hb (g/dl)	13.6	13.2	14.7	**13.8**	0.8
Hct (l/l)	0.418	0.418	0.444	**0.427**	0.02
MCV (fl)	61.1	62.6	59.5	**61.1**	1.6
MCH (pg)	19.9	19.8	19.7	**19.8**	0.1
MCHC (g/dl)	32.5	31.6	33.1	**32.4**	0.8
Plt (x10^9/l)	804	485	865	**718**	204.1
Neu (x10^9/l)	0.36	0.36	0.87	**0.53**	0.3
Ly (x10^9/l)	4.77	1.74	4.94	**3.82**	1.8
Mo (x10^9/l)	0.23	0.11	0.02	**0.12**	0.1
Eos (x10^9/l)	0.02	0.49	0.03	**0.18**	0.3
Bas (x10^9/l)	0.00	0.00	0.00	**0.00**	0.0

SD = standard deviation; S = saline; LMS = levamisole; D = days; WCC = white cell count; RCC = red cell count; Hb = haemoglobin; Hct = haematocrit; MCV = mean corpuscular volume; MCH = mean corpuscular haemoglobin; MCHC = mean corpuscular haemoglobin concentration; Plt = platelets; Neu = neutrophils; Ly = lymphocytes; Mo = monocytes; Eos = eosinophils; Bas = basophils; * = blood clotted

Table D-2b: Full blood count results of the S+LMS group after 28 and 42 days

Group (n = 3)	Rat 1	Rat 2	Rat 3	Mean	SD
S+LMS-28D (28 Days)					
WCC (x10^9/l)	6.11	3.06	6.21	**5.13**	1.8
RCC (x10^{12}/l)	7.25	7.57	7.42	**7.41**	0.2
Hb (g/dl)	14.5	14.9	15.2	**14.9**	0.4
Hct (l/l)	0.428	0.446	0.435	**0.436**	0.01
MCV (fl)	59.0	58.9	58.6	**58.8**	0.2
MCH (pg)	20.2	19.7	20.5	**20.1**	0.4
MCHC (g/dl)	33.9	33.4	34.9	**34.1**	0.8
Plt (x10^9/l)	526	562	647	**578**	62.1
Neu (x10^9/l)	0.84	0.56	0.73	**0.71**	0.1
Ly (x10^9/l)	5.13	2.44	5.24	**4.27**	1.6
Mo (x10^9/l)	0.11	0.02	0.18	**0.10**	0.1
Eos (x10^9/l)	0.02	0.04	0.05	**0.04**	0.0
Bas (x10^9/l)	0.01	0.00	0.01	**0.01**	0.0
S+LMS-42D (42 Days)					
WCC (x10^9/l)	5.93	6.78	7.56	**6.76**	0.8
RCC (x10^{12}/l)	7.74	8.09	7.91	**7.91**	0.2
Hb (g/dl)	15.3	16.0	15.4	**15.6**	0.4
Hct (l/l)	0.440	0.471	0.454	**0.455**	0.02
MCV (fl)	56.8	58.2	57.4	**57.5**	0.7
MCH (pg)	19.8	19.8	19.5	**19.7**	0.2
MCHC (g/dl)	34.8	34.0	33.9	**34.2**	0.5
Plt (x10^9/l)	802	573	727	**701**	116.7
Neu (x10^9/l)	0.77	0.94	0.85	**0.85**	0.1
Ly (x10^9/l)	4.87	5.63	6.58	**5.69**	0.9
Mo (x10^9/l)	0.25	0.16	0.08	**0.16**	0.1
Eos (x10^9/l)	0.04	0.05	0.04	**0.04**	0.0
Bas (x10^9/l)	0.00	0.00	0.01	**0.00**	0.0

SD = standard deviation; S = saline; LMS = levamisole; D = days; WCC = white cell count; RCC = red cell count; Hb = haemoglobin; Hct = haematocrit; MCV = mean corpuscular volume; MCH = mean corpuscular haemoglobin; MCHC = mean corpuscular haemoglobin concentration; Plt = platelets; Neu = neutrophils; Ly = lymphocytes; Mo = monocytes; Eos = eosinophils; Bas = basophils

Table D-3a: Full blood count results of the S+CBZ group after 2, 7 and 14 days

Group (n = 3)	Rat 1	Rat 2	Rat 3	Mean	SD
S+CBZ-2D (2 Days)					
WCC (x10^9/l)	5.29	7.00	7.16	**6.48**	1.0
RCC (x10^{12}/l)	6.95	6.55	6.71	**6.74**	0.2
Hb (g/dl)	13.3	13.2	13.1	**13.2**	0.1
Hct (l/l)	0.406	0.408	0.405	**0.406**	0.00
MCV (fl)	58.4	62.3	60.4	**60.4**	2.0
MCH (pg)	19.1	20.2	19.5	**19.6**	0.6
MCHC (g/dl)	32.8	32.4	21.3	**28.8**	6.5
Plt (x10^9/l)	887	909	1063	**953**	95.9
Neu (x10^9/l)	0.52	-*	0.50	**0.51**	0.0
Ly (x10^9/l)	4.68	-*	6.23	**5.46**	1.1
Mo (x10^9/l)	0.05	-*	0.43	**0.24**	0.3
Eos (x10^9/l)	0.03	-*	0.00	**0.02**	0.0
Bas (x10^9/l)	0.01	-*	0.00	**0.01**	0.0
S+CBZ-7D (7 Days)					
WCC (x10^9/l)	4.58	8.48	8.91	**7.32**	2.4
RCC (x10^{12}/l)	6.62	7.02	6.23	**6.62**	0.4
Hb (g/dl)	12.9	13.8	12.6	**13.1**	0.6
Hct (l/l)	0.406	0.438	0.398	**0.414**	0.02
MCV (fl)	61.3	62.4	63.9	**62.5**	1.3
MCH (pg)	19.5	19.7	20.2	**19.8**	0.4
MCHC (g/dl)	31.8	31.5	31.7	**31.7**	0.2
Plt (x10^9/l)	789	587	962	**779**	187.7
Neu (x10^9/l)	0.39	0.68	0.69	**0.59**	0.2
Ly (x10^9/l)	3.88	6.89	7.82	**6.20**	2.1
Mo (x10^9/l)	0.13	0.37	0.32	**0.27**	0.1
Eos (x10^9/l)	0.18	0.56	0.06	**0.27**	0.3
Bas (x10^9/l)	0.00	0.01	0.01	**0.01**	0.0
S+CBZ-14D (14 Days)					
WCC (x10^9/l)	8.10	9.22	7.92	**8.41**	0.7
RCC (x10^{12}/l)	7.16	7.38	7.41	**7.32**	0.1
Hb (g/dl)	14.7	15.0	14.5	**14.7**	0.3
Hct (l/l)	0.452	0.448	0.426	**0.442**	0.01
MCV (fl)	63.1	60.7	57.5	**60.4**	2.8
MCH (pg)	20.5	20.3	19.6	**20.1**	0.5
MCHC (g/dl)	32.5	33.5	34.0	**33.3**	0.8
Plt (x10^9/l)	759	791	1032	**861**	149.2
Neu (x10^9/l)	0.80	0.75	0.72	**0.76**	0.0
Ly (x10^9/l)	6.99	7.89	6.95	**7.28**	0.5
Mo (x10^9/l)	0.26	0.53	0.19	**0.33**	0.2
Eos (x10^9/l)	0.04	0.05	0.05	**0.05**	0.0
Bas (x10^9/l)	0.01	0.01	0.01	**0.01**	0.0

SD = standard deviation; S = saline; CBZ = carbamazepine; D = Days; WCC = white cell count; RCC = red cell count; Hb = haemoglobin; Hct = haematocrit; MCV = mean corpuscular volume; MCH = mean corpuscular haemoglobin; MCHC = mean corpuscular haemoglobin concentration; Plt = platelets; Neu = neutrophils; Ly = lymphocytes; Mo = monocytes; Eos = eosinophils; Bas = basophils; * = differential count failed

Table D-3b: Full blood count results of the S+CBZ group after 28 and 42 days

Group (n = 3)	Rat 1	Rat 2	Rat 3	Mean	SD
S+CBZ-28D (28 Days)					
WCC (x10^9/l)	5.48	4.17	6.78	**5.48**	1.3
RCC (x10^{12}/l)	6.48	7.13	8.19	**7.27**	0.9
Hb (g/dl)	13.1	14.0	16.3	**14.5**	1.7
Hct (l/l)	0.375	0.423	0.475	**0.424**	0.05
MCV (fl)	57.9	59.3	58.0	**58.4**	0.8
MCH (pg)	20.2	19.6	19.9	**19.9**	0.3
MCHC (g/dl)	34.9	33.1	34.3	**34.1**	0.9
Plt (x10^9/l)	572	612	726	**637**	79.9
Neu (x10^9/l)	0.76	0.62	1.15	**0.84**	0.3
Ly (x10^9/l)	4.12	3.40	5.43	**4.32**	1.0
Mo (x10^9/l)	0.52	0.04	0.14	**0.23**	0.3
Eos (x10^9/l)	0.08	0.11	0.06	**0.08**	0.0
Bas (x10^9/l)	0.00	0.00	0.00	**0.00**	0.0
S+CBZ-42D (42 Days)					
WCC (x10^9/l)	6.15	7.18	6.08	**6.47**	0.6
RCC (x10^{12}/l)	7.84	8.04	7.81	**7.90**	0.1
Hb (g/dl)	15.6	15.7	15.0	**15.4**	0.4
Hct (l/l)	0.458	0.460	0.435	**0.451**	0.01
MCV (fl)	58.4	57.2	55.7	**57.1**	1.4
MCH (pg)	19.9	19.5	19.2	**19.5**	0.4
MCHC (g/dl)	34.1	34.1	34.5	**34.2**	0.2
Plt (x10^9/l)	718	780	684	**727**	48.7
Neu (x10^9/l)	0.64	0.73	0.60	**0.66**	0.1
Ly (x10^9/l)	5.27	6.14	5.24	**5.55**	0.5
Mo (x10^9/l)	0.18	0.26	0.19	**0.21**	0.0
Eos (x10^9/l)	0.05	0.05	0.05	**0.05**	0.0
Bas (x10^9/l)	0.01	0.01	0.00	**0.01**	0.0

SD = standard deviation; S = saline; CBZ = carbamazepine; D = Days; WCC = white cell count; RCC = red cell count; Hb = haemoglobin; Hct = haematocrit; MCV = mean corpuscular volume; MCH = mean corpuscular haemoglobin; MCHC = mean corpuscular haemoglobin concentration; Plt = platelets; Neu = neutrophils; Ly = lymphocytes; Mo = monocytes; Eos = eosinophils; Bas = basophils

Appendix D-2: Full blood count results of the INH, INH+LMS and INH+CBZ groups

Table D-4a: Full blood count results of the INH group after 2, 7 and 14 days

Group (n = 3)	Rat 1	Rat 2	Rat 3	Mean	SD
INH-2D (2 Days)					
WCC (x10^9/l)	6.76	4.63	6.19	**5.86**	1.1
RCC (x10^{12}/l)	6.39	6.42	6.89	**6.57**	0.3
Hb (g/dl)	13.2	13.2	13.9	**13.4**	0.4
Hct (l/l)	0.410	0.416	0.421	**0.416**	0.01
MCV (fl)	64.2	64.8	61.1	**63.4**	2.0
MCH (pg)	20.7	20.6	20.2	**20.5**	0.3
MCHC (g/dl)	32.2	31.7	33.0	**32.3**	0.7
Plt (x10^9/l)	926	820	895	**880**	54.5
Neu (x10^9/l)	0.55	0.63	0.60	**0.59**	0.0
Ly (x10^9/l)	5.92	3.83	5.31	**5.02**	1.1
Mo (x10^9/l)	0.25	0.14	0.25	**0.21**	0.1
Eos (x10^9/l)	0.04	0.03	0.02	**0.03**	0.0
Bas (x10^9/l)	0.00	0.00	0.01	**0.00**	0.0
INH-7D (7 Days)					
WCC (x10^9/l)	6.73	6.05	5.16	**5.98**	0.8
RCC (x10^{12}/l)	6.43	5.41	6.30	**6.05**	0.6
Hb (g/dl)	13.5	11.4	13.1	**12.7**	1.1
Hct (l/l)	0.409	0.341	0.394	**0.381**	0.04
MCV (fl)	63.6	63.0	62.5	**63.0**	0.6
MCH (pg)	21.0	21.1	20.8	**21.0**	0.2
MCHC (g/dl)	33.0	33.4	33.2	**33.3**	0.2
Plt (x10^9/l)	650	464	491	**478**	19.1
Neu (x10^9/l)	0.60	0.00	0.51	**0.37**	0.3
Ly (x10^9/l)	5.95	4.87	4.47	**5.10**	0.8
Mo (x10^9/l)	0.14	0.28	0.15	**0.19**	0.1
Eos (x10^9/l)	0.04	0.00	0.03	**0.02**	0.0
Bas (x10^9/l)	0.00	0.02	0.00	**0.01**	0.0
INH-14D (14 Days)					
WCC (x10^9/l)	6.76	4.66	5.93	**5.78**	1.1
RCC (x10^{12}/l)	6.85	5.97	7.12	**6.65**	0.6
Hb (g/dl)	13.9	11.8	13.7	**13.1**	1.2
Hct (l/l)	0.426	0.350	0.405	**0.394**	0.04
MCV (fl)	62.2	58.6	56.9	**59.2**	2.7
MCH (pg)	20.3	19.8	19.2	**19.8**	0.6
MCHC (g/dl)	32.6	33.7	33.8	**33.4**	0.7
Plt (x10^9/l)	609	458	529	**494**	50.2
Neu (x10^9/l)	0.72	0.49	0.63	**0.61**	0.1
Ly (x10^9/l)	5.64	3.97	5.13	**4.91**	0.9
Mo (x10^9/l)	0.35	0.18	0.15	**0.23**	0.1
Eos (x10^9/l)	0.05	0.02	0.02	**0.03**	0.0
Bas (x10^9/l)	0.00	0.00	0.00	**0.00**	0.0

SD = standard deviation; INH = isoniazid; D = days; WCC = white cell count; RCC = red cell count; Hb = haemoglobin; Hct = haematocrit; MCV = mean corpuscular volume; MCH = mean corpuscular haemoglobin; MCHC = mean corpuscular haemoglobin concentration; Plt = platelets; Neu = neutrophils; Ly = lymphocytes; Mo = monocytes; Eos = eosinophils; Bas = basophils

Table D-4b: Full blood count results of the INH group after 28 and 42 days

Group (n = 3)	Rat 1	Rat 2	Rat 3	Mean	SD
INH-28D (28 Days)					
WCC (x10^9/l)	4.97	5.51	5.85	**5.44**	0.4
RCC (x10^{12}/l)	6.80	7.47	7.54	**7.27**	0.4
Hb (g/dl)	13.4	14.7	14.9	**14.3**	0.8
Hct (l/l)	0.380	0.421	0.417	**0.406**	0.02
MCV (fl)	55.9	56.4	55.3	**55.9**	0.6
MCH (pg)	19.7	19.7	19.8	**19.7**	0.1
MCHC (g/dl)	35.3	34.9	35.7	**35.3**	0.4
Plt (x10^9/l)	712	898	925	**845**	116.0
Neu (x10^9/l)	0.57	0.72	0.69	**0.66**	0.1
Ly (x10^9/l)	4.10	4.60	4.99	**4.56**	0.5
Mo (x10^9/l)	0.25	0.14	0.13	**0.17**	0.1
Eos (x10^9/l)	0.04	0.04	0.04	**0.04**	0.0
Bas (x10^9/l)	0.01	0.01	0.00	**0.01**	0.0
INH-42D (42 Days)					
WCC (x10^9/l)	5.07	5.99	4.28	**5.11**	0.9
RCC (x10^{12}/l)	7.82	7.40	6.53	**7.25**	0.7
Hb (g/dl)	14.7	14.5	12.5	**13.9**	1.2
Hct (l/l)	0.415	0.414	0.342	**0.390**	0.04
MCV (fl)	53.1	55.9	52.4	**53.8**	1.9
MCH (pg)	18.8	19.6	19.1	**19.2**	0.4
MCHC (g/dl)	35.4	35.0	36.5	**35.6**	0.8
Plt (x10^9/l)	1096	893	677	**785**	152.7
Neu (x10^9/l)	0.78	1.00	0.75	**0.84**	0.1
Ly (x10^9/l)	4.02	4.66	3.47	**4.05**	0.6
Mo (x10^9/l)	0.21	0.23	0.00	**0.15**	0.1
Eos (x10^9/l)	0.05	0.09	0.06	**0.07**	0.0
Bas (x10^9/l)	0.01	0.01	0.00	**0.01**	0.0

SD = standard deviation; INH = isoniazid; D = days; WCC = white cell count; RCC = red cell count; Hb = haemoglobin; Hct = haematocrit; MCV = mean corpuscular volume; MCH = mean corpuscular haemoglobin; MCHC = mean corpuscular haemoglobin concentration; Plt = platelets; Neu = neutrophils; Ly = lymphocytes; Mo = monocytes; Eos = eosinophils; Bas = basophils

Table D-5a: Full blood count results of the INH+LMS group after 2, 7 and 14 days

Group (n = 3)	Rat 1	Rat 2	Rat 3	Mean	SD
INH+LMS-2D (2 Days)					
WCC (x10^9/l)	7.65	6.58	8.42	**7.55**	0.9
RCC (x10^{12}/l)	6.34	6.67	7.17	**6.73**	0.4
Hb (g/dl)	12.9	13.6	14.2	**13.6**	0.7
Hct (l/l)	0.382	0.408	0.423	**0.404**	0.02
MCV (fl)	60.3	61.2	59.0	**60.2**	1.1
MCH (pg)	20.3	20.4	19.8	**20.2**	0.3
MCHC (g/dl)	33.8	33.3	33.6	**33.6**	0.3
Plt (x10^9/l)	1233	1156	1153	**1181**	45.3
Neu (x10^9/l)	0.86	0.92	0.93	**0.90**	0.0
Ly (x10^9/l)	6.41	5.40	7.24	**6.35**	0.9
Mo (x10^9/l)	0.31	0.20	0.17	**0.23**	0.1
Eos (x10^9/l)	0.05	0.07	0.08	**0.07**	0.0
Bas (x10^9/l)	0.01	0.00	0.00	**0.00**	0.0
INH+LMS-7D (7 Days)					
WCC (x10^9/l)	5.81	8.67	6.39	**6.96**	1.5
RCC (x10^{12}/l)	6.66	6.47	6.35	**6.49**	0.2
Hb (g/dl)	13.4	13.2	12.6	**13.1**	0.4
Hct (l/l)	0.405	0.403	0.382	**0.397**	0.01
MCV (fl)	60.8	62.3	60.2	**61.1**	1.1
MCH (pg)	20.1	20.4	19.8	**20.1**	0.3
MCHC (g/dl)	33.1	32.8	33.0	**33.0**	0.2
Plt (x10^9/l)	880	771	768	**806**	63.8
Neu (x10^9/l)	0.73	0.89	0.58	**0.73**	0.2
Ly (x10^9/l)	4.78	7.03	5.10	**5.64**	1.2
Mo (x10^9/l)	0.24	0.57	0.20	**0.34**	0.2
Eos (x10^9/l)	0.05	0.16	0.52	**0.24**	0.2
Bas (x10^9/l)	0.01	0.02	0.00	**0.01**	0.0
INH+LMS-14D (14 Days)					
WCC (x10^9/l)	6.31	4.92	7.33	**6.19**	1.2
RCC (x10^{12}/l)	6.70	6.86	6.23	**6.60**	0.3
Hb (g/dl)	13.4	14.0	12.5	**13.3**	0.8
Hct (l/l)	0.412	0.435	0.378	**0.408**	0.03
MCV (fl)	61.5	63.4	60.7	**61.9**	1.4
MCH (pg)	20.0	20.4	20.1	**20.2**	0.2
MCHC (g/dl)	32.5	32.2	33.1	**32.6**	0.5
Plt (x10^9/l)	866	757	826	**816**	55.1
Neu (x10^9/l)	0.66	0.57	0.70	**0.64**	0.1
Ly (x10^9/l)	5.53	4.20	6.04	**5.26**	1.0
Mo (x10^9/l)	0.07	0.13	0.53	**0.24**	0.3
Eos (x10^9/l)	0.04	0.02	0.06	**0.04**	0.0
Bas (x10^9/l)	0.01	0.00	0.01	**0.01**	0.0

SD = standard deviation; INH = isoniazid; LMS = levamisole; D = days; WCC = white cell count; RCC = red cell count; Hb = haemoglobin; Hct = haematocrit; MCV = mean corpuscular volume; MCH = mean corpuscular haemoglobin; MCHC = mean corpuscular haemoglobin concentration; Plt = platelets; Neu = neutrophils; Ly = lymphocytes; Mo = monocytes; Eos = eosinophils; Bas = basophils

Table D-5b: Full blood count results of the INH+LMS group after 28 and 42 days

Group (n = 3)	Rat 1	Rat 2	Rat 3	Mean	SD
INH+LMS-28D (28 Days)					
WCC (x10^9/l)	5.86	5.92	6.30	**6.03**	0.2
RCC (x10^{12}/l)	7.14	7.26	6.98	**7.13**	0.1
Hb (g/dl)	15.5	16.5	16.5	**16.2**	0.6
Hct (l/l)	0.421	0.420	0.421	**0.421**	0.00
MCV (fl)	59.0	57.9	60.3	**59.1**	1.2
MCH (pg)	21.7	22.7	23.6	**22.7**	1.0
MCHC (g/dl)	36.8	39.3	39.2	**38.4**	1.4
Plt (x10^9/l)	540	564	581	**562**	20.6
Neu (x10^9/l)	0.97	0.00	0.95	**0.64**	0.6
Ly (x10^9/l)	4.60	4.62	5.20	**4.81**	0.3
Mo (x10^9/l)	0.23	0.19	0.09	**0.17**	0.1
Eos (x10^9/l)	0.06	0.00	0.06	**0.04**	0.0
Bas (x10^9/l)	0.00	0.00	0.00	**0.00**	0.0
INH+LMS-42D (42 Days)					
WCC (x10^9/l)	5.45	6.56	5.73	**5.91**	0.6
RCC (x10^{12}/l)	7.99	7.67	7.83	**7.83**	0.2
Hb (g/dl)	15.3	14.8	15.2	**15.1**	0.3
Hct (l/l)	0.450	0.438	0.448	**0.445**	0.01
MCV (fl)	56.3	57.1	57.2	**56.9**	0.5
MCH (pg)	19.1	19.3	19.4	**19.3**	0.2
MCHC (g/dl)	34.0	33.8	33.9	**33.9**	0.1
Plt (x10^9/l)	575	700	653	**643**	63.1
Neu (x10^9/l)	0.76	0.72	0.60	**0.69**	0.1
Ly (x10^9/l)	4.48	5.68	4.83	**5.00**	0.6
Mo (x10^9/l)	0.19	0.13	0.21	**0.18**	0.0
Eos (x10^9/l)	0.02	0.02	0.08	**0.04**	0.0
Bas (x10^9/l)	0.00	0.01	0.01	**0.01**	0.0

SD = standard deviation; INH = isoniazid; LMS = levamisole; D = Days; WCC = white cell count; RCC = red cell count; Hb = haemoglobin; Hct = haematocrit; MCV = mean corpuscular volume; MCH = mean corpuscular haemoglobin; MCHC = mean corpuscular haemoglobin concentration; Plt = platelets; Neu = neutrophils; Ly = lymphocytes; Mo = monocytes; Eos = eosinophils; Bas = basophils

Table D-6a: Full blood count results of the INH+CBZ group after 2, 7 and 14 days

Group (n = 3)	Rat 1	Rat 2	Rat 3	Mean	SD
INH+CBZ-2D (2 Days)					
WCC (x10^9/l)	6.09	8.96	6.89	**7.31**	1.5
RCC (x10^{12}/l)	6.86	7.10	6.38	**6.78**	0.4
Hb (g/dl)	14.0	13.7	13.5	**13.7**	0.3
Hct (l/l)	0.420	0.407	0.406	**0.411**	0.01
MCV (fl)	61.2	56.9	63.6	**60.6**	3.4
MCH (pg)	20.4	19.2	21.2	**20.3**	1.0
MCHC (g/dl)	33.3	33.7	33.3	**33.4**	0.2
Plt (x10^9/l)	369	1175	820	**788**	404.0
Neu (x10^9/l)	0.47	1.60	0.60	**0.89**	0.6
Ly (x10^9/l)	5.15	6.68	6.10	**5.98**	0.8
Mo (x10^9/l)	0.41	0.63	0.18	**0.41**	0.2
Eos (x10^9/l)	0.05	0.04	0.01	**0.03**	0.0
Bas (x10^9/l)	0.01	0.01	0.01	**0.01**	0.0
INH+CBZ-7D (7 Days)					
WCC (x10^9/l)	9.01	6.26	6.28	**7.18**	1.6
RCC (x10^{12}/l)	6.77	6.92	5.86	**6.52**	0.6
Hb (g/dl)	13.2	14.2	12.0	**13.1**	1.1
Hct (l/l)	0.397	0.431	0.371	**0.400**	0.03
MCV (fl)	58.6	62.3	63.3	**61.4**	2.5
MCH (pg)	19.5	20.5	20.5	**20.2**	0.6
MCHC (g/dl)	33.2	32.9	32.3	**32.8**	0.5
Plt (x10^9/l)	849	682	754	**762**	83.8
Neu (x10^9/l)	0.60	0.66	0.92	**0.73**	0.2
Ly (x10^9/l)	8.02	4.85	4.99	**5.95**	1.8
Mo (x10^9/l)	0.30	0.30	0.33	**0.31**	0.0
Eos (x10^9/l)	0.08	0.44	0.02	**0.18**	0.2
Bas (x10^9/l)	0.01	0.01	0.01	**0.01**	0.0
INH+CBZ-14D (14 Days)					
WCC (x10^9/l)	6.32	5.94	5.38	**5.88**	0.5
RCC (x10^{12}/l)	6.75	6.20	6.78	**6.58**	0.3
Hb (g/dl)	13.2	12.7	13.5	**13.1**	0.4
Hct (l/l)	0.407	0.391	0.414	**0.404**	0.01
MCV (fl)	60.3	63.1	61.1	**61.5**	1.4
MCH (pg)	19.6	20.5	19.9	**20.0**	0.5
MCHC (g/dl)	32.4	32.5	32.6	**32.5**	0.1
Plt (x10^9/l)	751	677	907	**778**	117.4
Neu (x10^9/l)	0.80	0.52	0.49	**0.60**	0.2
Ly (x10^9/l)	5.15	4.81	4.67	**4.88**	0.3
Mo (x10^9/l)	0.32	0.27	0.20	**0.26**	0.1
Eos (x10^9/l)	0.04	0.33	0.02	**0.13**	0.2
Bas (x10^9/l)	0.01	0.01	0.00	**0.01**	0.0

SD = standard deviation; INH = isoniazid; CBZ = carbamazepine; D = Days; WCC = white cell count; RCC = red cell count; Hb = haemoglobin; Hct = haematocrit; MCV = mean corpuscular volume; MCH = mean corpuscular haemoglobin; MCHC = mean corpuscular haemoglobin concentration; Plt = platelets; Neu = neutrophils; Ly = lymphocytes; Mo = monocytes; Eos = eosinophils; Bas = basophils

Table D-6b: Full blood count results of the INH+CBZ group after 28 and 42 days

Group (n = 3)	Rat 1	Rat 2	Rat 3	Mean	SD
INH+CBZ-28D (28 Days)					
WCC (x10^9/l)	5.23	6.59	3.72	**5.18**	1.4
RCC (x10^{12}/l)	7.77	7.56	7.44	**7.59**	0.2
Hb (g/dl)	15.1	14.5	14.7	**14.8**	0.3
Hct (l/l)	0.449	0.430	0.440	**0.440**	0.01
MCV (fl)	57.8	56.9	59.1	**57.9**	1.1
MCH (pg)	19.4	19.2	19.8	**19.5**	0.3
MCHC (g/dl)	33.6	33.7	33.4	**33.6**	0.2
Plt (x10^9/l)	580	625	633	**613**	28.6
Neu (x10^9/l)	0.58	0.77	0.65	**0.67**	0.1
Ly (x10^9/l)	4.49	5.45	2.97	**4.30**	1.3
Mo (x10^9/l)	0.14	0.33	0.05	**0.17**	0.1
Eos (x10^9/l)	0.02	0.04	0.05	**0.04**	0.0
Bas (x10^9/l)	0.00	0.00	0.00	**0.00**	0.0
INH+CBZ-42D (42 Days)					
WCC (x10^9/l)	6.52	5.50	7.41	**6.48**	1.0
RCC (x10^{12}/l)	7.50	7.25	7.72	**7.49**	0.2
Hb (g/dl)	14.8	14.7	15.3	**14.9**	0.3
Hct (l/l)	0.429	0.436	0.448	**0.438**	0.01
MCV (fl)	57.2	60.1	58.0	**58.4**	1.5
MCH (pg)	19.7	20.3	19.8	**19.9**	0.3
MCHC (g/dl)	34.5	33.7	34.2	**34.1**	0.4
Plt (x10^9/l)	560	549	626	**578**	41.6
Neu (x10^9/l)	0.59	0.75	0.56	**0.63**	0.1
Ly (x10^9/l)	5.78	4.47	6.55	**5.60**	1.1
Mo (x10^9/l)	0.10	0.24	0.24	**0.19**	0.1
Eos (x10^9/l)	0.04	0.02	0.05	**0.04**	0.0
Bas (x10^9/l)	0.01	0.01	0.01	**0.01**	0.0

SD = standard deviation; INH = isoniazid; CBZ = carbamazepine; D = Days; WCC = white cell count; RCC = red cell count; Hb = haemoglobin; Hct = haematocrit; MCV = mean corpuscular volume; MCH = mean corpuscular haemoglobin; MCHC = mean corpuscular haemoglobin concentration; Plt = platelets; Neu = neutrophils; Ly = lymphocytes; Mo = monocytes; Eos = eosinophils; Bas = basophils

Appendix D-3: Full blood count results of the NVP, NVP+LMS and NVP+CBZ groups

Table D-7a: Full blood count results of the NVP group after 2, 7 and 14 days

Group (n = 3)	Rat 1	Rat 2	Rat 3	Mean	SD
NVP-2D (2 Days)					
WCC (x10^9/l)	4.03	2.92	-*	**3.48**	0.8
RCC (x10^{12}/l)	6.37	6.65	-*	**6.51**	0.2
Hb (g/dl)	13.3	13.8	-*	**13.6**	0.4
Hct (l/l)	0.403	0.413	-*	**0.408**	0.01
MCV (fl)	63.3	62.1	-*	**62.7**	0.8
MCH (pg)	20.9	20.8	-*	**20.9**	0.1
MCHC (g/dl)	33.0	33.4	-*	**33.2**	0.3
Plt (x10^9/l)	706	1002	-*	**854**	209.3
Neu (x10^9/l)	1.09	0.95	-*	**1.02**	0.1
Ly (x10^9/l)	2.12	1.64	-*	**1.88**	0.3
Mo (x10^9/l)	0.07	0.13	-*	**0.10**	0.0
Eos (x10^9/l)	0.75	0.20	-*	**0.48**	0.4
Bas (x10^9/l)	0.00	0.00	-*	**0.00**	0.0
NVP-7D (7 Days)					
WCC (x10^9/l)	4.08	6.70	5.84	**5.54**	1.3
RCC (x10^{12}/l)	7.03	7.44	6.94	**7.14**	0.3
Hb (g/dl)	14.5	14.5	13.6	**14.2**	0.5
Hct (l/l)	0.431	0.441	0.401	**0.424**	0.02
MCV (fl)	61.3	59.3	57.8	**59.5**	1.8
MCH (pg)	20.6	19.5	19.6	**19.9**	0.6
MCHC (g/dl)	33.6	32.9	33.9	**33.5**	0.5
Plt (x10^9/l)	789	915	633	**779**	141.3
Neu (x10^9/l)	0.54	1.05	0.69	**0.76**	0.3
Ly (x10^9/l)	2.94	4.81	3.67	**3.81**	0.9
Mo (x10^9/l)	0.27	0.48	0.71	**0.49**	0.2
Eos (x10^9/l)	0.33	0.36	0.74	**0.48**	0.2
Bas (x10^9/l)	0.00	0.00	0.03	**0.01**	0.0
NVP-14D (14 Days)					
WCC (x10^9/l)	4.94	5.47	4.16	**4.86**	0.7
RCC (x10^{12}/l)	6.64	7.17	7.10	**6.97**	0.3
Hb (g/dl)	13.6	14.3	13.8	**13.9**	0.4
Hct (l/l)	0.409	0.428	0.407	**0.415**	0.01
MCV (fl)	61.6	59.7	57.3	**59.5**	2.2
MCH (pg)	20.5	19.9	19.4	**19.9**	0.6
MCHC (g/dl)	33.3	33.4	33.9	**33.5**	0.3
Plt (x10^9/l)	931	1071	1037	**1013**	73.0
Neu (x10^9/l)	0.97	1.35	0.91	**1.08**	0.2
Ly (x10^9/l)	3.62	3.59	2.92	**3.38**	0.4
Mo (x10^9/l)	0.33	0.50	0.29	**0.37**	0.1
Eos (x10^9/l)	0.02	0.02	0.03	**0.02**	0.0
Bas (x10^9/l)	0.00	0.01	0.01	**0.01**	0.0

SD = standard deviation; NVP = nevirapine; D = days; WCC = white cell count; RCC = red cell count; Hb = haemoglobin; Hct = haematocrit; MCV = mean corpuscular volume; MCH = mean corpuscular haemoglobin; MCHC = mean corpuscular haemoglobin concentration; Plt = platelets; Neu = neutrophils; Ly = lymphocytes; Mo = monocytes; Eos = eosinophils; Bas = basophils; * = blood clotted

Table D-7b: Full blood count results of the NVP group after 28 and 42 days

Group (n = 3)	Rat 1	Rat 2	Rat 3	Mean	SD
NVP-28D (28 Days)					
WCC (x10^9/l)	6.44	5.35	6.41	**6.07**	0.6
RCC (x10^{12}/l)	6.73	7.46	7.89	**7.36**	0.6
Hb (g/dl)	13.1	14.2	15.2	**14.2**	1.1
Hct (l/l)	0.372	0.413	0.428	**0.404**	0.03
MCV (fl)	55.3	55.4	54.2	**55.0**	0.7
MCH (pg)	19.5	19.0	19.3	**19.3**	0.3
MCHC (g/dl)	35.2	34.4	35.5	**35.0**	0.6
Plt (x10^9/l)	879	858	892	**876**	17.2
Neu (x10^9/l)	1.22	1.17	0.87	**1.09**	0.2
Ly (x10^9/l)	4.58	3.94	5.33	**4.62**	0.7
Mo (x10^9/l)	0.46	0.21	0.16	**0.28**	0.2
Eos (x10^9/l)	0.17	0.03	0.04	**0.08**	0.1
Bas (x10^9/l)	0.01	0.00	0.01	**0.01**	0.0
NVP-42D (42 Days)					
WCC (x10^9/l)	5.56	5.41	5.29	**5.42**	0.1
RCC (x10^{12}/l)	8.11	7.61	7.46	**7.73**	0.3
Hb (g/dl)	14.8	14.4	14.3	**14.5**	0.3
Hct (l/l)	0.406	0.400	0.408	**0.405**	0.00
MCV (fl)	50.1	52.6	54.7	**52.5**	2.3
MCH (pg)	18.2	18.9	19.2	**18.8**	0.5
MCHC (g/dl)	36.5	36.0	35.0	**35.8**	0.8
Plt (x10^9/l)	1155	1074	927	**1052**	115.6
Neu (x10^9/l)	1.25	2.16	1.15	**1.52**	0.6
Ly (x10^9/l)	3.93	2.85	3.78	**3.52**	0.6
Mo (x10^9/l)	0.33	0.36	0.31	**0.33**	0.0
Eos (x10^9/l)	0.04	0.04	0.04	**0.04**	0.0
Bas (x10^9/l)	0.01	0.00	0.00	**0.00**	0.0

SD = standard deviation; NVP = nevirapine; D = days; WCC = white cell count; RCC = red cell count; Hb = haemoglobin; Hct = haematocrit; MCV = mean corpuscular volume; MCH = mean corpuscular haemoglobin; MCHC = mean corpuscular haemoglobin concentration; Plt = platelets; Neu = neutrophils; Ly = lymphocytes; Mo = monocytes; Eos = eosinophils; Bas = basophils

Table D-8a: Full blood count results of the NVP+LMS group after 2, 7 and 14 days

Group (n = 3)	Rat 1	Rat 2	Rat 3	Mean	SD
NVP+LMS-2D (2 Days)					
WCC (x10^9/l)	5.16	-*	4.68	**4.92**	0.3
RCC (x10^{12}/l)	4.60	-*	5.26	**4.93**	0.5
Hb (g/dl)	9.5	-*	10.6	**10.1**	0.8
Hct (l/l)	0.296	-*	0.331	**0.314**	0.02
MCV (fl)	64.3	-*	62.9	**63.6**	1.0
MCH (pg)	20.7	-*	20.2	**20.5**	0.4
MCHC (g/dl)	32.1	-*	32.0	**32.1**	0.1
Plt (x10^9/l)	558	-*	805	**682**	174.7
Neu (x10^9/l)	1.20	-*	1.24	**1.22**	0.0
Ly (x10^9/l)	3.26	-*	3.27	**3.27**	0.0
Mo (x10^9/l)	0.64	-*	0.14	**0.39**	0.4
Eos (x10^9/l)	0.04	-*	0.02	**0.03**	0.0
Bas (x10^9/l)	0.02	-*	0.01	**0.02**	0.0
NVP+LMS-7D (7 Days)					
WCC (x10^9/l)	4.21	4.45	6.42	**5.03**	1.2
RCC (x10^{12}/l)	7.41	6.98	7.39	**7.26**	0.2
Hb (g/dl)	14.0	14.4	14.1	**14.2**	0.2
Hct (l/l)	0.419	0.422	0.412	**0.418**	0.01
MCV (fl)	56.5	60.5	55.8	**57.6**	2.5
MCH (pg)	18.9	20.6	19.1	**19.5**	0.9
MCHC (g/dl)	33.4	34.1	34.2	**33.9**	0.4
Plt (x10^9/l)	833	741	1009	**861**	136.2
Neu (x10^9/l)	0.59	0.73	2.62	**1.31**	1.1
Ly (x10^9/l)	3.39	2.89	3.08	**3.12**	0.3
Mo (x10^9/l)	0.23	0.43	0.69	**0.45**	0.2
Eos (x10^9/l)	0.00	0.39	0.02	**0.14**	0.2
Bas (x10^9/l)	0.00	0.01	0.01	**0.01**	0.0
NVP+LMS-14D (14 Days)					
WCC (x10^9/l)	9.36	4.78	9.74	**7.96**	2.8
RCC (x10^{12}/l)	7.59	6.99	7.36	**7.31**	0.3
Hb (g/dl)	14.9	13.6	14.5	**14.3**	0.7
Hct (l/l)	0.455	0.407	0.443	**0.435**	0.02
MCV (fl)	59.9	58.2	60.2	**59.4**	1.1
MCH (pg)	19.6	19.5	19.7	**19.6**	0.1
MCHC (g/dl)	32.7	33.4	32.7	**32.9**	0.4
Plt (x10^9/l)	1032	940	1027	**1000**	51.7
Neu (x10^9/l)	1.43	1.02	1.44	**1.30**	0.2
Ly (x10^9/l)	7.31	3.21	7.82	**6.11**	2.5
Mo (x10^9/l)	0.59	0.51	0.45	**0.52**	0.1
Eos (x10^9/l)	0.02	0.03	0.02	**0.02**	0.0
Bas (x10^9/l)	0.01	0.01	0.01	**0.01**	0.0

SD = standard deviation; NVP = nevirapine; LMS = levamisole; D = Days; WCC = white cell count; RCC = red cell count; Hb = haemoglobin; Hct = haematocrit; MCV = mean corpuscular volume; MCH = mean corpuscular haemoglobin; MCHC = mean corpuscular haemoglobin concentration; Plt = platelets; Neu = neutrophils; Ly = lymphocytes; Mo = monocytes; Eos = eosinophils; Bas = basophils; * = blood clotted

Table D-8b: Full blood count results of the NVP+LMS group after 28 and 42 days

Group (n = 3)	Rat 1	Rat 2	Rat 3	Mean	SD
NVP+LMS-28D (28 Days)					
WCC (x10^9/l)	5.81	6.21	3.78	**5.27**	1.3
RCC (x10^{12}/l)	7.40	7.79	7.60	**7.60**	0.2
Hb (g/dl)	14.8	14.9	14.8	**14.8**	0.1
Hct (l/l)	0.433	0.447	0.438	**0.439**	0.01
MCV (fl)	58.5	57.4	57.6	**57.8**	0.6
MCH (pg)	20.0	19.1	19.5	**19.5**	0.5
MCHC (g/dl)	34.2	33.3	33.8	**33.8**	0.5
Plt (x10^9/l)	621	702	797	**707**	88.1
Neu (x10^9/l)	1.14	1.06	0.70	**0.97**	0.2
Ly (x10^9/l)	4.36	4.86	2.94	**4.05**	1.0
Mo (x10^9/l)	0.28	0.26	0.11	**0.22**	0.1
Eos (x10^9/l)	0.03	0.02	0.03	**0.03**	0.0
Bas (x10^9/l)	0.00	0.01	0.00	**0.00**	0.0
NVP+LMS-42D (42 Days)					
WCC (x10^9/l)	3.75	5.62	3.79	**4.39**	1.1
RCC (x10^{12}/l)	8.36	7.97	7.23	**7.85**	0.6
Hb (g/dl)	15.3	15.6	14.0	**15.0**	0.9
Hct (l/l)	0.442	0.450	0.414	**0.435**	0.02
MCV (fl)	52.9	56.5	57.3	**55.6**	2.3
MCH (pg)	18.3	19.6	19.4	**19.1**	0.7
MCHC (g/dl)	34.6	34.7	33.8	**34.4**	0.5
Plt (x10^9/l)	711	715	569	**665**	83.2
Neu (x10^9/l)	0.72	0.97	0.72	**0.80**	0.1
Ly (x10^9/l)	2.76	4.23	2.97	**3.32**	0.8
Mo (x10^9/l)	0.25	0.36	0.07	**0.23**	0.1
Eos (x10^9/l)	0.02	0.06	0.03	**0.04**	0.0
Bas (x10^9/l)	0.00	0.00	0.00	**0.00**	0.0

SD = standard deviation; NVP = nevirapine; LMS = levamisole; D = Days; WCC = white cell count; RCC = red cell count; Hb = haemoglobin; Hct = haematocrit; MCV = mean corpuscular volume; MCH = mean corpuscular haemoglobin; MCHC = mean corpuscular haemoglobin concentration; Plt = platelets; Neu = neutrophils; Ly = lymphocytes; Mo = monocytes; Eos = eosinophils; Bas = basophils

Table D-9a: Full blood count results of the NVP+CBZ group after 2, 7 and 14 days

Group (n = 3)	Rat 1	Rat 2	Rat 3	Mean	SD
NVP+CBZ-2D (2 Days)					
WCC (x10^9/l)	6.61	4.74	3.63	**4.99**	1.5
RCC (x10^{12}/l)	5.96	6.60	6.20	**6.25**	0.3
Hb (g/dl)	12.2	13.5	12.6	**12.8**	0.7
Hct (l/l)	0.380	0.415	0.389	**0.395**	0.02
MCV (fl)	63.8	62.9	62.7	**63.1**	0.6
MCH (pg)	20.5	20.5	20.3	**20.4**	0.1
MCHC (g/dl)	32.1	32.5	32.4	**32.3**	0.2
Plt (x10^9/l)	873	872	934	**893**	35.5
Neu (x10^9/l)	2.63	1.63	1.46	**1.91**	0.6
Ly (x10^9/l)	3.49	2.88	2.03	**2.80**	0.7
Mo (x10^9/l)	0.44	0.22	0.13	**0.26**	0.2
Eos (x10^9/l)	0.03	0.01	0.01	**0.02**	0.0
Bas (x10^9/l)	0.01	0.00	0.00	**0.00**	0.0
NVP+CBZ-7D (7 Days)					
WCC (x10^9/l)	5.02	3.82	5.10	**4.65**	0.7
RCC (x10^{12}/l)	6.65	6.62	6.93	**6.73**	0.2
Hb (g/dl)	13.1	13.4	13.8	**13.4**	0.4
Hct (l/l)	0.433	0.421	0.428	**0.427**	0.01
MCV (fl)	65.1	63.6	61.8	**63.5**	1.7
MCH (pg)	19.7	20.2	19.9	**19.9**	0.3
MCHC (g/dl)	30.3	31.8	32.2	**31.4**	1.0
Plt (x10^9/l)	891	694	930	**838**	126.5
Neu (x10^9/l)	0.66	1.00	1.25	**0.97**	0.3
Ly (x10^9/l)	3.95	2.34	3.41	**3.23**	0.8
Mo (x10^9/l)	0.39	0.45	0.42	**0.42**	0.0
Eos (x10^9/l)	0.01	0.01	0.02	**0.01**	0.0
Bas (x10^9/l)	0.01	0.01	0.00	**0.01**	0.0
NVP+CBZ-14D (14 Days)					
WCC (x10^9/l)	4.53	6.18	6.61	**5.77**	1.1
RCC (x10^{12}/l)	7.27	7.63	7.05	**7.32**	0.3
Hb (g/dl)	14.4	14.6	13.5	**14.2**	0.6
Hct (l/l)	0.425	0.450	0.403	**0.426**	0.02
MCV (fl)	58.5	59.0	57.2	**58.2**	0.9
MCH (pg)	19.8	19.1	19.1	**19.3**	0.4
MCHC (g/dl)	33.9	32.4	33.5	**33.3**	0.8
Plt (x10^9/l)	814	871	869	**851**	32.3
Neu (x10^9/l)	0.81	0.70	0.98	**0.83**	0.1
Ly (x10^9/l)	3.47	5.01	5.13	**4.54**	0.9
Mo (x10^9/l)	0.23	0.46	0.15	**0.28**	0.2
Eos (x10^9/l)	0.01	0.01	0.35	**0.12**	0.2
Bas (x10^9/l)	0.01	0.00	0.00	**0.00**	0.0

SD = standard deviation; NVP = nevirapine; CBZ = carbamazepine; D = Days; WCC = white cell count; RCC = red cell count; Hb = haemoglobin; Hct = haematocrit; MCV = mean corpuscular volume; MCH = mean corpuscular haemoglobin; MCHC = mean corpuscular haemoglobin concentration; Plt = platelets; Neu = neutrophils; Ly = lymphocytes; Mo = monocytes; Eos = eosinophils; Bas = basophils

Table D-9b: Full blood count results of the NVP+CBZ group after 28 and 42 days

Group (n = 3)	Rat 1	Rat 2	Rat 3	Mean	SD
NVP+CBZ-28D (28 Days)					
WCC (x10^9/l)	6.74	4.76	4.57	**5.36**	1.2
RCC (x10^{12}/l)	7.01	7.31	7.45	**7.26**	0.2
Hb (g/dl)	13.9	15.0	14.8	**14.6**	0.6
Hct (l/l)	0.403	0.439	0.444	**0.429**	0.02
MCV (fl)	57.5	60.1	59.6	**59.1**	1.4
MCH (pg)	19.8	20.5	19.9	**20.1**	0.4
MCHC (g/dl)	35.4	34.2	33.3	**34.3**	1.1
Plt (x10^9/l)	699	655	841	**732**	97.2
Neu (x10^9/l)	1.52	0.95	0.65	**1.04**	0.4
Ly (x10^9/l)	4.84	3.58	3.88	**4.10**	0.7
Mo (x10^9/l)	0.35	0.22	0.02	**0.20**	0.2
Eos (x10^9/l)	0.03	0.01	0.02	**0.02**	0.0
Bas (x10^9/l)	0.00	0.00	0.00	**0.00**	0.0
NVP+CBZ-42D (42 Days)					
WCC (x10^9/l)	5.83	4.37	5.67	**5.29**	0.8
RCC (x10^{12}/l)	7.68	8.21	7.74	**7.88**	0.3
Hb (g/dl)	14.9	15.4	13.7	**14.7**	0.9
Hct (l/l)	0.403	0.431	0.386	**0.407**	0.02
MCV (fl)	52.5	52.5	49.9	**51.6**	1.5
MCH (pg)	19.4	18.8	17.7	**18.6**	0.9
MCHC (g/dl)	37.0	35.7	35.5	**36.1**	0.8
Plt (x10^9/l)	934	1011	754	**900**	131.9
Neu (x10^9/l)	1.09	1.35	2.06	**1.50**	0.5
Ly (x10^9/l)	4.28	2.65	2.68	**3.20**	0.9
Mo (x10^9/l)	0.43	0.33	0.26	**0.34**	0.1
Eos (x10^9/l)	0.02	0.04	0.66	**0.24**	0.4
Bas (x10^9/l)	0.01	0.00	0.01	**0.01**	0.0

SD = standard deviation; NVP = nevirapine; CBZ = carbamazepine; D = Days; WCC = white cell count; RCC = red cell count; Hb = haemoglobin; Hct = haematocrit; MCV = mean corpuscular volume; MCH = mean corpuscular haemoglobin; MCHC = mean corpuscular haemoglobin concentration; Plt = platelets; Neu = neutrophils; Ly = lymphocytes; Mo = monocytes; Eos = eosinophils; Bas = basophils

Appendix D-4: Full blood count results of the PAR, PAR+LMS and PAR+CBZ groups

Table D-10a: Full blood count results of the PAR groups after 2, 7 and 14 days

Group (n = 3)	Rat 1	Rat 2	Rat 3	Mean	SD
PAR-2D (2 Days)					
WCC (x10^9/l)	7.54	-*	-*	**7.54**	0.0
RCC (x10^{12}/l)	6.77	-*	-*	**6.77**	0.0
Hb (g/dl)	14.8	-*	-*	**14.8**	0.0
Hct (l/l)	0.461	-*	-*	**0.461**	0.00
MCV (fl)	68.1	-*	-*	**68.1**	0.0
MCH (pg)	21.9	-*	-*	**21.9**	0.0
MCHC (g/dl)	32.1	-*	-*	**32.1**	0.0
Plt (x10^9/l)	419	-*	-*	**419**	0.0
Neu (x10^9/l)	0.75	-*	-*	**0.75**	0.0
Ly (x10^9/l)	5.96	-*	-*	**5.96**	0.0
Mo (x10^9/l)	0.37	-*	-*	**0.37**	0.0
Eos (x10^9/l)	0.46	-*	-*	**0.46**	0.0
Bas (x10^9/l)	0.01	-*	-*	**0.01**	0.0
PAR-7D (7 Days)					
WCC (x10^9/l)	6.37	7.76	7.82	**7.32**	0.8
RCC (x10^{12}/l)	7.02	6.61	6.63	**6.75**	0.2
Hb (g/dl)	13.9	14.0	13.9	**13.9**	0.1
Hct (l/l)	0.424	0.429	0.426	**0.426**	0.00
MCV (fl)	60.4	64.9	64.3	**63.2**	2.4
MCH (pg)	19.8	21.2	21.0	**20.7**	0.8
MCHC (g/dl)	32.8	32.6	32.6	**32.7**	0.1
Plt (x10^9/l)	787	803	832	**807**	22.8
Neu (x10^9/l)	0.79	0.61	0.99	**0.80**	0.2
Ly (x10^9/l)	5.27	6.88	6.50	**6.22**	0.8
Mo (x10^9/l)	0.25	0.20	0.23	**0.23**	0.0
Eos (x10^9/l)	0.05	0.06	0.10	**0.07**	0.0
Bas (x10^9/l)	0.01	0.01	0.01	**0.01**	0.0
PAR-14D (14 Days)					
WCC (x10^9/l)	4.27	4.55	3.76	**4.19**	0.4
RCC (x10^{12}/l)	6.44	6.80	6.73	**6.66**	0.2
Hb (g/dl)	13.4	14.0	14.0	**13.8**	0.3
Hct (l/l)	0.400	0.421	0.412	**0.411**	0.01
MCV (fl)	62.1	61.9	61.2	**61.7**	0.5
MCH (pg)	20.8	20.6	20.8	**20.7**	0.1
MCHC (g/dl)	33.5	33.3	34.0	**33.6**	0.4
Plt (x10^9/l)	857	860	784	**834**	43.0
Neu (x10^9/l)	0.59	0.54	0.51	**0.55**	0.0
Ly (x10^9/l)	3.57	3.97	2.72	**3.42**	0.6
Mo (x10^9/l)	0.09	0.02	0.17	**0.09**	0.1
Eos (x10^9/l)	0.02	0.02	0.36	**0.13**	0.2
Bas (x10^9/l)	0.00	0.00	0.00	**0.00**	0.0

SD = standard deviation; PAR = paracetamol; D = days; WCC = white cell count; RCC = red cell count; Hb = haemoglobin; Hct = haematocrit; MCV = mean corpuscular volume; MCH = mean corpuscular haemoglobin; MCHC = mean corpuscular haemoglobin concentration; Plt = platelets; Neu = neutrophils; Ly = lymphocytes; Mo = monocytes; Eos = eosinophils; Bas = basophils; * = blood clotted

Table D-10b: Full blood count results of the PAR group after 28 and 42 days

Group (n = 3)	Rat 1	Rat 2	Rat 3	Mean	SD
PAR-28D (28 Days)					
WCC (x10^9/l)	3.97	5.86	6.13	**5.32**	1.2
RCC (x10^{12}/l)	7.35	6.96	6.98	**7.10**	0.2
Hb (g/dl)	13.9	14.3	14.0	**14.1**	0.2
Hct (l/l)	0.393	0.399	0.399	**0.397**	0.00
MCV (fl)	53.5	57.3	57.2	**56.0**	2.2
MCH (pg)	18.9	20.5	20.1	**19.8**	0.8
MCHC (g/dl)	35.4	35.8	35.1	**35.4**	0.4
Plt (x10^9/l)	856	807	976	**880**	87.0
Neu (x10^9/l)	0.84	0.86	0.94	**0.88**	0.1
Ly (x10^9/l)	2.91	4.60	5.05	**4.19**	1.1
Mo (x10^9/l)	0.21	0.34	0.08	**0.21**	0.1
Eos (x10^9/l)	0.01	0.05	0.06	**0.04**	0.0
Bas (x10^9/l)	0.00	0.01	0.00	**0.00**	0.0
PAR-42D (42 Days)					
WCC (x10^9/l)	5.02	4.53	5.18	**4.91**	0.3
RCC (x10^{12}/l)	7.00	7.55	7.43	**7.33**	0.3
Hb (g/dl)	14.2	14.6	14.7	**14.5**	0.3
Hct (l/l)	0.396	0.400	0.407	**0.401**	0.01
MCV (fl)	56.6	53.0	54.8	**54.8**	1.8
MCH (pg)	20.3	19.3	19.8	**19.8**	0.5
MCHC (g/dl)	35.9	36.5	36.1	**36.2**	0.3
Plt (x10^9/l)	868	1037	978	**961**	85.8
Neu (x10^9/l)	0.64	0.82	0.94	**0.80**	0.2
Ly (x10^9/l)	4.16	3.45	3.96	**3.86**	0.4
Mo (x10^9/l)	0.16	0.21	0.23	**0.20**	0.0
Eos (x10^9/l)	0.05	0.05	0.04	**0.05**	0.0
Bas (x10^9/l)	0.01	0.00	0.01	**0.01**	0.0

SD = standard deviation; PAR = paracetamol; D = days; WCC = white cell count; RCC = red cell count; Hb = haemoglobin; Hct = haematocrit; MCV = mean corpuscular volume; MCH = mean corpuscular haemoglobin; MCHC = mean corpuscular haemoglobin concentration; Plt = platelets; Neu = neutrophils; Ly = lymphocytes; Mo = monocytes; Eos = eosinophils; Bas = basophils

Table D-11a: Full blood count results of the PAR+LMS group after 2, 7 and 14 days

Group (n = 3)	Rat 1	Rat 2	Rat 3	Mean	SD
PAR+LMS-2D (2 Days)					
WCC (x10^9/l)	7.14	5.37	8.16	**6.89**	1.4
RCC (x10^{12}/l)	6.79	7.29	6.78	**6.95**	0.3
Hb (g/dl)	14.3	14.1	14.2	**14.2**	0.1
Hct (l/l)	0.458	0.429	0.438	**0.442**	0.01
MCV (fl)	67.5	58.8	64.6	**63.6**	4.4
MCH (pg)	21.1	19.3	20.9	**20.4**	1.0
MCHC (g/dl)	31.2	32.9	32.4	**32.2**	0.9
Plt (x10^9/l)	724	1053	232	**670**	413.2
Neu (x10^9/l)	0.60	1.09	0.84	**0.84**	0.2
Ly (x10^9/l)	5.58	4.04	6.75	**5.46**	1.4
Mo (x10^9/l)	0.30	0.22	0.48	**0.33**	0.1
Eos (x10^9/l)	0.65	0.01	0.07	**0.24**	0.4
Bas (x10^9/l)	0.01	0.01	0.02	**0.01**	0.0
PAR+LMS-7D (7 Days)					
WCC (x10^9/l)	8.16	6.05	4.83	**6.35**	1.7
RCC (x10^{12}/l)	6.70	6.49	7.19	**6.79**	0.4
Hb (g/dl)	14.2	13.0	14.4	**13.9**	0.8
Hct (l/l)	0.446	0.421	0.465	**0.444**	0.02
MCV (fl)	66.6	64.9	64.7	**65.4**	1.0
MCH (pg)	21.2	20.0	20.0	**20.4**	0.7
MCHC (g/dl)	31.8	30.9	31.0	**31.2**	0.5
Plt (x10^9/l)	981	703	901	**862**	143.1
Neu (x10^9/l)	0.76	0.79	0.53	**0.69**	0.1
Ly (x10^9/l)	7.04	4.82	4.05	**5.30**	1.6
Mo (x10^9/l)	0.32	0.38	0.20	**0.30**	0.1
Eos (x10^9/l)	0.04	0.04	0.04	**0.04**	0.0
Bas (x10^9/l)	0.00	0.01	0.01	**0.01**	0.0
PAR+LMS-14D (14 Days)					
WCC (x10^9/l)	7.11	6.78	7.78	**7.22**	0.5
RCC (x10^{12}/l)	7.07	6.63	7.23	**6.98**	0.3
Hb (g/dl)	14.5	13.5	14.8	**14.3**	0.7
Hct (l/l)	0.432	0.411	0.443	**0.429**	0.02
MCV (fl)	61.1	62.0	61.3	**61.5**	0.5
MCH (pg)	20.5	20.4	20.5	**20.5**	0.1
MCHC (g/dl)	33.6	32.8	33.4	**33.3**	0.4
Plt (x10^9/l)	724	713	669	**702**	29.1
Neu (x10^9/l)	0.67	0.61	0.88	**0.72**	0.1
Ly (x10^9/l)	6.02	5.85	6.53	**6.13**	0.4
Mo (x10^9/l)	0.37	0.27	0.34	**0.33**	0.1
Eos (x10^9/l)	0.05	0.04	0.03	**0.04**	0.0
Bas (x10^9/l)	0.00	0.01	0.00	**0.00**	0.0

SD = standard deviation; PAR = paracetamol; LMS = levamisole; D = days; WCC = white cell count; RCC = red cell count; Hb = haemoglobin; Hct = haematocrit; MCV = mean corpuscular volume; MCH = mean corpuscular haemoglobin; MCHC = mean corpuscular haemoglobin concentration; Plt = platelets; Neu = neutrophils; Ly = lymphocytes; Mo = monocytes; Eos = eosinophils; Bas = basophils

Table D-11b: Full blood count results of the PAR+LMS group after 28 and 42 days

Group (n = 3)	Rat 1	Rat 2	Rat 3	Mean	SD
PAR+LMS-28D (28 Days)					
WCC (x10^9/l)	6.63	3.92	5.81	**5.45**	1.4
RCC (x10^{12}/l)	7.16	7.08	7.41	**7.22**	0.2
Hb (g/dl)	14.6	14.4	14.9	**14.6**	0.3
Hct (l/l)	0.436	0.422	0.441	**0.433**	0.01
MCV (fl)	60.9	59.6	59.5	**60.0**	0.8
MCH (pg)	20.4	20.3	20.1	**20.3**	0.2
MCHC (g/dl)	33.5	34.1	33.8	**33.8**	0.3
Plt (x10^9/l)	518	633	683	**611**	84.6
Neu (x10^9/l)	0.67	0.62	0.53	**0.61**	0.1
Ly (x10^9/l)	5.66	3.22	5.12	**4.67**	1.3
Mo (x10^9/l)	0.26	0.06	0.14	**0.15**	0.1
Eos (x10^9/l)	0.04	0.02	0.02	**0.03**	0.0
Bas (x10^9/l)	0.00	0.00	0.00	**0.00**	0.0
PAR+LMS-42D (42 Days)					
WCC (x10^9/l)	4.04	3.09	7.52	**4.88**	2.3
RCC (x10^{12}/l)	7.28	7.02	7.11	**7.14**	0.1
Hb (g/dl)	14.8	14.0	14.0	**14.3**	0.5
Hct (l/l)	0.424	0.400	0.401	**0.408**	0.01
MCV (fl)	58.2	57.0	56.4	**57.2**	0.9
MCH (pg)	20.3	19.9	19.7	**20.0**	0.3
MCHC (g/dl)	34.9	35.0	34.9	**34.9**	0.1
Plt (x10^9/l)	591	601	634	**609**	22.5
Neu (x10^9/l)	0.60	0.60	1.02	**0.74**	0.2
Ly (x10^9/l)	3.24	2.36	6.11	**3.90**	2.0
Mo (x10^9/l)	0.16	0.11	0.37	**0.21**	0.1
Eos (x10^9/l)	0.03	0.02	0.02	**0.02**	0.0
Bas (x10^9/l)	0.01	0.00	0.00	**0.00**	0.0

SD = standard deviation; PAR = paracetamol; LMS = levamisole; D = Days; WCC = white cell count; RCC = red cell count; Hb = haemoglobin; Hct = haematocrit; MCV = mean corpuscular volume; MCH = mean corpuscular haemoglobin; MCHC = mean corpuscular haemoglobin concentration; Plt = platelets; Neu = neutrophils; Ly = lymphocytes; Mo = monocytes; Eos = eosinophils; Bas = basophils

Table D-12a: Full blood count results of the PAR+CBZ group after 2, 7 and 14 days

Group (n = 3)	Rat 1	Rat 2	Rat 3	Mean	SD
PAR+CBZ-2D (2 Days)					
WCC (x10^9/l)	4.70	9.47	7.27	**7.15**	2.4
RCC (x10^{12}/l)	4.94	6.44	6.51	**5.96**	0.9
Hb (g/dl)	10.5	13.2	13.5	**12.4**	1.7
Hct (l/l)	0.296	0.423	0.415	**0.378**	0.07
MCV (fl)	59.9	65.7	63.7	**63.1**	2.9
MCH (pg)	21.3	20.5	20.7	**20.8**	0.4
MCHC (g/dl)	35.5	31.2	32.5	**33.1**	2.2
Plt (x10^9/l)	852	876	1004	**911**	81.7
Neu (x10^9/l)	0.48	1.46	0.73	**0.89**	0.5
Ly (x10^9/l)	4.01	7.36	6.22	**5.86**	1.7
Mo (x10^9/l)	0.15	0.39	0.27	**0.27**	0.1
Eos (x10^9/l)	0.05	0.26	0.04	**0.12**	0.1
Bas (x10^9/l)	0.01	0.01	0.01	**0.01**	0.0
PAR+CBZ-7D (7 Days)					
WCC (x10^9/l)	6.24	5.58	8.14	**6.65**	1.3
RCC (x10^{12}/l)	6.46	6.48	6.73	**6.56**	0.2
Hb (g/dl)	13.1	13.3	13.8	**13.4**	0.4
Hct (l/l)	0.420	0.424	0.436	**0.427**	0.01
MCV (fl)	65.0	65.4	64.8	**65.1**	0.3
MCH (pg)	20.3	20.5	20.5	**20.4**	0.1
MCHC (g/dl)	31.2	31.4	31.7	**31.4**	0.3
Plt (x10^9/l)	798	927	739	**821**	96.1
Neu (x10^9/l)	0.75	0.65	0.64	**0.68**	0.1
Ly (x10^9/l)	5.08	4.69	7.21	**5.66**	1.4
Mo (x10^9/l)	0.36	0.20	0.23	**0.26**	0.1
Eos (x10^9/l)	0.04	0.04	0.05	**0.04**	0.0
Bas (x10^9/l)	0.01	0.00	0.01	**0.01**	0.0
PAR+CBZ-14D (14 Days)					
WCC (x10^9/l)	6.56	8.27	7.26	**7.36**	0.9
RCC (x10^{12}/l)	7.11	7.49	6.77	**7.12**	0.4
Hb (g/dl)	14.2	14.7	13.6	**14.2**	0.6
Hct (l/l)	0.431	0.437	0.410	**0.426**	0.01
MCV (fl)	60.6	58.3	60.6	**59.8**	1.3
MCH (pg)	20.0	19.6	20.1	**19.9**	0.3
MCHC (g/dl)	32.9	33.6	33.2	**33.2**	0.4
Plt (x10^9/l)	917	938	844	**900**	49.3
Neu (x10^9/l)	0.66	1.02	0.81	**0.83**	0.2
Ly (x10^9/l)	5.49	6.69	6.17	**6.12**	0.6
Mo (x10^9/l)	0.33	0.46	0.23	**0.34**	0.1
Eos (x10^9/l)	0.08	0.09	0.04	**0.07**	0.0
Bas (x10^9/l)	0.00	0.01	0.00	**0.00**	0.0

SD = standard deviation; PAR = paracetamol; CBZ = carbamazepine; D = Days; WCC = white cell count; RCC = red cell count; Hb = haemoglobin; Hct = haematocrit; MCV = mean corpuscular volume; MCH = mean corpuscular haemoglobin; MCHC = mean corpuscular haemoglobin concentration; Plt = platelets; Neu = neutrophils; Ly = lymphocytes; Mo = monocytes; Eos = eosinophils; Bas = basophils

Table D-12b: Full blood count results of the PAR+CBZ group after 28 and 42 days

Group (n = 3)	Rat 1	Rat 2	Rat 3	Mean	SD
PAR+CBZ-28D (28 Days)					
WCC (x10^9/l)	5.31	5.02	6.56	**5.63**	0.8
RCC (x10^{12}/l)	7.73	7.37	7.55	**7.55**	0.2
Hb (g/dl)	15.7	15.2	15.9	**15.6**	0.4
Hct (l/l)	0.461	0.444	0.450	**0.452**	0.01
MCV (fl)	59.6	60.2	59.6	**59.8**	0.3
MCH (pg)	20.3	20.6	21.1	**20.7**	0.4
MCHC (g/dl)	34.1	34.2	35.3	**34.5**	0.7
Plt (x10^9/l)	838	709	762	**770**	64.8
Neu (x10^9/l)	0.64	0.93	0.86	**0.81**	0.2
Ly (x10^9/l)	4.59	3.83	5.48	**4.63**	0.8
Mo (x10^9/l)	0.05	0.23	0.15	**0.14**	0.1
Eos (x10^9/l)	0.03	0.02	0.07	**0.04**	0.0
Bas (x10^9/l)	0.00	0.01	0.00	**0.00**	0.0
PAR+CBZ-42D (42 Days)					
WCC (x10^9/l)	6.45	5.89	6.86	**6.40**	0.5
RCC (x10^{12}/l)	8.32	7.74	7.63	**7.90**	0.4
Hb (g/dl)	16.7	15.6	15.4	**15.9**	0.7
Hct (l/l)	0.458	0.429	0.427	**0.438**	0.02
MCV (fl)	55.0	55.4	56.0	**55.5**	0.5
MCH (pg)	20.1	20.2	20.2	**20.2**	0.1
MCHC (g/dl)	36.5	36.4	36.1	**36.3**	0.2
Plt (x10^9/l)	961	813	990	**921**	94.9
Neu (x10^9/l)	0.99	0.92	0.73	**0.88**	0.1
Ly (x10^9/l)	4.88	3.94	5.92	**4.91**	1.0
Mo (x10^9/l)	0.31	0.33	0.14	**0.26**	0.1
Eos (x10^9/l)	0.26	0.70	0.06	**0.34**	0.3
Bas (x10^9/l)	0.01	0.00	0.01	**0.01**	0.0

SD = standard deviation; PAR = paracetamol; CBZ = carbamazepine; D = Days; WCC = white cell count; RCC = red cell count; Hb = haemoglobin; Hct = haematocrit; MCV = mean corpuscular volume; MCH = mean corpuscular haemoglobin; MCHC = mean corpuscular haemoglobin concentration; Plt = platelets; Neu = neutrophils; Ly = lymphocytes; Mo = monocytes; Eos = eosinophils; Bas = basophils

APPENDIX E: RENAL FUNCTION TEST RESULTS

Appendix E-1: Renal function test results of the S, S+LMS and S+CBZ groups

Table E-1: BUN (mmol/l) and creatinine (µmol/l) results of the S group

Group (n = 3)	RFT	Rat 1 (mmol/l & µmol/l)	Rat 2 (mmol/l & µmol/l)	Rat 3 (mmol/l & µmol/l)	Mean	SD
Untreated						
0 Days	**BUN**	8.2	6.9	6.5	**7.20**	0.9
	Cr	46	31	33	**36.67**	8.1
S-2D						
2 Days	**BUN**	6.6	7.7	7.6	**7.30**	0.6
	Cr	38	37	41	**38.67**	2.1
S-7D						
7 Days	**BUN**	8.3	8.2	7.8	**8.10**	0.3
	Cr	49	50	38	**45.67**	6.7
S-14D						
14 Days	**BUN**	8.3	7.1	7.1	**7.50**	0.7
	Cr	40	41	36	**39.00**	2.7
S-28D						
28 Days	**BUN**	12.5	8.1	11.1	**10.57**	2.3
	Cr	56	74	90	**73.33**	17.0
S-42D						
42 Days	**BUN**	6.0	6.8	4.6	**5.80**	1.1
	Cr	31	48	35	**38.00**	8.9

SD = standard deviation; S = saline; D = days; BUN = blood urea nitrogen; Cr = creatinine

Table E-2: BUN (mmol/l) and creatinine (µmol/l) results of the S+LMS group

Group (n = 3)	RFT	Rat 1 (mmol/l & µmol/l)	Rat 2 (mmol/l & µmol/l)	Rat 3 (mmol/l & µmol/l)	Mean	SD
S+LMS-2D						
2 Days	**BUN**	9.5	8.0	7.5	**8.33**	1.0
	Cr	33	43	38	**38.00**	5.0
S+LMS-7D						
7 Days	**BUN**	8.4	7.8	7.1	**7.77**	0.7
	Cr	61	33	27	**40.33**	18.2
S+LMS-14D						
14 Days	**BUN**	6.4	7.6	5.6	**6.53**	1.0
	Cr	30	40	34	**34.67**	5.0
S+LMS-28D						
28 Days	**BUN**	6.3	5.8	6.1	**6.07**	0.3
	Cr	58	60	63	**60.33**	2.5
S+LMS-42D						
42 Days	**BUN**	5.8	6.7	7.0	**6.50**	0.6
	Cr	22	20	21	**21.00**	1.0

SD = standard deviation; S = saline; LMS = levamisole; D = days; BUN = blood urea nitrogen; Cr = creatinine

Table E-3: BUN (mmol/l) and creatinine (µmol/l) results of the S+CBZ group

Group (n = 3)	RFT	Rat 1 (mmol/l & µmol/l)	Rat 2 (mmol/l & µmol/l)	Rat 3 (mmol/l & µmol/l)	Mean	SD
S+CBZ-2D						
2 Days	**BUN**	6.8	6.7	6.0	**6.50**	0.4
	Cr	39	35	35	**36.33**	2.3
S+CBZ-7D						
7 Days	**BUN**	5.8	7.1	7.5	**6.80**	0.9
	Cr	26	37	37	**33.33**	6.4
S+CBZ-14D						
14 Days	**BUN**	7.0	6.4	5.8	**6.40**	0.6
	Cr	40	30	39	**36.33**	5.5
S+CBZ-28D						
28 Days	**BUN**	8.8	6.5	6.3	**7.20**	1.4
	Cr	76	65	52	**64.33**	12.0
S+CBZ-42D						
42 Days	**BUN**	7.7	7.6	6.4	**7.23**	0.7
	Cr	11	13	1	**8.33**	6.4

SD = standard deviation; S = saline; CBZ = carbamazepine; D = days; BUN = blood urea nitrogen; Cr = creatinine

Appendix E-2: Renal function test results of the INH, INH+LMS and INH+CBZ groups

Table E-4: BUN (mmol/l) and creatinine (µmol/l) results of the INH group

Group (n = 3)	RFT	Rat 1 (mmol/l & µmol/l)	Rat 2 (mmol/l & µmol/l)	Rat 3 (mmol/l & µmol/l)	Mean	SD
INH-2D						
2 Days	**BUN**	6.6	6.9	6.6	**6.70**	0.2
	Cr	34	39	28	**33.67**	5.5
INH-7D						
7 Days	**BUN**	6.4	6.9	6.5	**6.60**	0.3
	Cr	38	34	37	**36.33**	2.1
INH-14D						
14 Days	**BUN**	6.1	6.8	5.8	**6.23**	0.5
	Cr	36	51	41	**42.67**	7.6
INH-28D						
28 Days	**BUN**	7.6	6.6	7.6	**7.27**	0.6
	Cr	69	72	65	**68.67**	3.5
INH-42D						
42 Days	**BUN**	5.6	6.0	5.8	**5.80**	0.2
	Cr	43	10	50	**34.33**	21.4

SD = standard deviation; INH = isoniazid; D = days; BUN = blood urea nitrogen; Cr = creatinine

Table E-5: BUN (mmol/l) and creatinine (µmol/l) results of the INH+LMS group

Group (n = 3)	RFT	Rat 1 (mmol/l & µmol/l)	Rat 2 (mmol/l & µmol/l)	Rat 3 (mmol/l & µmol/l)	Mean	SD
INH+LMS-2D						
2 Days	**BUN**	7.5	5.6	7.5	**6.87**	1.1
	Cr	37	29	46	**37.33**	8.5
INH+LMS-7D						
7 Days	**BUN**	7.8	6.7	6.8	**7.10**	0.6
	Cr	35	33	30	**32.67**	2.5
INH+LMS-14D						
14 Days	**BUN**	6.7	6.0	6.5	**6.40**	0.4
	Cr	34	37	33	**34.67**	2.1
INH+LMS-28D						
28 Days	**BUN**	6.3	6.9	5.7	**6.30**	0.6
	Cr	63	65	61	**63.00**	2.0
INH+LMS-42D						
42 Days	**BUN**	6.1	5.9	5.6	**5.87**	0.3
	Cr	24	24	23	**23.67**	0.6

SD = standard deviation; INH = isoniazid; LMS = levamisole; D = days; BUN = blood urea nitrogen; Cr = creatinine

Table E-6: BUN (mmol/l) and creatinine (µmol/l) results of the INH+CBZ group

Group (n = 3)	RFT	Rat 1 (mmol/l & µmol/l)	Rat 2 (mmol/l & µmol/l)	Rat 3 (mmol/l & µmol/l)	Mean	SD
INH+CBZ-2D						
2 Days	**BUN**	6.1	6.8	6.6	**6.50**	0.4
	Cr	29	47	44	**40.00**	9.6
INH+CBZ-7D						
7 Days	**BUN**	5.6	7.1	6.5	**6.40**	0.8
	Cr	37	28	35	**33.33**	4.7
INH+CBZ-14D						
14 Days	**BUN**	6.2	6.6	5.4	**6.07**	0.6
	Cr	31	29	35	**31.67**	3.1
INH+CBZ-28D						
28 Days	**BUN**	6.8	5.6	4.7	**5.70**	1.1
	Cr	63	65	65	**64.33**	1.2
INH+CBZ-42D						
42 Days	**BUN**	6.2	6.7	6.8	**6.57**	0.3
	Cr	16	13	4	**11.00**	6.2

SD = standard deviation; INH = isoniazid; CBZ = carbamazepine; D = days; BUN = blood urea nitrogen; Cr = creatinine

Appendix E-3: Renal function test results of the NVP, NVP+LMS and NVP+CBZ groups

Table E-7: BUN (mmol/l) and creatinine (µmol/l) results of the NVP group

Group (n = 3)	RFT	Rat 1 (mmol/l & µmol/l)	Rat 2 (mmol/l & µmol/l)	Rat 3 (mmol/l & µmol/l)	Mean	SD
NVP-2D						
2 Days	**BUN**	7.7	7.3	8.6	**7.87**	0.7
	Cr	35	36	37	**36.00**	1.0
NVP-7D						
7 Days	**BUN**	8.9	8.9	8.4	**8.73**	0.3
	Cr	44	47	-*	**45.50**	2.1
NVP-14D						
14 Days	**BUN**	9.0	10.3	8.1	**9.13**	1.1
	Cr	45	47	30	**40.67**	9.3
NVP-28D						
28 Days	**BUN**	9.9	8.3	7.3	**8.50**	1.3
	Cr	69	64	56	**63.00**	6.6
NVP-42D						
42 Days	**BUN**	7.3	6.8	6.9	**7.00**	0.3
	Cr	24	30	26	**26.67**	3.1

SD = standard deviation; NVP = nevirapine; D = days; BUN = blood urea nitrogen; Cr = creatinine; * = too low to detect

Table E-8: BUN (mmol/l) and creatinine (µmol/l) results of the NVP+LMS group

Group (n = 3)	RFT	Rat 1 (mmol/l & µmol/l)	Rat 2 (mmol/l & µmol/l)	Rat 3 (mmol/l & µmol/l)	Mean	SD
NVP+LMS-2D						
2 Days	**BUN**	9.7	7.9	7.5	**8.37**	1.2
	Cr	42	29	27	**32.67**	8.1
NVP+LMS-7D						
7 Days	**BUN**	7.9	7.9	6.8	**7.53**	0.6
	Cr	42	27	31	**33.33**	7.8
NVP+LMS-14D						
14 Days	**BUN**	7.5	6.0	6.4	**6.63**	0.8
	Cr	43	30	20	**31.00**	11.5
NVP+LMS-28D						
28 Days	**BUN**	5.7	5.9	5.9	**5.83**	0.1
	Cr	51	55	63	**56.33**	6.1
NVP+LMS-42D						
42 Days	**BUN**	7.1	6.9	8.2	**7.40**	0.7
	Cr	11	10	10	**10.33**	0.6

SD = standard deviation; NVP = nevirapine; LMS = levamisole; D = days; BUN = blood urea nitrogen; Cr = creatinine

Table E-9: BUN (mmol/l) and creatinine (µmol/l) results of the NVP+CBZ group

Group (n = 3)	RFT	Rat 1 (mmol/l & µmol/l)	Rat 2 (mmol/l & µmol/l)	Rat 3 (mmol/l & µmol/l)	Mean	SD
NVP+CBZ-2D						
2 Days	**BUN**	6.4	7.8	7.5	**7.23**	0.7
	Cr	38	38	34	**36.67**	2.3
NVP+CBZ-7D						
7 Days	**BUN**	6.5	7.2	7.6	**7.10**	0.6
	Cr	24	29	37	**30.00**	6.6
NVP+CBZ-14D						
14 Days	**BUN**	7.9	7.3	8.1	**7.77**	0.4
	Cr	32	36	37	**35.00**	2.7
NVP+CBZ-28D						
28 Days	**BUN**	9.0	8.1	7.0	**8.03**	1.0
	Cr	75	63	54	**64.00**	10.5
NVP+CBZ-42D						
42 Days	**BUN**	8.2	7.3	8.4	**7.97**	0.6
	Cr	21	12	20	**17.67**	4.9

SD = standard deviation; NVP = nevirapine; CBZ = carbamazepine; D = days; BUN = blood urea nitrogen; Cr = creatinine

Appendix E-4: Renal function test results of the PAR, PAR+LMS and PAR+CBZ groups

Table E-10: BUN (mmol/l) and creatinine (µmol/l) results of the PAR group

Group (n = 3)	RFT	Rat 1 (mmol/l & µmol/l)	Rat 2 (mmol/l & µmol/l)	Rat 3 (mmol/l & µmol/l)	Mean	SD
PAR-2D						
2 Days	**BUN**	7.3	6.5	6.4	**6.73**	0.5
	Cr	39	40	29	**36.00**	6.1
PAR-7D						
7 Days	**BUN**	7.3	8.4	7.0	**7.57**	0.7
	Cr	41	39	32	**37.33**	4.7
PAR-14D						
14 Days	**BUN**	7.9	7.6	6.4	**7.30**	0.8
	Cr	38	36	35	**36.33**	1.5
PAR-28D						
28 Days	**BUN**	6.7	8.3	7.0	**7.33**	0.9
	Cr	69	74	77	**73.33**	4.0
PAR-42D						
42 Days	**BUN**	5.7	7.7	7.3	**6.90**	1.1
	Cr	26	28	19	**24.33**	4.7

SD = standard deviation; PAR = paracetamol; D = days; BUN = blood urea nitrogen; Cr = creatinine

Table E-11: BUN (mmol/l) and creatinine (µmol/l) results of the PAR+LMS group

Group (n = 3)	RFT	Rat 1 (mmol/l & µmol/l)	Rat 2 (mmol/l & µmol/l)	Rat 3 (mmol/l & µmol/l)	Mean	SD
PAR+LMS-2D						
2 Days	**BUN**	6.2	5.7	6.6	**6.17**	0.5
	Cr	25	36	19	**26.67**	8.6
PAR+LMS-7D						
7 Days	**BUN**	6.0	5.8	5.7	**5.83**	0.2
	Cr	44	23	32	**33.00**	10.5
PAR+LMS-14D						
14 Days	**BUN**	7.6	7.6	5.6	**6.93**	1.2
	Cr	39	33	34	**35.33**	3.2
PAR+LMS-28D						
28 Days	**BUN**	5.2	5.5	5.5	**5.40**	0.2
	Cr	60	64	64	**62.67**	2.3
PAR+LMS-42D						
42 Days	**BUN**	6.8	7.1	5.8	**6.57**	0.7
	Cr	10	21	9	**13.33**	6.7

SD = standard deviation; PAR = paracetamol; LMS = levamisole; D = days; BUN = blood urea nitrogen; Cr = creatinine

Table E-12: BUN (mmol/l) and creatinine (µmol/l) results of the PAR+CBZ group

Group (n = 3)	RFT	Rat 1 (mmol/l & µmol/l)	Rat 2 (mmol/l & µmol/l)	Rat 3 (mmol/l & µmol/l)	Mean	SD
PAR+CBZ-2D						
2 Days	**BUN**	6.1	6.9	6.0	**6.33**	0.5
	Cr	15	20	27	**20.67**	6.0
PAR+CBZ-7D						
7 Days	**BUN**	6.7	6.8	6.6	**6.70**	0.1
	Cr	33	17	35	**28.33**	9.9
PAR+CBZ-14D						
14 Days	**BUN**	10.4	9.6	12.5	**10.83**	1.5
	Cr	17	36	43	**32.00**	13.5
PAR+CBZ-28D						
28 Days	**BUN**	10.4	7.3	9.6	**9.10**	1.6
	Cr	53	50	58	**53.67**	4.0
PAR+CBZ-42D						
42 Days	**BUN**	7.8	7.8	6.2	**7.27**	0.9
	Cr	15	23	21	**19.67**	4.2

SD = standard deviation; PAR = paracetamol; CBZ = carbamazepine; D = days; BUN = blood urea nitrogen; Cr = creatinine

APPENDIX F: LIVER FUNCTION TEST RESULTS

Appendix F-1: Liver function test results of the S, S+LMS and S+CBZ groups

Table F-1: Liver function test results in u/l of the S group

Group (n = 3)	Enzyme	Rat 1 (u/l)	Rat 2 (u/l)	Rat 3 (u/l)	Mean	SD
Untreated	**ALT**	55	48	46	**49.67**	4.7
0 Days	**AST**	104	79	81	**88.00**	13.9
	ALP	436	289	332	**352.33**	75.6
S-2D	**ALT**	46	48	44	**46.00**	2.0
2 Days	**AST**	96	91	83	**90.00**	6.6
	ALP	393	406	400	**399.67**	6.5
S-7D	**ALT**	61	44	42	**49.00**	10.4
7 Days	**AST**	130	99	80	**103.00**	25.2
	ALP	299	294	318	**303.67**	12.7
S-14D	**ALT**	63	56	56	**58.33**	4.0
14 Days	**AST**	168	119	95	**127.33**	37.2
	ALP	511	470	544	**508.33**	37.1
S-28D	**ALT**	48	49	45	**47.33**	2.1
28 Days	**AST**	66	128	152	**115.33**	44.4
	ALP	201	237	211	**216.33**	18.6
S-42D	**ALT**	45	52	41	**46.00**	5.6
42 Days	**AST**	61	108	59	**76.00**	27.7
	ALP	94	191	42	**109.00**	75.6

SD = standard deviation; S = saline; D = days; ALT = alanine aminotransferase; AST = aspartate aminotransferase; ALP = alkaline phosphatase

Table F-2: Liver function test results in u/l of the S+LMS group

Group (n = 3)	Enzyme	Rat 1 (u/l)	Rat 2 (u/l)	Rat 3 (u/l)	Mean	SD
S+LMS-2D	**ALT**	38	38	43	**39.67**	2.9
2 Days	**AST**	174	76	90	**113.33**	53.0
	ALP	551	540	533	**541.33**	9.1
S+LMS-7D	**ALT**	47	69	41	**52.33**	14.7
7 Days	**AST**	110	102	59	**90.33**	27.4
	ALP	583	531	337	**483.67**	129.7
S+LMS-14D	**ALT**	47	37	61	**48.33**	12.1
14 Days	**AST**	71	58	90	**73.00**	16.1
	ALP	432	404	599	**478.33**	105.4
S+LMS-28D	**ALT**	58	47	46	**50.33**	6.7
28 Days	**AST**	95	74	57	**75.33**	19.0
	ALP	54	161	166	**127.00**	63.3
S+LMS-42D	**ALT**	44	48	47	**46.33**	2.1
42 Days	**AST**	75	69	80	**74.67**	5.5
	ALP	14	36	22	**24.00**	11.1

SD = standard deviation; S = saline; LMS = levamisole; D = days; ALT = alanine aminotransferase; AST = aspartate aminotransferase; ALP = alkaline phosphatase

Table F-3: Liver function test results in u/l of the S+CBZ group

Group (n = 3)	Enzyme	Rat 1 (u/l)	Rat 2 (u/l)	Rat 3 (u/l)	Mean	SD
S+CBZ-2D	**ALT**	48	55	52	**51.67**	3.5
2 Days	**AST**	79	102	89	**90.00**	11.5
	ALP	333	314	350	**332.33**	18.0
S+CBZ-7D	**ALT**	48	45	37	**43.33**	5.7
7 Days	**AST**	95	84	79	**86.00**	8.2
	ALP	350	364	310	**341.33**	28.0
S+CBZ-14D	**ALT**	46	49	46	**47.00**	1.7
14 Days	**AST**	84	87	83	**84.67**	2.1
	ALP	357	357	355	**356.33**	1.2
S+CBZ-28D	**ALT**	52	47	56	**51.67**	4.5
28 Days	**AST**	104	96	91	**97.00**	6.6
	ALP	221	166	68	**151.67**	77.5
S+CBZ-42D	**ALT**	43	44	47	**44.67**	2.1
42 Days	**AST**	95	69	109	**91.00**	20.3
	ALP	160	132	20	**104.00**	74.1

SD = standard deviation; S = saline; CBZ = carbamazepine; D = days; ALT = alanine aminotransferase; AST = aspartate aminotransferase; ALP = alkaline phosphatase

Appendix F-2: Liver function test results of the INH, INH+LMS and INH+CBZ groups

Table F-4: Liver function test results in u/l of the INH group

Group (n = 3)	Enzyme	Rat 1 (u/l)	Rat 2 (u/l)	Rat 3 (u/l)	Mean	SD
INH-2D	**ALT**	42	46	50	**46.00**	4.0
2 Days	**AST**	100	100	112	**104.00**	6.9
	ALP	301	339	365	**335.00**	32.2
INH-7D	**ALT**	46	68	46	**53.33**	12.7
7 Days	**AST**	117	490	91	**232.67**	223.2
	ALP	358	376	373	**369.00**	9.6
INH-14D	**ALT**	46	43	39	**42.67**	3.5
14 Days	**AST**	88	130	75	**97.67**	28.8
	ALP	377	394	321	**364.00**	38.2
INH-28D	**ALT**	44	50	44	**46.00**	3.5
28 Days	**AST**	134	113	183	**143.33**	35.9
	ALP	222	190	247	**219.67**	28.6
INH-42D	**ALT**	49	53	50	**50.67**	2.1
42 Days	**AST**	70	93	96	**86.33**	14.2
	ALP	170	37	173	**126.67**	77.7

SD = standard deviation; INH = isoniazid; D = days; ALT = alanine aminotransferase; AST = aspartate aminotransferase; ALP = alkaline phosphatase

Table F-5: Liver function test results in u/l of the INH+LMS group

Group (n = 3)	Enzyme	Rat 1 (u/l)	Rat 2 (u/l)	Rat 3 (u/l)	Mean	SD
INH+LMS-2D	**ALT**	51	51	42	**48.00**	5.2
2 Days	**AST**	169	94	98	**120.33**	42.2
	ALP	391	347	369	**369.00**	22.0
INH+LMS-7D	**ALT**	48	45	46	**46.33**	1.5
7 Days	**AST**	128	128	211	**155.67**	47.9
	ALP	321	341	327	**329.67**	10.3
INH+LMS-14D	**ALT**	40	45	53	**46.00**	6.6
14 Days	**AST**	90	142	137	**123.00**	28.7
	ALP	399	413	365	**392.33**	24.7
INH+LMS-28D	**ALT**	44	42	12	**32.67**	17.9
28 Days	**AST**	139	88	76	**101.00**	33.5
	ALP	175	217	161	**184.33**	29.1
INH+LMS-42D	**ALT**	38	42	34	**38.00**	4.0
42 Days	**AST**	68	57	68	**64.33**	6.4
	ALP	27	53	16	**32.00**	19.0

SD = standard deviation; INH = isoniazid; LMS = levamisole; D = days; ALT = alanine aminotransferase; AST = aspartate aminotransferase; ALP = alkaline phosphatase

Table F-6: Liver function test results in u/l of the INH+CBZ group

Group (n = 3)	Enzyme	Rat 1 (u/l)	Rat 2 (u/l)	Rat 3 (u/l)	Mean	SD
INH+CBZ-2D	**ALT**	56	53	58	**55.67**	2.5
2 Days	**AST**	149	120	156	**141.67**	19.1
	ALP	294	401	412	**369.00**	65.2
INH+CBZ-7D	**ALT**	42	48	50	**46.67**	4.2
7 Days	**AST**	121	192	167	**160.00**	36.0
	ALP	375	343	358	**358.67**	16.0
INH+CBZ-14D	**ALT**	45	49	54	**49.33**	4.5
14 Days	**AST**	100	258	187	**181.67**	79.1
	ALP	368	335	331	**344.67**	20.3
INH+CBZ-28D	**ALT**	38	57	48	**47.67**	9.5
28 Days	**AST**	74	66	68	**69.33**	4.2
	ALP	149	161	116	**142.00**	23.3
INH+CBZ-42D	**ALT**	45	44	51	**46.67**	3.8
42 Days	**AST**	67	70	66	**67.67**	2.1
	ALP	78	34	54	**55.33**	22.0

SD = standard deviation; INH = isoniazid; CBZ = carbamazepine; D = days; ALT = alanine aminotransferase; AST = aspartate aminotransferase; ALP = alkaline phosphatase

Appendix F-3: Liver function test results of the NVP, NVP+LMS and NVP+CBZ groups

Table F-7: Liver function test results in u/l of the NVP group

Group (n = 3)	Enzyme	Rat 1 (u/l)	Rat 2 (u/l)	Rat 3 (u/l)	Mean	SD
NVP-2D	**ALT**	55	69	64	**62.67**	7.1
2 Days	**AST**	97	106	117	**106.67**	10.0
	ALP	374	392	310	**358.67**	43.1
NVP-7D	**ALT**	67	66	129	**87.33**	36.1
7 Days	**AST**	105	100	302	**169.00**	115.2
	ALP	490	495	357	**447.33**	78.3
NVP-14D	**ALT**	74	72	69	**71.67**	2.5
14 Days	**AST**	88	93	147	**109.33**	32.7
	ALP	484	445	399	**442.67**	42.6
NVP-28D	**ALT**	56	48	55	**53.00**	4.4
28 Days	**AST**	178	95	112	**128.33**	43.8
	ALP	208	142	147	**165.67**	36.8
NVP-42D	**ALT**	53	52	56	**53.67**	2.1
42 Days	**AST**	73	72	65	**70.00**	4.4
	ALP	12	24	7	**14.33**	8.7

SD = standard deviation; NVP = nevirapine; D = days; ALT = alanine aminotransferase; AST = aspartate aminotransferase; ALP = alkaline phosphatase

Table F-8: Liver function test results in u/l of the NVP+LMS group

Group (n = 3)	Enzyme	Rat 1 (u/l)	Rat 2 (u/l)	Rat 3 (u/l)	Mean	SD
NVP+LMS-2D	**ALT**	67	107	52	**75.33**	28.4
2 Days	**AST**	101	382	64	**182.33**	173.9
	ALP	548	589	434	**523.67**	80.3
NVP+LMS-7D	**ALT**	46	59	65	**56.67**	9.7
7 Days	**AST**	78	72	59	**69.67**	9.7
	ALP	447	465	356	**422.67**	58.4
NVP+LMS-14D	**ALT**	51	61	44	**52.00**	8.5
14 Days	**AST**	81	124	70	**91.67**	28.5
	ALP	461	340	334	**378.33**	71.7
NVP+LMS-28D	**ALT**	54	52	48	**51.33**	3.1
28 Days	**AST**	82	64	56	**67.33**	13.3
	ALP	141	37	24	**67.33**	64.1
NVP+LMS-42D	**ALT**	48	55	44	**49.00**	5.6
42 Days	**AST**	51	81	78	**70.00**	16.5
	ALP	15	10	10	**11.67**	2.9

SD = standard deviation; NVP = nevirapine; LMS = levamisole; D = days; ALT = alanine aminotransferase; AST = aspartate aminotransferase; ALP = alkaline phosphatase

Table F-9: Liver function test results in u/l of the NVP+CBZ group

Group (n = 3)	Enzyme	Rat 1 (u/l)	Rat 2 (u/l)	Rat 3 (u/l)	Mean	SD
NVP+CBZ-2D	**ALT**	42	61	59	**54.00**	10.4
2 Days	**AST**	125	192	121	**146.00**	39.9
	ALP	322	435	440	**399.00**	66.7
NVP+CBZ-7D	**ALT**	47	53	71	**57.00**	12.5
7 Days	**AST**	138	144	124	**135.33**	10.3
	ALP	301	330	317	**316.00**	14.5
NVP+CBZ-14D	**ALT**	53	45	47	**48.33**	4.2
14 Days	**AST**	114	89	79	**94.00**	18.0
	ALP	284	249	258	**263.67**	18.2
NVP+CBZ-28D	**ALT**	55	53	50	**52.67**	2.5
28 Days	**AST**	105	94	75	**91.33**	15.2
	ALP	196	160	156	**170.67**	22.0
NVP+CBZ-42D	**ALT**	51	55	64	**56.67**	6.7
42 Days	**AST**	108	67	195	**123.33**	65.4
	ALP	151	122	181	**151.33**	29.5

SD = standard deviation; NVP = nevirapine; CBZ = carbamazepine; D = days; ALT = alanine aminotransferase; AST = aspartate aminotransferase; ALP = alkaline phosphatase;

Appendix F-4: Liver function test results of the PAR, PAR+LMS and PAR+CBZ groups

Table F-10: Liver function test results in u/l of the PAR group

Group (n = 3)	Enzyme	Rat 1 (u/l)	Rat 2 (u/l)	Rat 3 (u/l)	Mean	SD
PAR-2D	**ALT**	58	52	51	**53.67**	3.8
2 Days	**AST**	103	113	112	**109.33**	5.5
	ALP	418	332	457	**402.33**	64.0
PAR-7D	**ALT**	61	57	57	**58.33**	2.3
7 Days	**AST**	90	138	83	**103.67**	29.9
	ALP	360	319	369	**349.33**	26.7
PAR-14D	**ALT**	53	51	54	**52.67**	1.5
14 Days	**AST**	84	108	92	**94.67**	12.2
	ALP	386	386	321	**364.33**	37.5
PAR-28D	**ALT**	45	52	50	**49.00**	3.6
28 Days	**AST**	72	130	135	**112.33**	35.0
	ALP	171	248	253	**224.00**	46.0
PAR-42D	**ALT**	54	46	45	**48.33**	4.9
42 Days	**AST**	65	66	61	**64.00**	2.7
	ALP	31	128	7	**55.33**	64.1

SD = standard deviation; PAR = paracetamol; D = days; ALT = alanine aminotransferase; AST = aspartate aminotransferase; ALP = alkaline phosphatase

Table F-11: Liver function test results in u/l of the PAR+LMS group

Group (n = 3)	Enzyme	Rat 1 (u/l)	Rat 2 (u/l)	Rat 3 (u/l)	Mean	SD
PAR+LMS-2D	**ALT**	61	40	57	**52.67**	11.2
2 Days	**AST**	154	117	142	**137.67**	18.9
	ALP	333	285	347	**321.67**	32.5
PAR+LMS-7D	**ALT**	65	49	60	**58.00**	8.2
7 Days	**AST**	121	86	91	**99.33**	18.9
	ALP	474	381	365	**406.67**	58.9
PAR+LMS-14D	**ALT**	49	48	48	**48.33**	0.6
14 Days	**AST**	84	114	81	**93.00**	18.3
	ALP	379	369	351	**366.33**	14.2
PAR+LMS-28D	**ALT**	49	42	44	**45.00**	3.6
28 Days	**AST**	61	61	60	**60.67**	0.6
	ALP	48	20	28	**32.00**	14.4
PAR+LMS-42D	**ALT**	44	45	49	**46.00**	2.7
42 Days	**AST**	78	63	80	**73.67**	9.3
	ALP	10	17	10	**12.33**	4.0

SD = standard deviation; PAR = paracetamol; LMS = levamisole; D = days; ALT = alanine aminotransferase; AST = aspartate aminotransferase; ALP = alkaline phosphatase

Table F-12: Liver function test results in u/l of the PAR+CBZ group

Group (n = 3)	Enzyme	Rat 1 (u/l)	Rat 2 (u/l)	Rat 3 (u/l)	Mean	SD
PAR+CBZ-2D	**ALT**	42	55	56	**51.00**	7.8
2 Days	**AST**	135	110	133	**126.00**	13.9
	ALP	317	315	442	**358.00**	72.8
PAR+CBZ-7D	**ALT**	55	59	56	**56.67**	2.1
7 Days	**AST**	102	157	116	**125.00**	28.6
	ALP	474	349	428	**417.00**	63.2
PAR+CBZ-14D	**ALT**	42	50	58	**50.00**	8.0
14 Days	**AST**	139	98	98	**111.67**	23.7
	ALP	251	282	298	**277.00**	23.9
PAR+CBZ-28D	**ALT**	35	66	35	**45.33**	17.9
28 Days	**AST**	70	122	67	**86.33**	30.9
	ALP	45	229	86	**120.00**	96.6
PAR+CBZ-42D	**ALT**	54	41	42	**45.67**	7.2
42 Days	**AST**	89	101	58	**82.67**	22.2
	ALP	224	216	194	**211.33**	15.5

SD = standard deviation; PAR = paracetamol; CBZ = carbamazepine; D = days; ALT = alanine aminotransferase; AST = aspartate aminotransferase; ALP = alkaline phosphatase

APPENDIX G: ISONIAZID, NEVIRAPINE, AND PARACETAMOL PLASMA MONITORING

Appendix G-1: Isoniazid plasma concentrations of the INH, INH+LMS and INH+CBZ groups

Table G-1: Isoniazid plasma concentrations in µg/ml of the INH group

Group (n = 5)	Rat 1 (µg/ml)	Rat 2 (µg/ml)	Rat 3 (µg/ml)	Rat 4 (µg/ml)	Rat 5 (µg/ml)	Mean	SD
INH-2D							
2 Days	2.572	2.147	1.476	1.370	-*	**1.891**	0.57
INH-7D							
7 Days	5.328	4.216	6.086	3.559	2.245	**4.287**	1.50
INH-14D							
14 Days	8.517	1.562	18.644	3.231	11.188	**8.628**	6.82
INH-28D							
28 Days	2.930	2.135	1.886	3.924	2.334	**2.642**	0.81
INH-42D							
42 Days	3.094	1.057	2.341	1.544	0.000	**1.607**	1.19

SD = standard deviation; INH = isoniazid; D = days; * = only 4 rats available

Table G-2: Isoniazid plasma concentrations in µg/ml of the INH+LMS group

Group (n = 5)	Rat 1 (µg/ml)	Rat 2 (µg/ml)	Rat 3 (µg/ml)	Rat 4 (µg/ml)	Rat 5 (µg/ml)	Mean	SD
INH+LMS-2D							
2 Days	2.285	3.009	2.564	1.339	-*	**2.299**	0.71
INH+LMS-7D							
7 Days	1.405	2.996	2.289	3.420	1.688	**2.360**	0.85
INH+LMS-14D							
14 Days	8.227	2.043	4.115	1.588	2.498	**3.694**	2.71
NH+LMS-28D							
28 Days	11.638	11.288	6.361	11.253	7.549	**9.618**	2.47
INH+LMS-42D							
42 Days	5.087	3.006	3.227	2.961	2.297	**3.316**	1.05

SD = standard deviation; INH = isoniazid; LMS = levamisole; D = days; * = only 4 rats available

Table G-3: Isoniazid plasma concentrations in µg/ml of the INH+CBZ group

Group (n = 5)	Rat 1 (µg/ml)	Rat 2 (µg/ml)	Rat 3 (µg/ml)	Rat 4 (µg/ml)	Rat 5 (µg/ml)	Mean	SD
INH+CBZ-2D							
2 Days	2.730	0.505	3.064	0.505	-*	**1.701**	1.39
INH+CBZ-7D							
7 Days	2.748	3.667	3.702	1.829	6.035	**3.596**	1.57
INH+CBZ-14D							
14 Days	4.469	2.548	9.421	1.437	3.862	**4.347**	3.07
INH+CBZ-28D							
28 Days	0.000	0.843	0.000	0.000	0.000	**0.169**	0.38
INH+CBZ-42D							
42 Days	10.712	0.000	0.000	0.000	-*	**2.678**	5.36

SD = standard deviation; INH = isoniazid; CBZ = carbamazepine; D = days; * = only 4 rats available

Appendix G-2: Nevirapine plasma concentrations of the NVP, NVP+LMS and NVP+CBZ groups

Table G-4: Nevirapine plasma concentrations in µg/ml of the NVP group

Group (n = 5)	Rat 1 (µg/ml)	Rat 2 (µg/ml)	Rat 3 (µg/ml)	Rat 4 (µg/ml)	Rat 5 (µg/ml)	Mean	SD
NVP-2D							
2 Days	8.868	3.243	3.920	1.763	3.666	**4.292**	2.69
NVP-7D							
7 Days	1.552	2.101	3.116	1.848	1.425	**2.008**	0.67
NVP-24D							
14 Days	2.101	1.467	2.440	1.298	2.609	**1.983**	0.58
NVP-28D							
28 Days	7.155	0.326	0.199	0.385	0.539	**1.721**	3.04
NVP-42D							
42 Days	2.486	0.000	0.000	0.000	0.000	**0.497**	1.11

SD = standard deviation; NVP = nevirapine; D = days

Table G-5: Nevirapine plasma concentrations in µg/ml of the NVP+LMS group

Group (n = 5)	Rat 1 (µg/ml)	Rat 2 (µg/ml)	Rat 3 (µg/ml)	Rat 4 (µg/ml)	Rat 5 (µg/ml)	Mean	SD
NVP+LMS-2D							
2 Days	6.154	0.770	1.239	0.985	-*	**2.287**	2.59
NVP+LMS-7D							
7 Days	0.561	1.027	4.078	3.146	12.849	**4.332**	4.98
NVP+LMS-14D							
14 Days	4.078	0.180	0.265	2.002	0.858	**1.477**	1.63
NVP+LMS-28D							
28 Days	1.562	1.809	1.624	1.469	0.000	**1.293**	0.73
NVP+LMS-42D							
42 Days	0.000	0.000	0.000	0.000	0.000	**0.000**	0.00

SD = standard deviation; NVP = nevirapine; LMS = levamisole; D = days; * = only 4 rats available

Table G-6: Nevirapine plasma concentrations in µg/ml of the NVP+CBZ group

Group (n = 5)	Rat 1 (µg/ml)	Rat 2 (µg/ml)	Rat 3 (µg/ml)	Rat 4 (µg/ml)	Rat 5 (µg/ml)	Mean	SD
NVP+CBZ-2D							
2 Days	0.515	0.087	0.371	0.299	0.491	**0.353**	0.17
NVP+CBZ-7D							
7 Days	0.779	0.851	0.683	0.731	0.803	**0.769**	0.06
NVP+CBZ-14D							
14 Days	2.076	0.707	0.851	0.515	0.107	**0.851**	0.74
NVP+CBZ-28D							
28 Days	0.080	0.000	0.000	0.000	0.896	**0.195**	0.39
NVP+CBZ-42D							
42 Days	0.000	0.469	0.000	0.000	3.175	**0.729**	1.38

SD = standard deviation; NVP = nevirapine; CBZ = carbamazepine; D = days

Appendix G-3: Paracetamol plasma concentrations of the PAR, PAR+LMS and PAR+CBZ groups

Table G-7: Paracetamol plasma concentrations in µg/ml of the PAR group

Group (n = 5)	Rat 1 (µg/ml)	Rat 2 (µg/ml)	Rat 3 (µg/ml)	Rat 4 (µg/ml)	Rat 5 (µg/ml)	Mean	SD
PAR-2							
2 Days	0.484	0.643	0.850	1.056	0.897	**0.786**	0.22
PAR-7D							
7 Days	1.088	0.619	0.929	0.381	0.588	**0.721**	0.28
PAR-14D							
14 Days	0.670	0.644	0.523	0.462	0.376	**0.535**	0.12
PAR-28D							
28 Days	1.309	0.097	0.873	0.235	0.671	**0.637**	0.49
PAR-42D							
42 Days	0.000	0.000	0.000	0.000	0.232	**0.046**	0.10

SD = standard deviation; PAR = paracetamol; D = days

Table G-8: Paracetamol plasma concentrations in µg/ml of the PAR+LMS group

Group (n = 5)	Rat 1 (µg/ml)	Rat 2 (µg/ml)	Rat 3 (µg/ml)	Rat 4 (µg/ml)	Rat 5 (µg/ml)	Mean	SD
PAR+LMS-2D							
2 Days	0.825	1.724	0.678	0.290	-*	**0.879**	0.61
PAR+LMS-7D							
7Days	0.488	0.229	0.341	0.151	0.186	**0.279**	0.14
PAR+LMS-14D							
14 Days	0.346	0.848	0.574	0.112	0.015	**0.379**	0.34
PAR+LMS-28D							
28 Days	0.963	0.831	0.000	0.000	0.000	**0.359**	0.49
PAR+LMS-42D							
42 Days	0.000	0.000	0.000	0.000	0.000	**0.000**	0.00

SD = standard deviation; PAR = paracetamol; LMS = levamisole; D = days; * = only 4 rats available

Table G-9: Paracetamol plasma concentrations in µg/ml of the PAR+CBZ group

Group (n = 5)	Rat 1 (µg/ml)	Rat 2 (µg/ml)	Rat 3 (µg/ml)	Rat 4 (µg/ml)	Rat 5 (µg/ml)	Mean	SD
PAR+CBZ-2D							
2 Days	0.283	0.363	0.182	0.584	0.102	**0.303**	0.19
PAR+CBZ-7D							
7 Days	0.279	0.166	0.052	0.052	0.065	**0.123**	0.10
PAR+CBZ-14D							
14 Days	0.059	0.273	0.169	0.126	-*	**0.157**	0.09
PAR+CBZ-28D							
28 Days	0.047	0.000	1.042	0.000	0.001	**0.218**	0.46
PAR+CBZ-42D							
42 Days	0.031	0.000	0.090	0.182	0.000	**0.061**	0.08

SD = standard deviation; PAR = paracetamol; CBZ = carbamazepine; D = days; * = only 4 rats available

APPENDIX H: RAT WEIGHTS

Appendix H-1: Rat weights of the S, S+LMS and S+CBZ groups

Table H-1: Rat weights in grams of the S group

Group (n = 5)	Rat 1 (g)	Rat 2 (g)	Rat 3 (g)	Rat 4 (g)	Rat 5 (g)	Mean	SD
Untreated							
0 Days	213.7	211.0	212.9	244.1	238.1	**223.96**	15.8
S-2D							
0 Days	229.4	228.1	218.4	230.3	205.1	**222.26**	10.7
2 Days	234.0	235.1	233.9	240.3	214.1	**231.48**	10.1
S-7D							
0 Days	207.4	218.6	222.3	212.8	210.1	**214.24**	6.1
7 Days	243.6	248.6	249.2	250.7	257.4	**249.90**	5.0
S-14D							
0 Days	232.2	210.9	218.4	215.0	231.4	**221.60**	9.7
14 Days	315.6	292.5	296.7	304.3	322.1	**306.24**	12.5
S-28D							
0 Days	212.3	216.3	209.7	208.0	201.5	**209.56**	5.5
14 Days	281.4	275.9	279.3	279.4	273.4	**277.88**	3.2
28 Days	305.0	321.7	323.2	314.8	322.3	**317.40**	7.7
S-42D							
0 Days	220.2	215.6	205.6	210.6	220.7	**214.54**	6.5
14 Days	292.4	305.0	288.5	292.9	305.1	**296.78**	7.7
28 Days	363.1	320.1	332.0	343.3	339.8	**339.66**	15.8
42 Days	379.5	353.5	415.6	389.9	391.2	**385.94**	22.5

SD = standard deviation; S = saline; D = days

Table H-2: Rat weights in grams of the S+LMS group

Group (n = 5)	Rat 1 (g)	Rat 2 (g)	Rat 3 (g)	Rat 4 (g)	Rat 5 (g)	Mean	SD
S+LMS-2D							
0 Days	222.4	232.7	240.4	-*	-*	**231.83**	9.0
2 Days	230.2	238.7	222.5	-*	-*	**230.47**	8.1
S+LMS-7D							
0 Days	247.3	234.0	248.2	243.4	223.6	**239.30**	10.4
7 Days	250.4	259.7	267.2	272.5	243.6	**258.68**	11.8
S+LMS-14D							
0 Days	245.0	235.5	206.6	237.6	216.3	**228.20**	16.1
14 Days	303.2	260.3	290.7	274.2	297.6	**285.20**	17.7
S+LMS-28D							
0 Days	246.1	243.8	240.1	244.3	235.4	**241.94**	4.3
14 Days	288.4	298.2	306.6	313.2	294.0	**300.08**	9.9
28 Days	350.9	333.3	322.2	357.3	326.6	**338.06**	15.3
S+LMS-42D							
0 Days	224.0	202.4	200.3	204.8	211.0	**208.50**	9.5
14 Days	300.7	267.5	286.7	267.0	298.8	**284.14**	16.3
28 Days	338.7	304.5	343.7	299.7	344.5	**326.22**	22.2
42 Days	372.2	384.9	340.1	330.8	381.4	**361.88**	24.8

SD = standard deviation; S = saline; LMS = levamisole; D = days; * = only 3 rats available

Table H-3: Rat weights in grams of the S+CBZ group

Group (n = 5)	Rat 1 (g)	Rat 2 (g)	Rat 3 (g)	Rat 4 (g)	Rat 5 (g)	Mean	SD
S+CBZ-2D							
0 Days	241.9	233.9	224.8	-*	-*	**233.53**	8.6
2 Days	237.8	223.2	227.9	-*	-*	**229.63**	7.5
S+CBZ-7D							
0 Days	231.3	215.0	205.5	-*	-*	**217.27**	13.0
7 Days	245.1	268.1	240.6	-*	-*	**251.27**	14.8
S+CBZ-14D							
0 Days	234.7	236.8	208.2	-*	-*	**226.57**	15.9
14 Days	300.4	306.8	271.1	-*	-*	**292.77**	19.0
S+CBZ-28D							
0 Days	220.3	221.3	218.6	205.7	226.0	**218.38**	7.6
14 Days	253.7	272.8	288.3	278.1	311.3	**280.84**	21.2
28 Days	300.8	273.7	328.9	322.8	352.4	**315.72**	29.8
S+CBZ-42D							
0 Days	209.7	205.3	207.7	207.9	-**	**207.65**	1.8
14 Days	275.1	259.6	279.7	268.6	-**	**270.75**	8.7
28 Days	306.0	308.5	309.5	326.5	-**	**312.63**	9.4
42 Days	336.7	338.9	339.1	365.7	-**	**345.10**	13.8

SD = standard deviation; S = saline; CBZ = carbamazepine; D = days; * = only 3 rats available; ** = only 4 rats available

Appendix H-2: Rat weights of the INH, INH+LMs and INH+CBZ groups

Table H-4: Rat weights in grams of the INH group

Group (n = 5)	Rat 1 (g)	Rat 2 (g)	Rat 3 (g)	Rat 4 (g)	Rat 5 (g)	Mean	SD
INH-2D							
0 Days	231.2	235.8	234.6	235.9	-*	**234.38**	2.2
2 Days	247.3	240.8	240.7	243.8	-*	**243.15**	3.1
INH-7D							
0 Days	223.3	238.9	229.8	212.5	218.3	**224.56**	10.2
7 Days	266.5	244.0	251.8	267.7	247.6	**255.52**	10.9
INH-14D							
0 Days	210.0	218.1	217.7	233.7	241.8	**224.26**	13.0
14 Days	272.9	256.8	275.1	295.9	295.7	**279.28**	16.7
INH-28D							
0 Days	209.1	216.8	211.8	213.2	208.1	**211.74**	3.4
14 Days	277.8	286.0	282.9	296.5	277.9	**284.22**	7.7
28 Days	323.0	320.9	315.9	318.4	340.1	**323.66**	9.6
INH-42D							
0 Days	210.1	215.5	212.4	212.9	226.3	**215.44**	6.4
14 Days	283.1	279.5	271.2	276.8	299.2	**281.96**	10.6
28 Days	299.3	316.0	321.9	337.4	315.4	**318.00**	13.7
42 Days	358.1	359.9	337.3	372.9	355.7	**356.78**	12.8

SD = standard deviation; INH = isoniazid; D = days; * = only 4 rats available

Table H-5: Rat weights in grams of the INH+LMS group

Group (n = 5)	Rat 1 (g)	Rat 2 (g)	Rat 3 (g)	Rat 4 (g)	Rat 5 (g)	Mean	SD
INH+LMS-2D							
0 Days	222.7	212.6	224.6	221.3	-*	**220.30**	5.3
2 Days	223.5	218.7	236.3	227.8	-*	**226.58**	7.5
INH+LMS-7D							
0 Days	226.3	241.1	219.0	241.3	243.5	**234.24**	10.9
7 Days	258.3	241.0	260.4	273.8	243.5	**255.40**	13.4
INH+LMS-14D							
0 Days	206.2	211.4	207.3	207.4	202.5	**206.96**	3.2
14 Days	293.2	270.4	268.3	278.6	284.8	**279.06**	10.3
INH+LMS-28D							
0 Days	233.6	229.1	238.9	229.6	249.1	**236.06**	8.3
14 Days	309.7	314.5	278.5	310.7	286.5	**299.98**	16.3
28 Days	362.6	359.0	307.7	354.9	328.0	**342.44**	23.7
INH+LMS-42D							
0 Days	220.4	204.5	200.8	212.9	220.6	**211.84**	9.0
14 Days	262.1	289.1	298.2	306.4	281.7	**287.50**	17.0
28 Days	344.6	308.5	334.4	364.7	332.5	**336.94**	20.4
42 Days	384.2	334.3	365.0	370.6	403.5	**371.52**	25.6

SD = standard deviation; INH = isoniazid; LMS = levamisole; D = days; * = only 4 rats available

Table H-6: Rat weights in grams of the INH+CBZ group

Group (n = 5)	Rat 1 (g)	Rat 2 (g)	Rat 3 (g)	Rat 4 (g)	Rat 5 (g)	Mean	SD
INH+CBZ-2D							
0 Days	233.7	230.4	204.0	207.3	-*	**218.85**	15.4
2 Days	213.4	215.1	248.4	243.2	-*	**230.03**	18.4
INH+CBZ-7D							
0 Days	231.1	244.5	213.4	246.9	228.3	**232.84**	13.6
7 Days	272.8	231.5	253.8	251.8	268.3	**255.64**	16.2
INH+CBZ-14D							
0 Days	212.3	221.1	242.7	213.6	211.2	**220.18**	13.2
14 Days	298.7	294.2	277.5	277.7	286.7	**286.96**	9.6
INH+CBZ-28D							
0 Days	216.7	208.1	241.8	239.8	205.3	**222.34**	17.4
14 Days	318.3	264.2	259.1	328.4	272.8	**288.56**	32.3
28 Days	366.4	297.1	307.8	373.0	315.7	**332.00**	35.1
INH+CBZ-42D							
0 Days	203.2	206.0	204.0	202.5	-*	**203.93**	1.5
14 Days	258.1	258.3	258.6	263.9	-*	**259.73**	2.8
28 Days	300.5	297.8	306.4	304.7	-*	**302.35**	3.9
42 Days	330.4	328.0	326.9	329.5	-*	**328.70**	1.6

SD = standard deviation; INH = isoniazid; CBZ = carbamazepine; D = days; * = only 4 rats available

Appendix H-3: Rat weights of the NVP, NVP+LMs and NVP+CBZ groups

Table H-7: Rat weights in grams of the NVP group

Group (n = 5)	Rat 1 (g)	Rat 2 (g)	Rat 3 (g)	Rat 4 (g)	Rat 5 (g)	Mean	SD
NVP-2D							
0 Days	223.3	219.7	220.2	209.8	220.9	**218.78**	5.2
2 Days	219.3	216.0	238.0	240.3	233.5	**229.42**	11.1
NVP-7D							
0 Days	213.5	217.5	219.2	222.2	213.8	**217.24**	3.7
7 Days	249.8	244.2	242.5	244.6	247.8	**245.78**	3.0
NVP-14D							
0 Days	201.1	204.4	204.9	202.3	216.3	**205.80**	6.1
14 Days	283.0	299.8	286.2	290.9	282.9	**288.56**	7.1
NVP-28D							
0 Days	219.3	225.2	207.9	204.9	210.2	**213.50**	8.5
14 Days	259.9	269.3	286.1	269.6	263.4	**269.66**	10.1
28 Days	294.2	332.8	317.1	320.1	301.7	**313.18**	15.3
NVP-42D							
0 Days	223.6	232.8	247.0	228.5	248.1	**236.00**	11.0
14 Days	288.5	296.9	296.3	266.1	276.5	**284.86**	13.3
28 Days	332.7	326.5	323.8	323.3	293.1	**319.88**	15.4
42 Days	350.1	342.2	353.7	311.4	350.5	**341.58**	17.4

SD = standard deviation; NVP = nevirapine; D = days

Table H-8: Rat weights in grams of the NVP+LMS group

Group (n = 5)	Rat 1 (g)	Rat 2 (g)	Rat 3 (g)	Rat 4 (g)	Rat 5 (g)	Mean	SD
NVP+LMS-2D							
0 Days	238.8	226.1	208.4	226.3	-*	**224.90**	12.5
2 Days	210.7	238.8	229.0	226.4	-*	**226.23**	11.6
NVP+LMS-7D							
0 Days	244.0	236.5	241.8	222.5	202.7	**229.56**	17.2
7 Days	259.8	271.0	268.9	247.1	210.4	**251.44**	24.8
NVP+LMS-14D							
0 Days	240.7	201.7	230.9	246.4	232.7	**230.48**	17.3
14 Days	293.9	282.7	302.5	246.2	275.2	**280.10**	21.6
NVP+LMS-28D							
0 Days	229.5	229.0	233.7	229.4	241.8	**232.68**	5.4
14 Days	285.0	290.1	285.6	296.2	312.9	**293.96**	11.5
28 Days	344.4	320.4	323.7	358.7	344.4	**338.32**	16.0
NVP+LMS-42D							
0 Days	215.4	200.0	213.5	210.6	221.0	**211.90**	8.2
14 Days	265.4	262.7	261.1	267.9	273.7	**266.16**	5.0
28 Days	289.4	308.2	291.4	305.5	317.9	**302.48**	12.0
42 Days	301.4	324.6	315.2	343.3	344.7	**325.84**	18.5

SD = standard deviation; NVP = nevirapine; LMS = levamisole; D = days; * = only 4 rats available

Table H-9: Rat weights in grams of the NVP+CBZ group

Group (n = 5)	Rat 1 (g)	Rat 2 (g)	Rat 3 (g)	Rat 4 (g)	Rat 5 (g)	Mean	SD
NVP+CBZ-2D							
0 Days	218.7	220.5	231.2	223.3	222.4	**223.22**	4.8
2 Days	225.2	242.2	229.9	229.9	229.4	**231.38**	6.3
NVP+CBZ-7D							
0 Days	238.5	219.6	217.3	220.3	222.3	**234.40**	22.0
7 Days	217.2	220.2	236.1	251.0	248.5	**234.60**	15.6
NVP+CBZ-14D							
0 Days	229.9	205.3	248.9	222.7	204.9	**222.34**	18.4
14 Days	243.7	283.6	301.2	266.2	275.3	**274.00**	21.3
NVP+CBZ-28D							
0 Days	240.6	246.1	218.7	218.1	246.2	**233.94**	14.4
14 Days	306.4	300.4	274.8	318.3	285.2	**297.02**	17.2
28 Days	305.8	316.3	307.1	333.9	343.2	**312.26**	16.6
NVP+CBZ-42D							
0 Days	205.5	203.1	200.9	204.7	210.8	**205.00**	3.7
14 Days	260.7	225.9	248.5	248.0	255.4	**247.70**	13.3
28 Days	298.0	239.6	283.6	289.8	278.6	**249.48**	27.9
42 Days	326.3	269.4	304.9	319.9	289.3	**301.96**	23.1

SD = standard deviation; NVP = nevirapine; CBZ = carbamazepine; D = days

Appendix H-4: Rat weights of the PAR, PAR+LMS and PAR+CBZ groups

Table H-10: Rat weights in grams of the PAR group

Group (n = 5)	Rat 1 (g)	Rat 2 (g)	Rat 3 (g)	Rat 4 (g)	Rat 5 (g)	Mean	SD
PAR-2D							
0 Days	215.3	208.7	231.4	229.9	224.9	**222.04**	9.8
2 Days	231.5	233.8	215.5	230.8	247.2	**231.76**	11.3
PAR-7D							
0 Days	233.1	235.1	246.0	222.3	211.0	**229.50**	13.3
7 Days	260.6	277.6	263.3	273.2	245.8	**264.10**	12.4
PAR-14D							
0 Days	204.3	202.3	209.9	200.2	205.7	**204.48**	3.7
14 Days	249.8	280.6	263.2	267.1	274.7	**267.08**	11.8
PAR-28D							
0 Days	306.3	201.2	210.3	214.8	219.1	**210.34**	7.0
14 Days	265.9	272.3	277.3	264.6	276.1	**271.24**	5.8
28 Days	319.4	314.3	317.1	320.6	297.9	**313.18**	9.2
PAR-42D							
0 Days	218.5	208.3	209.1	231.0	200.7	**213.52**	11.6
14 Days	290.2	267.9	280.5	273.3	284.1	**279.20**	8.8
28 Days	339.3	318.2	316.3	324.0	337.5	**327.06**	10.8
42 Days	353.6	361.4	353.4	358.2	378.4	**361.00**	10.3

SD = standard deviation; PAR = paracetamol; D = days

Table H-11: Rat weights in grams of the PAR+LMS group

Group (n = 5)	Rat 1 (g)	Rat 2 (g)	Rat 3 (g)	Rat 4 (g)	Rat 5 (g)	Mean	SD
PAR+LMS-2D							
0 Days	222.6	243.8	235.0	242.8	-*	**236.05**	9.8
2 Days	215.6	236.4	238.6	233.4	-*	**231.00**	10.5
PAR+LMS-7D							
0 Days	247.2	218.8	207.2	206.8	237.1	**223.42**	18.1
7 Days	267.6	232.1	248.8	241.3	274.1	**252.78**	17.7
PAR+LMS-14D							
0 Days	212.5	214.2	208.8	200.7	200.5	**207.14**	6.7
14 Days	256.5	266.7	275.3	261.4	270.4	**266.04**	7.4
PAR+LMS-28D							
0 Days	245.5	229.8	226.7	238.1	222.1	**232.44**	9.3
14 Days	326.6	280.3	290.7	308.9	273.1	**295.92**	21.8
28 Days	320.3	321.6	375.7	360.9	310.5	**337.80**	28.7
PAR+LMS-42D							
0 Days	203.1	219.9	222.5	207.2	205.5	**211.64**	8.9
14 Days	229.2	266.0	268.5	268.0	263.8	**259.10**	16.8
28 Days	309.8	279.9	311.0	305.9	303.3	**301.98**	12.7
42 Days	321.6	304.1	339.5	329.3	325.4	**323.98**	13.0

SD = standard deviation; PAR = paracetamol; LMS = levamisole; D = days; * = only 4 rats available

Table H-12: Rat weights in grams of the PAR+CBZ group

Group (n = 5)	Rat 1 (g)	Rat 2 (g)	Rat 3 (g)	Rat 4 (g)	Rat 5 (g)	Mean	SD
PAR+CBZ-2D							
0 Days	221.6	215.7	214.7	215.3	200.2	**213.50**	7.9
2 Days	220.5	219.0	220.6	224.7	203.2	**217.60**	8.3
PAR+CBZ-7D							
0 Days	225.2	236.4	224.0	238.8	221.8	**229.24**	7.8
7 Days	271.9	246.8	246.5	264.3	251.9	**256.28**	11.3
PAR+CBZ-14D							
0 Days	202.5	216.0	201.2	217.7	-*	**209.35**	8.7
14 Days	263.2	244.8	260.3	250.7	-*	**254.75**	8.5
PAR+CBZ-28D							
0 Days	244.1	218.2	230.3	236.5	230.0	**213.82**	9.5
14 Days	290.9	273.1	317.1	291.6	292.2	**293.18**	15.7
28 Days	319.8	325.5	350.5	329.5	320.5	**329.16**	12.6
PAR+CBZ-42D							
0 Days	237.0	243.9	229.2	232.6	243.7	**237.28**	6.6
14 Days	295.3	307.4	300.3	292.6	318.2	**302.76**	10.3
28 Days	343.6	344.3	341.9	363.1	341.3	**346.84**	9.2
42 Days	367.4	370.0	337.6	377.0	385.1	**367.42**	18.0

SD = standard deviation; PAR = paracetamol; CBZ = carbamazepine; D = days; * = only 4 rats available

APPENDIX I: CYTOKINE CONCENTRATION RESULTS

Appendix I-1: IL-2 and IL-10 concentrations of the S, S+LMS and S+CBZ groups

Table I-1: IL-2 and IL-10 serum concentrations in pg/ml of the S group

Group (n = 3)	Cytokine	Rat 1 (pg/ml)	Rat 2 (pg/ml)	Rat 3 (pg/ml)	Mean	SD
Untreated						
0 Days	**IL-2**	66.098	63.229	67.056	**65.461**	1.99
	IL-10	29.742	31.962	31.549	**31.084**	1.18
S-2D						
2 Days	**IL-2**	71.840	70.405	82.364	**74.870**	6.53
	IL-10	26.784	31.120	31.962	**29.955**	2.78
S-7D						
7 Days	**IL-2**	83.322	76.624	71.840	**77.262**	5.77
	IL-10	34.978	34.921	33.811	**34.570**	0.66
S-14D						
14 Days	**IL-2**	73.754	85.236	73.754	**77.581**	6.63
	IL-10	41.834	33.262	31.962	**35.686**	5.36
S-28D						
28 Days	**IL-2**	73.624	80.452	82.364	**78.813**	4.59
	IL-10	29.002	37.140	31.222	**32.455**	4.21
S-42D						
42 Days	**IL-2**	80.930	70.883	71.362	**74.392**	5.67
	IL-10	30.692	30.482	34.922	**32.032**	2.51

SD = standard deviation; S = saline; D = days

Table I-2: IL-2 and IL-10 serum concentrations in pg/ml of the S+LMS group

Group (n = 3)	Cytokine	Rat 1 (pg/ml)	Rat 2 (pg/ml)	Rat 3 (pg/ml)	Mean	SD
S+LMS-2D						
2 Days	**IL-2**	60.383	63.730	64.566	**62.893**	2.21
	IL-10	31.506	29.822	30.383	**30.570**	0.86
S+LMS-7D						
7 Days	**IL-2**	62.056	62.893	63.730	**62.893**	0.84
	IL-10	31.484	29.260	33.189	**31.311**	1.97
S+LMS-14D						
14 Days	**IL-2**	72.934	54.526	60.382	**62.614**	9.40
	IL-10	31.506	39.362	26.454	**32.441**	6.50
S+LMS-28D						
28 Days	**IL-2**	61.638	68.331	67.495	**65.821**	3.65
	IL-10	29.822	31.506	28.138	**29.822**	1.68
S+LMS-42D						
42 Days	**IL-2**	65.404	72.098	70.842	**69.448**	3.56
	IL-10	25.892	29.822	32.066	**29.260**	3.13

SD = standard deviation; S = saline; LMS = levamisole; D = days

Table I-3: IL-2 and IL-10 serum concentrations in pg/ml of the S+CBZ group

Group (n = 3)	Cytokine	Rat 1 (pg/ml)	Rat 2 (pg/ml)	Rat 3 (pg/ml)	Mean	SD
S+CBZ-2D						
2 Days	**IL-2**	50.000	39.000	68.000	**52.333**	14.64
	IL-10	35.995	39.924	35.995	**37.305**	2.27
S+CBZ-7D						
7 Days	**IL-2**	107.000	118.000	-*	**112.500**	7.78
	IL-10	43.852	41.608	34.872	**40.111**	4.67
S+CBZ-14D						
14 Days	**IL-2**	125.000	131.000	106.500	**120.833**	12.77
	IL-10	37.118	39.924	43.292	**40.111**	3.09
S+CBZ-28D						
28 Days	**IL-2**	101.000	134.500	-*	**117.750**	23.69
	IL-10	44.414	33.750	43.852	**40.672**	6.00
S+CBZ-42D						
42 Days	**IL-2**	125.000	60.000	-*	**92.500**	45.96
	IL-10	33.190	33.190	41.608	**35.996**	4.86

SD = standard deviation; S = saline; CBZ = carbamazepine; D = days; * = sample out of range

Appendix I-2: IL-2 and IL-10 concentrations of the INH, INH+LMS and INH+CBZ groups

Table I-4: IL-2 and IL-10 serum concentrations in pg/ml of the INH groups

Group (n = 3)	Cytokine	Rat 1 (pg/ml)	Rat 2 (pg/ml)	Rat 3 (pg/ml)	Mean	SD
INH-2D						
2 Days	**IL-2**	71.840	70.884	68.969	**70.564**	1.46
	IL-10	35.720	31.222	23.834	**30.259**	6.00
INH-7D						
7 Days	**IL-2**	81.886	84.278	85.236	**83.800**	1.73
	IL-10	31.962	32.702	31.222	**31.962**	0.74
INH-14D						
14 Days	**IL-2**	80.452	71.840	82.843	**78.378**	5.79
	IL-10	30.482	37.880	29.002	**32.455**	4.76
INH-28D						
28 Days	**IL-2**	73.275	74.710	84.278	**77.421**	5.98
	IL-10	23.084	29.002	33.442	**28.509**	5.20
INH-42D						
42 Days	**IL-2**	76.624	74.710	65.621	**72.318**	5.88
	IL-10	28.120	41.580	33.262	**34.321**	6.79

SD = standard deviation; INH = isoniazid; D = days

Table I-5: IL-2 and IL-10 serum concentrations in pg/ml of the INH+LMS group

Group (n = 3)	Cytokine	Rat 1 (pg/ml)	Rat 2 (pg/ml)	Rat 3 (pg/ml)	Mean	SD
INH+LMS-2D						
2 Days	**IL-2**	63.000	78.000	86.000	**75.667**	11.68
	IL-10	39.082	38.240	32.628	**36.650**	3.51
INH+LMS-7D						
7 Days	**IL-2**	48.000	52.000	48.000	**49.333**	2.31
	IL-10	34.592	38.240	44.976	**39.269**	5.27
INH+LMS-14D						
14 Days	**IL-2**	33.000	48.000	60.000	**47.000**	13.53
	IL-10	42.170	34.312	41.608	**39.363**	4.38
INH+LMS-28D						
28 Days	**IL-2**	71.000	52.000	86.000	**69.667**	17.04
	IL-10	43.292	35.434	33.190	**37.305**	5.30
INH+LMS-42D						
42 Days	**IL-2**	44.000	52.000	41.000	**45.667**	5.69
	IL-10	35.434	41.608	37.118	**38.053**	3.19

SD = standard deviation; INH = isoniazid; LMS = levamisole; D = days

Table I-6: IL-2 and IL-10 serum concentrations in pg/ml of the INH+CBZ group

Group (n = 3)	Cytokine	Rat 1 (pg/ml)	Rat 2 (pg/ml)	Rat 3 (pg/ml)	Mean	SD
INH+CBZ-2D						
2 Days	**IL-2**	88.000	84.000	-*	**86.000**	2.83
	IL-10	41.608	38.802	44.976	**41.795**	3.09
INH+CBZ-7D						
7 Days	**IL-2**	62.000	62.000	188.000	**104.000**	72.75
	IL-10	38.802	33.750	33.190	**35.247**	3.09
INH+CBZ-14D						
14 Days	**IL-2**	163.000	194.000	150.000	**169.000**	22.61
	IL-10	37.118	37.680	38.240	**37.679**	0.56
INH+CBZ-28D						
28 Days	**IL-2**	79.000	61.000	46.000	**62.000**	16.52
	IL-10	36.556	39.924	39.362	**38.614**	1.80
INH+CBZ-42D						
42 Days	**IL-2**	50.000	49.000	34.000	**44.333**	8.96
	IL-10	29.260	31.506	34.312	**31.693**	2.53

SD = standard deviation; INH = isoniazid; CBZ = carbamazepine; D = days; * = sample out of range

Appendix I-3: IL-2 and IL-10 concentrations of the NVP, NVP+LMS and NVP+CBZ groups

Table I-7: IL-2 and IL-10 serum concentrations in pg/ml of the NVP group

Group (n = 3)	Cytokine	Rat 1 (pg/ml)	Rat 2 (pg/ml)	Rat 3 (pg/ml)	Mean	SD
NVP-2D						
2 Days	**IL-2**	77.850	75.667	76.146	**76.554**	1.15
	IL-10	36.692	35.660	31.962	**34.771**	2.49
NVP-7D						
7 Days	**IL-2**	78.059	87.150	81.408	**82.206**	4.60
	IL-10	37.548	32.702	39.643	**36.631**	3.56
NVP-14D						
14 Days	**IL-2**	82.138	68.332	68.750	**73.073**	7.85
	IL-10	42.730	30.944	30.944	**34.873**	6.80
NVP-28D						
28 Days	**IL-2**	73.770	81.302	60.382	**71.818**	10.60
	IL-10	35.715	42.450	40.766	**39.644**	3.50
NVP-42D						
42 Days	**IL-2**	67.076	59.546	59.128	**61.917**	4.47
	IL-10	48.342	41.889	35.434	**41.888**	6.45

SD = standard deviation; NVP = nevirapine; D = days

Table I-8: IL-2 and IL-10 serum concentrations in pg/ml of the NVP+LMS group

Group (n = 3)	Cytokine	Rat 1 (pg/ml)	Rat 2 (pg/ml)	Rat 3 (pg/ml)	Mean	SD
NVP+LMS-2D						
2 Days	**IL-2**	44.000	52.000	-*	**48.000**	5.66
	IL-10	36.838	35.715	38.802	**37.118**	1.56
NVP+LMS-7D						
7 Days	**IL-2**	59.500	46.000	56.000	**53.833**	7.01
	IL-10	38.240	35.996	44.976	**39.737**	4.67
NVP+LMS-14D						
14 Days	**IL-2**	56.000	53.500	56.000	**55.167**	1.44
	IL-10	34.312	34.872	37.680	**35.621**	1.80
NVP+LMS-28D						
28 Days	**IL-2**	34.000	59.000	66.500	**53.167**	17.02
	IL-10	38.240	32.628	34.312	**35.060**	2.88
NVP+LMS-42D						
42 Days	**IL-2**	32.000	26.000	37.000	**31.667**	2.51
	IL-10	36.276	33.470	36.556	**35.434**	1.71

SD = standard deviation; NVP = nevirapine; LMS = levamisole; D = days; * = sample out of range

Table I-9: IL-2 and IL-10 serum concentrations in pg/ml of the NVP+CBZ group

Group (n = 3)	Cytokine	Rat 1 (pg/ml)	Rat 2 (pg/ml)	Rat 3 (pg/ml)	Mean	SD
NVP+CBZ-2D						
2 Days	**IL-2**	68.000	73.000	-*	**70.500**	3.54
	IL-10	35.995	36.276	30.102	**34.124**	3.49
NVP+CBZ-7D						
7 Days	**IL-2**	57.000	68.000	-*	**62.500**	7.78
	IL-10	34.120	33.262	40.978	**36.120**	4.23
NVP+CBZ-14D						
14 Days	**IL-2**	62.500	118.000	93.000	**91.167**	27.80
	IL-10	44.414	32.406	35.834	**37.551**	6.19
NVP+CBZ-28D						
28 Days	**IL-2**	150.000	60.000	72.500	**94.167**	48.76
	IL-10	30.692	36.692	27.016	**31.467**	4.88
NVP+CBZ-42D						
42 Days	**IL-2**	150.000	144.000	-*	**147.000**	4.24
	IL-10	28.548	27.691	38.834	**31.691**	6.20

SD = standard deviation; NVP = nevirapine; CBZ = carbamazepine; D = days; * = sample out of range

Appendix I-4: IL-2 and IL-10 concentrations of the PAR, PAR+LMS and PAR+CBZ groups

Table I-10: IL-2 and IL-10 serum concentrations in pg/ml of the PAR group

Group (n = 3)	Cytokine	Rat 1 (pg/ml)	Rat 2 (pg/ml)	Rat 3 (pg/ml)	Mean	SD
PAR-2D						
2 Days	**IL-2**	66.240	67.495	66.240	**66.658**	0.72
	IL-10	27.577	26.735	31.225	**28.512**	2.39
PAR-7D						
7 Days	**IL-2**	62.894	55.362	63.730	**60.662**	4.61
	IL-10	22.526	33.750	28.700	**28.325**	5.62
PAR-14D						
14 Days	**IL-2**	59.965	56.199	57.873	**58.012**	1.89
	IL-10	28.138	27.576	31.786	**29.167**	2.29
PAR-28D						
28 Days	**IL-2**	59.546	62.894	57.036	**59.825**	2.94
	IL-10	28.138	27.858	32.066	**29.354**	2.35
PAR-42D						
42 Days	**IL-2**	72.934	61.220	62.056	**65.403**	6.54
	IL-10	26.454	27.576	32.348	**28.793**	3.13

SD = standard deviation; PAR = paracetamol; D = days

Table I-11: IL-2 and IL-10 serum concentrations in pg/ml of the PAR+LMS group

Group (n = 3)	Cytokine	Rat 1 (pg/ml)	Rat 2 (pg/ml)	Rat 3 (pg/ml)	Mean	SD
PAR+LMS-2D						
2 Days	**IL-2**	48.000	45.000	30.000	**41.000**	9.64
	IL-10	37.680	37.960	39.643	**38.428**	1.06
PAR+LMS-7D						
7 Days	**IL-2**	63.000	44.000	30.000	**45.667**	16.56
	IL-10	35.434	39.644	40.485	**38.521**	2.71
PAR+LMS-14D						
14 Days	**IL-2**	37.000	52.000	50.000	**46.333**	8.14
	IL-10	38.802	41.608	39.924	**40.111**	1.41
PAR+LMS-28D						
28 Days	**IL-2**	30.000	38.000	47.500	**38.500**	8.76
	IL-10	37.680	34.872	31.506	**34.686**	3.09
PAR+LMS-42D						
42 Days	**IL-2**	34.000	42.000	-*	**38.000**	5.66
	IL-10	34.872	27.016	-*	**30.944**	5.56

SD = standard deviation; PAR = paracetamol; LMS = levamisole; D = days; * = sample out of range

Table I-12: IL-2 and IL-10 serum concentrations in pg/ml of the PAR+CBZ group

Group (n = 3)	Cytokine	Rat 1 (pg/ml)	Rat 2 (pg/ml)	Rat 3 (pg/ml)	Mean	SD
PAR+CBZ-2D						
2 Days	**IL-2**	106.000	74.000	-*	**90.000**	22.63
	IL-10	33.262	32.978	46.658	**37.633**	7.82
PAR+CBZ-7D						
7 Days	**IL-2**	106.000	73.000	131.000	**103.333**	29.09
	IL-10	47.834	40.486	40.978	**43.099**	4.11
PAR+CBZ-14D						
14 Days	**IL-2**	238.000	213.000	-*	**225.500**	17.68
	IL-10	36.692	46.120	34.120	**38.977**	6.32
PAR+CBZ-28D						
28 Days	**IL-2**	63.000	51.000	-*	**57.000**	8.49
	IL-10	41.046	40.978	36.556	**39.527**	2.57
PAR+CBZ-42D						
42 Days	**IL-2**	109.000	207.000	257.000	**191.000**	75.29
	IL-10	39.262	44.406	38.406	**40.691**	3.25

SD = standard deviation; PAR = paracetamol; CBZ = carbamazepine; D = days; * = sample out of range

APPENDIX J: CD4 and CD8 COUNT RESULTS

Appendix J-1: CD4 and CD8 count results of the S, S+LMS and S+CBZ groups

Table J-1: CD4 and CD8 count results of the S group

Group (n = 3)	T-Ly	Rat 1	Rat 2	Rat 3	Mean	SD
Untreated						
0 Days	**CD4**	1.295	-*	3.155	**2.225**	1.32
	CD8	0.914	-*	1.934	**1.424**	0.72
S-2D						
2 Days	**CD4**	2.075	2.118	2.629	**2.274**	0.31
	CD8	1.100	1.414	1.525	**1.347**	0.22
S-7D						
7 Days	**CD4**	2.651	1.112	1.387	**1.717**	0.82
	CD8	1.677	0.843	0.679	**1.066**	0.54
S-14D						
14 Days	**CD4**	1.888	1.431	1.742	**1.687**	0.23
	CD8	1.427	1.051	1.035	**1.171**	0.22
S-28D						
28 Days	**CD4**	2.267	2.572	2.495	**2.445**	0.16
	CD8	1.886	1.543	1.319	**1.583**	0.29
S-42D						
42 Days	**CD4**	1.405	1.522	1.494	**1.474**	0.06
	CD8	0.925	0.576	0.852	**0.785**	0.18

T-Ly = T lymphocyte; SD = standard deviation; S = saline; D = days; CD = cluster of differentiation; * = no lymphocyte count

Table J-2: CD4 and CD8 count results of the S+LMS group

Group (n = 3)	T-Ly	Rat 1	Rat 2	Rat 3	Mean	SD
S+LMS-2D						
2 Days	**CD4**	2.203	-*	2.293	**2.248**	0.06
	CD8	1.560	-*	1.604	**1.582**	0.03
S+LMS-7D						
7 Days	**CD4**	-*	-*	2.093	**2.093**	0.00
	CD8	-*	-*	1.339	**1.339**	0.00
S+LMS-14D						
14 Days	**CD4**	2.152	0.711	2.063	**1.642**	0.81
	CD8	1.161	0.412	1.331	**0.968**	0.49
S+LMS-28D						
28 Days	**CD4**	2.056	0.997	2.026	**1.693**	0.60
	CD8	1.428	0.608	1.400	**1.145**	0.47
S+LMS-42D						
42 Days	**CD4**	1.985	2.265	2.535	**2.261**	0.28
	CD8	0.013	0.006	0.007	**0.009**	0.00

T-Ly = T lymphocyte; SD = standard deviation; S = saline; LMS = levamisole; D = days; CD = cluster of differentiation; * = no lymphocyte count

Table J-3: CD4 and CD8 count results of the S+CBZ group

Group (n = 3)	T-Ly	Rat 1	Rat 2	Rat 3	Mean	SD
S+CBZ-2D						
2 Days	**CD4**	1.759	-*	2.162	**1.961**	0.28
	CD8	1.290	-*	1.480	**1.385**	0.13
S+CBZ-7D						
7 Days	**CD4**	1.478	2.554	2.955	**2.329**	0.76
	CD8	0.950	1.764	1.522	**1.412**	0.42
S+CBZ-14D						
14 Days	**CD4**	2.799	2.876	2.429	**2.701**	0.24
	CD8	1.511	1.773	1.475	**1.587**	0.16
S+CBZ-28D						
28 Days	**CD4**	1.783	1.398	2.180	**1.787**	0.39
	CD8	0.002	0.003	0.000	**0.002**	0.00
S+CBZ-42D						
42 Days	**CD4**	1.620	1.964	1.603	**1.729**	0.20
	CD8	1.605	1.700	1.325	**1.543**	0.20

T-Ly = T lymphocyte; SD = standard deviation; S = saline; CBZ = carbamazepine; D = days; CD = cluster of differentiation; * = no lymphocyte count

Appendix J-2: CD4 and CD8 count results of the INH, INH+LMS and INH+CBZ groups

Table J-4: CD4 and CD8 count results of the INH group

Group (n = 3)	T-Ly	Rat 1	Rat 2	Rat 3	Mean	SD
INH-2D						
2 Days	**CD4**	2.044	1.499	2.303	**1.949**	0.41
	CD8	1.373	1.056	1.373	**1.267**	0.18
INH-7D						
7 Days	**CD4**	2.742	1.852	1.992	**2.196**	0.48
	CD8	1.639	1.205	0.991	**1.278**	0.33
INH-14D						
14 Days	**CD4**	2.318	1.855	2.673	**2.282**	0.41
	CD8	1.638	0.661	0.931	**1.077**	0.50
INH-28D						
28 Days	**CD4**	1.339	2.041	1.907	**1.762**	0.37
	CD8	1.225	1.139	1.350	**1.238**	0.11
INH-42D						
42 Days	**CD4**	1.488	1.686	1.332	**1.502**	0.18
	CD8	1.042	1.291	0.854	**1.063**	0.22

T-Ly = T lymphocyte; SD = standard deviation; INH = isoniazid; D = days; CD = cluster of differentiation

Table J-5: CD4 and CD8 count results of the INH+LMS group

Group (n = 3)	T-Ly	Rat 1	Rat 2	Rat 3	Mean	SD
INH+LMS-2D						
2 Days	**CD4**	2.631	1.949	2.815	**2.465**	0.46
	CD8	1.399	1.333	2.011	**1.581**	0.37
INH+LMS-7D						
7 Days	**CD4**	2.337	2.569	2.166	**2.358**	0.20
	CD8	0.939	1.628	1.061	**1.209**	0.37
INH+LMS-14D						
14 Days	**CD4**	2.700	1.732	2.399	**2.277**	0.50
	CD8	1.023	1.146	1.541	**1.237**	0.27
INH+LMS-28D						
28 Days	**CD4**	2.000	1.712	1.858	**1.857**	0.14
	CD8	1.297	1.139	1.556	**1.331**	0.21
INH+LMS-42D						
42 Days	**CD4**	1.571	1.897	1.623	**1.697**	0.18
	CD8	0.005	0.012	0.004	**0.007**	0.00

T-Ly = T lymphocyte; SD = standard deviation; INH = isoniazid; LMS = levamisole; D = days; CD = cluster of differentiation

Table J-6: CD4 and CD8 count results of all INH+CBZ groups

Group (n = 3)	T-Ly	Rat 1	Rat 2	Rat 3	Mean	SD
INH+CBZ-2D						
2 Days	**CD4**	2.422	2.623	2.290	**2.445**	0.17
	CD8	1.729	1.211	1.534	**1.491**	0.26
INH +CBZ-7D						
7 Days	**CD4**	2.970	1.741	2.059	**2.257**	0.64
	CD8	1.612	1.224	1.325	**1.387**	0.20
INH +CBZ-14D						
14 Days	**CD4**	2.496	2.090	1.588	**2.058**	0.46
	CD8	1.022	1.152	1.047	**1.074**	0.07
INH +CBZ-28D						
28 Days	**CD4**	2.007	2.759	1.358	**2.041**	0.70
	CD8	0.000	0.000	0.002	**0.002**	0.00
INH +CBZ-42D						
42 Days	**CD4**	2.213	1.750	2.619	**2.194**	0.43
	CD8	1.452	1.200	1.744	**1.465**	0.27

T-Ly = T lymphocyte; SD = standard deviation; INH = isoniazid; CBZ = carbamazepine; D = days; CD = cluster of differentiation

Appendix J-3: CD4 and CD8 count results of the NVP, NVP+LMS and NVP+CBZ groups

Table J-7: CD4 and CD8 count results of the NVP group

Group (n = 3)	T-Ly	Rat 1	Rat 2	Rat 3	Mean	SD
NVP-2D						
2 Days	**CD4**	1.184	0.958	-*	**1.071**	0.16
	CD8	0.668	0.518	-*	**0.593**	0.11
NVP-7D						
7 Days	**CD4**	1.396	1.899	1.447	**1.581**	0.28
	CD8	0.822	1.357	1.030	**1.070**	0.27
NVP-14D						
14 Days	**CD4**	1.416	1.348	1.514	**1.426**	0.08
	CD8	1.002	0.901	0.641	**0.848**	0.19
NVP-28D						
28 Days	**CD4**	1.738	1.425	2.116	**1.775**	0.35
	CD8	1.401	1.011	1.382	**1.264**	0.22
NVP-42D						
42 Days	**CD4**	1.368	0.958	1.353	**1.226**	0.23
	CD8	0.875	0.632	0.912	**0.806**	0.15

T-Ly = T lymphocyte; SD = standard deviation; NVP = nevirapine; D = days; CD = cluster of differentiation; * = no lymphocyte count

Table J-8: CD4 and CD8 count results of the NVP+LMS group

Group (n = 3)	T-Ly	Rat 1	Rat 2	Rat 3	Mean	SD
NVP+LMS-2D						
2 Days	**CD4**	1.424	-*	1.554	**1.489**	0.09
	CD8	0.844	-*	0.910	**0.877**	0.05
NVP+LMS-7D						
7 Days	**CD4**	1.765	1.204	1.018	**1.329**	0.39
	CD8	0.654	0.810	0.535	**0.666**	0.14
NVP+LMS-14D						
14 Days	**CD4**	3.204	1.080	2.765	**2.350**	1.12
	CD8	1.873	0.787	2.003	**1.554**	0.67
NVP+LMS-28D						
28 Days	**CD4**	1.100	1.702	1.102	**1.301**	0.35
	CD8	0.789	1.298	0.668	**0.918**	0.33
NVP+LMS-42D						
42 Days	**CD4**	1.033	1.596	1.277	**1.302**	0.28
	CD8	0.006	0.003	0.001	**0.003**	0.00

T-Ly = T lymphocyte; SD = standard deviation; NVP = nevirapine; LMS = levamisole; D = days; CD = cluster of differentiation; * = no lymphocyte count

Table J-9: CD4 and CD8 count results of the NVP+CBZ group

Group (n = 3)	T-Ly	Rat 1	Rat 2	Rat 3	Mean	SD
NVP+CBZ-2D						
2 Days	**CD4**	1.725	1.401	1.068	**1.398**	0.33
	CD8	1.022	0.788	0.559	**0.789**	0.23
NVP+CBZ-7D						
7 Days	**CD4**	1.421	1.011	1.680	**1.371**	0.34
	CD8	0.687	0.567	0.995	**0.749**	0.22
NVP+CBZ-14D						
14 Days	**CD4**	1.236	1.752	1.721	**1.570**	0.29
	CD8	0.702	0.943	1.045	**0.897**	0.18
NVP+CBZ-28D						
28 Days	**CD4**	1.539	1.400	1.420	**1.453**	0.07
	CD8	0.945	0.889	0.908	**0.914**	0.03
NVP+CBZ-42D						
42 Days	**CD4**	1.450	1.091	1.095	**1.212**	0.21
	CD8	1.112	0.608	0.627	**0.782**	0.29

T-Ly = T lymphocyte; SD = standard deviation; NVP = nevirapine; CBZ = carbamazepine; D = days; CD = cluster of differentiation

Appendix J-4: CD4 and CD8 count results of the PAR, PAR+LMS and PAR+CBZ groups

Table J-10: CD4 and CD8 count results of the PAR group

Group (n = 3)	T-Ly	Rat 1	Rat 2	Rat 3	Mean	SD
PAR-2D						
2 Days	**CD4**	3.041	-*	-*	**3.041**	0.00
	CD8	1.511	-*	-*	**1.511**	0.00
PAR-7D						
7 Days	**CD4**	2.175	2.709	2.712	**2.532**	0.31
	CD8	1.392	1.982	1.612	**1.662**	0.30
PAR-14D						
14 Days	**CD4**	1.835	1.999	1.142	**1.659**	0.46
	CD8	0.875	1.050	0.837	**0.921**	0.11
PAR-28D						
28 Days	**CD4**	1.761	1.807	1.964	**1.844**	0.11
	CD8	0.477	1.289	1.251	**1.006**	0.46
PAR-42D						
42 Days	**CD4**	1.648	1.348	1.599	**1.532**	0.16
	CD8	1.231	0.969	1.205	**1.135**	0.14

T-Ly = T lymphocyte; SD = standard deviation; PAR = paracetamol; D = days; CD = cluster of differentiation; * = no lymphocyte count

Table J-11: CD4 and CD8 count results of the PAR+LMS group

Group (n = 3)	T-Ly	Rat 1	Rat 2	Rat 3	Mean	SD
PAR+LMS-2D						
2 Days	**CD4**	2.503	1.621	3.024	**2.383**	0.71
	CD8	1.368	1.141	1.455	**1.321**	0.16
PAR+LMS-7D						
7 Days	**CD4**	3.108	2.154	1.483	**2.248**	0.82
	CD8	1.366	1.344	1.004	**1.238**	0.20
PAR+LMS-14D						
14 Days	**CD4**	2.345	2.587	2.854	**2.596**	0.25
	CD8	1.512	1.547	1.612	**1.557**	0.05
PAR+LMS-28D						
28 Days	**CD4**	2.188	1.231	2.315	**1.911**	0.59
	CD8	1.381	0.991	1.309	**1.227**	0.21
PAR+LMS-42D						
42 Days	**CD4**	1.439	1.122	2.470	**1.677**	0.71
	CD8	0.002	0.001	0.000	**0.001**	0.00

T-Ly = T lymphocyte; SD = standard deviation; PAR = paracetamol; LMS = levamisole; D = days; CD = cluster of differentiation

Table J-12: CD4 and CD8 count results of the PAR+CBZ group

Group (n = 3)	T-Ly	Rat 1	Rat 2	Rat 3	Mean	SD
PAR+CBZ-2D						
2 Days	**CD4**	1.781	2.765	2.338	**2.295**	0.49
	CD8	0.691	1.858	1.556	**1.368**	0.61
PAR+CBZ-7D						
7 Days	**CD4**	2.331	1.995	3.055	**2.460**	0.54
	CD8	1.173	1.135	1.829	**1.379**	0.39
PAR+CBZ-14D						
14 Days	**CD4**	1.838	2.083	1.820	**1.913**	0.15
	CD8	1.071	1.432	1.243	**1.248**	0.18
PAR+CBZ-28D						
28 Days	**CD4**	1.847	1.962	2.006	**1.938**	0.08
	CD8	1.087	0.871	1.423	**1.127**	0.28
PAR+CBZ-42D						
42 Days	**CD4**	1.933	1.624	2.049	**1.869**	0.22
	CD8	1.370	1.018	1.461	**1.283**	0.23

T-Ly = T lymphocyte; SD = standard deviation; PAR = paracetamol; CBZ = carbamazepine; D = days; CD = cluster of differentiation

APPENDIX K: IMMUNOGLOBULIN SERUM CONCENTRATION RESULTS

Appendix K-1: IgM and IgG serum concentrations of the S, S+LMS and S+CBZ groups

Table K-1: IgM and IgG serum concentrations in mg/ml of the S group

Group (n = 3)	Ig	Rat 1 (mg/ml)	Rat 2 (mg/ml)	Rat 3 (mg/ml)	Mean	SD
Untreated						
0 Days	**IgM**	0.095	0.106	0.126	**0.109**	0.02
	IgG	15.424	14.632	13.246	**14.434**	1.10
S-2D						
2 Days	**IgM**	0.112	0.144	0.057	**0.104**	0.04
	IgG	14.334	13.146	14.930	**14.137**	0.91
S-7D						
7 Days	**IgM**	0.066	0.110	0.154	**0.110**	0.04
	IgG	14.434	13.542	14.390	**14.302**	0.70
S-14D						
14 Days	**IgM**	0.142	0.093	0.096	**0.110**	0.03
	IgG	12.948	12.452	12.452	**12.617**	0.29
S-28D						
28 Days	**IgM**	0.111	0.069	0.043	**0.075**	0.03
	IgG	17.506	15.722	15.822	**16.350**	1.00
S-42D						
42 Days	**IgM**	0.036	0.058	0.042	**0.046**	0.01
	IgG	17.010	17.406	16.910	**17.109**	0.26

Ig = immunoglobulin; SD = standard deviation; S = saline; D= days

Table K-2: IgM and IgG serum concentrations in mg/ml of the S+LMS group

Group (n = 3)	Ig	Rat 1 (mg/ml)	Rat 2 (mg/ml)	Rat 3 (mg/ml)	Mean	SD
S+LMS-2D						
2 Days	**IgM**	0.039	0.041	0.041	**0.040**	0.00
	IgG	9.084	10.568	8.806	**9.486**	0.95
S+LMS-7D						
7 Days	**IgM**	0.060	0.026	0.049	**0.045**	0.02
	IgG	11.312	12.426	13.724	**12.487**	1.21
S+LMS-14D						
14Days	**IgM**	0.038	0.060	0.081	**0.059**	0.02
	IgG	10.200	13.630	12.054	**11.961**	1.72
S+LMS-28D						
28 Days	**IgM**	0.067	0.037	0.057	**0.053**	0.02
	IgG	13.168	10.876	12.240	**12.095**	1.15
S+LMS-42D						
42 Days	**IgM**	0.056	0.045	0.039	**0.046**	0.01
	IgG	12.612	14.464	11.962	**13.013**	1.30

Ig = immunoglobulin; SD = standard deviation; S = saline; LMS = levamisole; D = days

Table K-3: IgM and IgG serum concentrations in mg/ml of the S+CBZ group

Group (n = 3)	Ig	Rat 1 (mg/ml)	Rat 2 (mg/ml)	Rat 3 (mg/ml)	Mean	SD
S+CBZ-2D						
2 Days	**IgM**	0.090	0.113	0.117	**0.107**	0.01
	IgG	11.164	8.880	9.424	**9.823**	1.19
S+CBZ-7D						
7 Days	**IgM**	0.109	0.105	0.111	**0.108**	0.00
	IgG	9.208	8.120	8.120	**8.483**	0.63
S+CBZ-14D						
14 Days	**IgM**	0.090	0.080	0.081	**0.083**	0.01
	IgG	9.316	7.904	12.032	**9.751**	2.10
S+CBZ-28D						
28 Days	**IgM**	0.082	0.091	0.080	**0.084**	0.01
	IgG	11.488	13.662	9.424	**11.525**	2.12
S+CBZ-42D						
42 Days	**IgM**	0.080	0.064	0.068	**0.071**	0.01
	IgG	10.728	14.204	11.488	**12.140**	1.83

Ig = immunoglobulin; SD = standard deviation; S = saline; CBZ = carbamazepine; D = days

Appendix K-2: IgM and IgG serum concentrations of the INH, INH+LMS and INH+CBZ groups

Table K-4: IgM and IgG serum concentrations in mg/ml of the INH group

Group (n = 3)	Ig	Rat 1 (mg/ml)	Rat 2 (mg/ml)	Rat 3 (mg/ml)	Mean	SD
INH-2D						
2 Days	**IgM**	0.083	0.028	0.058	**0.057**	0.03
	IgG	12.652	13.246	12.650	**12.849**	0.34
INH-7D						
7 Days	**IgM**	0.061	0.018	0.041	**0.040**	0.02
	IgG	15.228	14.534	14.532	**14.765**	0.40
INH-14D						
14 Days	**IgM**	0.039	0.053	0.046	**0.046**	0.01
	IgG	13.742	11.758	11.462	**12.321**	1.24
INH-28D						
28 Days	**IgM**	0.022	0.036	0.031	**0.029**	0.01
	IgG	12.948	16.612	11.560	**13.707**	2.61
INH-42D						
42 Days	**IgM**	0.020	0.018	0.045	**0.027**	0.01
	IgG	17.308	19.190	18.398	**18.299**	0.94

Ig = immunoglobulin; SD = standard deviation; INH = isoniazid; D = days

Table K-5: IgM and IgG serum concentrations in mg/ml of the INH+LMS group

Group (n = 3)	Ig	Rat 1 (mg/ml)	Rat 2 (mg/ml)	Rat 3 (mg/ml)	Mean	SD
INH+LMS-2D						
2 Days	**IgM**	0.101	0.093	0.083	**0.092**	0.01
	IgG	13.510	11.422	12.852	**12.595**	1.07
INH+LMS-7D						
7 Days	**IgM**	0.108	0.091	0.170	**0.123**	0.04
	IgG	12.410	14.830	10.872	**12.704**	2.00
INH+LMS-14D						
14 Days	**IgM**	0.110	0.053	0.093	**0.085**	0.03
	IgG	11.422	12.960	8.564	**10.982**	2.23
INH+LMS-28D						
28 Days	**IgM**	0.119	0.157	0.131	**0.135**	0.02
	IgG	15.160	11.640	14.720	**13.840**	1.92
INH+LMS-42D						
42 Days	**IgM**	0.071	0.075	0.103	**0.083**	0.02
	IgG	12.522	16.150	15.820	**14.831**	2.01

Ig = immunoglobulin; SD = standard deviation; INH = isoniazid; LMS = levamisole; D = days

Table K-6: IgM and IgG serum concentrations in mg/ml of the INH+CBZ group

Group (n = 3)	Ig	Rat 1 (mg/ml)	Rat 2 (mg/ml)	Rat 3 (mg/ml)	Mean	SD
INH+CBZ-2D						
2 Days	**IgM**	0.116	0.056	0.102	**0.091**	0.03
	IgG	8.446	8.554	7.468	**8.156**	0.60
INH+CBZ-7D						
7 Days	**IgM**	0.083	0.069	0.048	**0.066**	0.02
	IgG	8.228	8.336	8.120	**8.228**	0.11
INH+CBZ-14D						
14 Days	**IgM**	0.029	0.043	0.061	**0.044**	0.02
	IgG	7.794	8.662	9.750	**8.735**	0.98
INH+CBZ-28D						
28 Days	**IgM**	0.012	0.071	0.035	**0.039**	0.03
	IgG	9.424	10.944	9.424	**9.931**	0.88
INH+CBZ-42D						
42 Days	**IgM**	0.048	0.075	0.025	**0.049**	0.03
	IgG	10.620	8.336	8.428	**9.128**	1.29

Ig = immunoglobulin; SD = standard deviation; INH = isoniazid; CBZ = carbamazepine; D = days

Appendix K-3: IgM and IgG serum concentrations of the NVP, NVP+LMS and NVP+CBZ groups

Table K-7: IgM and IgG serum concentrations in mg/ml of the NVP group

Group (n = 3)	Ig	Rat 1 (mg/ml)	Rat 2 (mg/ml)	Rat 3 (mg/ml)	Mean	SD
NVP-2D						
2 Days	**IgM**	0.016	0.016	0.001	**0.011**	0.01
	IgG	15.820	13.344	12.750	**13.971**	1.63
NVP-7D						
7 Days	**IgM**	0.008	0.011	0.018	**0.012**	0.00
	IgG	12.652	13.640	13.642	**13.311**	0.57
NVP-14D						
14 Days	**IgM**	0.142	0.092	0.114	**0.116**	0.03
	IgG	13.724	12.054	11.774	**12.517**	1.05
NVP-28D						
28 Days	**IgM**	0.094	0.104	0.063	**0.087**	0.02
	IgG	11.498	12.516	12.052	**12.022**	0.51
NVP-42D						
42 Days	**IgM**	0.054	0.134	0.080	**0.089**	0.04
	IgG	17.620	18.364	14.188	**16.724**	2.23

Ig = immunoglobulin; SD = standard deviation; NVP = nevirapine; D = days

Table K-8: IgM and IgG serum concentrations in mg/ml of the NVP+LMS group

Group (n = 3)	Ig	Rat 1 (mg/ml)	Rat 2 (mg/ml)	Rat 3 (mg/ml)	Mean	SD
NVP+LMS-2D						
2 Days	**IgM**	0.049	0.046	0.040	**0.045**	0.00
	IgG	12.140	11.202	10.324	**11.222**	0.91
NVP+LMS-7D						
7 Days	**IgM**	0.090	0.083	0.066	**0.080**	0.01
	IgG	14.390	13.400	12.080	**13.290**	1.16
NVP+LMS-14D						
14 Days	**IgM**	0.016	0.036	0.033	**0.028**	0.01
	IgG	10.762	10.324	10.214	**10.433**	0.29
NVP+LMS-28D						
28 Days	**IgM**	0.001	0.024	0.016	**0.014**	0.01
	IgG	10.212	13.620	11.860	**11.897**	1.70
NVP+LMS-42D						
42 Days	**IgM**	0.051	0.022	0.011	**0.028**	0.02
	IgG	11.640	14.610	12.740	**12.997**	1.50

Ig = immunoglobulin; SD = standard deviation; NVP = nevirapine; LMS = levamisole; D = days

Table K-9: IgM and IgG serum concentrations in mg/ml of the NVP+CBZ group

Group (n = 3)	Ig	Rat 1 (mg/ml)	Rat 2 (mg/ml)	Rat 3 (mg/ml)	Mean	SD
NVP+CBZ-2D						
2 Days	**IgM**	0.025	0.032	0.037	**0.031**	0.01
	IgG	11.596	13.768	13.116	**12.827**	1.11
NVP+CBZ-7D						
7 Days	**IgM**	0.120	0.129	0.193	**0.147**	0.04
	IgG	9.096	6.318	7.540	**7.651**	1.39
NVP+CBZ-14D						
14 Days	**IgM**	0.077	0.111	0.100	**0.096**	0.02
	IgG	9.652	8.428	7.096	**8.392**	1.28
NVP+CBZ-28D						
28 Days	**IgM**	0.079	0.063	0.075	**0.072**	0.01
	IgG	8.208	9.320	12.876	**10.135**	2.44
NVP+CBZ-42D						
42 Days	**IgM**	0.118	0.044	0.061	**0.074**	0.04
	IgG	8.208	10.876	7.984	**9.023**	1.61

Ig = immunoglobulin; SD = standard deviation; NVP = nevirapine; CBZ = carbamazepine; D = days

Appendix K-4: IgM and IgG serum concentrations of the PAR, PAR+LMS and PAR+CBZ groups

Table K-10: IgM and IgG serum concentrations in mg/ml of the PAR group

Group (n = 3)	Ig	Rat 1 (mg/ml)	Rat 2 (mg/ml)	Rat 3 (mg/ml)	Mean	SD
PAR-2D						
2 Days	**IgM**	0.146	0.122	0.100	**0.123**	0.02
	IgG	11.590	11.590	12.054	**11.745**	0.27
PAR-7D						
7 Days	**IgM**	0.076	0.086	0.100	**0.088**	0.01
	IgG	13.352	9.362	12.796	**11.837**	2.16
PAR-14D						
14 Days	**IgM**	0.092	0.067	0.114	**0.091**	0.02
	IgG	14.836	11.124	12.796	**12.919**	1.86
PAR-28D						
28 Days	**IgM**	0.065	0.086	0.071	**0.074**	0.01
	IgG	15.952	12.426	11.220	**13.199**	2.46
PAR-42D						
42 Days	**IgM**	0.060	0.049	0.062	**0.057**	0.01
	IgG	13.538	12.424	13.444	**13.135**	0.62

Ig = immunoglobulin; SD = standard deviation; PAR = paracetamol; D = days

Table K-11: IgM and IgG serum concentrations in mg/ml of the PAR+LMS group

Group (n = 3)	Ig	Rat 1 (mg/ml)	Rat 2 (mg/ml)	Rat 3 (mg/ml)	Mean	SD
PAR+LMS-2D						
2 Days	**IgM**	0.066	0.065	0.016	**0.049**	0.03
	IgG	9.664	10.104	9.774	**9.847**	0.23
PAR+LMS-7D						
7 Days	**IgM**	0.025	0.034	0.057	**0.039**	0.02
	IgG	9.774	9.772	9.884	**9.810**	0.06
PAR+LMS-14D						
14 Days	**IgM**	0.049	0.052	0.039	**0.047**	0.01
	IgG	11.640	12.740	12.522	**12.301**	0.58
PAR+LMS-28D						
28 Days	**IgM**	0.109	0.051	0.146	**0.102**	0.05
	IgG	9.098	6.652	5.096	**6.949**	2.02
PAR+LMS-42D						
42 Days	**IgM**	0.126	0.081	0.116	**0.108**	0.02
	IgG	13.334	13.988	14.746	**14.023**	0.71

Ig = immunoglobulin; SD = standard deviation; PAR = paracetamol; LMS = levamisole; D = days

Table K-12: IgM and IgG serum concentrations in mg/ml of the PAR+CBZ group

Group (n = 3)	Ig	Rat 1 (mg/ml)	Rat 2 (mg/ml)	Rat 3 (mg/ml)	Mean	SD
PAR+CBZ-2D						
2 Days	**IgM**	0.135	0.130	0.086	**0.117**	0.03
	IgG	8.542	9.320	8.764	**8.875**	0.40
PAR+CBZ-7D						
7 Days	**IgM**	0.110	0.098	0.107	**0.105**	0.01
	IgG	8.096	8.318	7.764	**8.059**	0.28
PAR+CBZ-14D						
14 Days	**IgM**	0.096	0.103	0.094	**0.097**	0.00
	IgG	9.096	8.096	7.096	**8.096**	1.00
PAR+CBZ-28D						
28 Days	**IgM**	0.076	0.086	0.097	**0.086**	0.01
	IgG	9.208	9.542	7.320	**8.690**	1.20
PAR+CBZ-42D						
42 Days	**IgM**	0.148	0.096	0.105	**0.116**	0.03
	IgG	8.652	8.652	10.098	**9.134**	0.83

Ig = immunoglobulin; SD = standard deviation; PAR = paracetamol; CBZ = carbamazepine; D = days

APPENDIX L: MICROSOMAL PROTEIN CONCENTRATIONS

Appendix L-1: Microsomal protein concentrations of untreated group from selected rat livers

Table L-1: Protein concentrations in mg/ml of the untreated group at 0 days

Group (n = 3)	Conc. 1 (mg/ml)	Conc. 2 (mg/ml)	Mean	SD	Abs. 1 (nm)	Abs. 2 (nm)	Mean	SD
Untreated								
Rat 1	44.580	54.210	**49.395**	6.81	0.120	0.144	**0.132**	0.02
Rat 2	54.210	55.820	**55.015**	1.14	0.144	0.148	**0.146**	0.00
Rat 4	54.610	47.390	**51.000**	5.11	0.145	0.127	**0.136**	0.01

Conc. = concentration; Abs. = absorption; SD = standard deviation

Appendix L-2: Microsomal protein concentrations of the INH and INH+CBZ groups from selected rat livers

Table L-2: Protein concentrations in mg/ml of the INH group after 2, 7 and 14 days

Group (n = 3)	Conc. 1 (mg/ml)	Conc. 2 (mg/ml)	Mean	SD	Abs. 1 (nm)	Abs. 2 (nm)	Mean	SD
INH-2D								
Rat 1	32.330	29.770	**31.050**	1.81	0.062	0.056	**0.059**	0.00
Rat 2	38.730	38.310	**38.520**	0.30	0.077	0.076	**0.077**	0.00
Rat 3	54.100	49.410	**51.755**	3.32	0.113	0.102	**0.108**	0.01
INH-7D								
Rat 1	31.300	31.690	**31.495**	0.28	0.077	0.078	**0.078**	0.00
Rat 3	40.650	43.760	**42.205**	2.20	0.101	0.109	**0.105**	0.01
Rat 5	41.820	38.310	**40.065**	2.48	0.104	0.095	**0.100**	0.01
INH-14D								
Rat 1	20.230	19.770	**20.000**	0.33	0.051	0.050	**0.051**	0.00
Rat 4	23.450	24.370	**23.910**	0.65	0.058	0.060	**0.059**	0.00
Rat 5	36.800	32.660	**34.730**	2.93	0.087	0.078	**0.083**	0.01

Conc. = concentration; Abs. = absorption; SD = standard deviation; INH = isoniazid; D = days

Table L-3: Protein concentrations in mg/ml of the INH+CBZ group after 2, 7 and 14 days

Group (n = 3)	Conc. 1 (mg/ml)	Conc. 2 (mg/ml)	Mean	SD	Abs. 1 (nm)	Abs. 2 (nm)	Mean	SD
INH+CBZ-2D								
Rat 2	19.170	16.220	**17.695**	2.09	0.038	0.031	**0.035**	0.00
Rat 3	31.480	29.770	**30.625**	1.21	0.060	0.056	**0.058**	0.00
Rat 4	31.050	31.480	**31.265**	0.30	0.059	0.060	**0.060**	0.00
INH+CBZ-7D								
Rat 2	38.310	37.920	**38.115**	0.28	0.095	0.094	**0.095**	0.00
Rat 4	35.980	27.570	**31.775**	5.95	0.078	0.058	**0.068**	0.01
Rat 5	44.150	35.980	**40.065**	5.78	0.110	0.089	**0.100**	0.01
INH+CBZ-14D								
Rat 2	61.210	54.760	**57.985**	4.56	0.140	0.126	**0.133**	0.01
Rat 3	47.390	51.080	**49.235**	2.61	0.110	0.118	**0.114**	0.01
Rat 4	46.490	46.910	**46.700**	0.30	0.103	0.104	**0.104**	0.00

Conc. = concentration; Abs. = absorption; SD = standard deviation; INH = isoniazid; CBZ = carbamazepine; D = days

Appendix L-3: Microsomal protein concentrations of the NVP and NVP+CBZ groups from selected rat livers

Table L-4: Protein concentrations in mg/ml of the NVP group after 2, 7 and 14 days

Group (n = 3)	Conc. 1 (mg/ml)	Conc. 2 (mg/ml)	Mean	SD	Abs. 1 (nm)	Abs. 2 (nm)	Mean	SD
NVP-2D								
Rat 2	46.510	41.500	**44.005**	3.54	0.092	0.082	**0.087**	0.01
Rat 3	62.890	47.750	**55.320**	10.71	0.142	0.106	**0.124**	0.03
Rat 4	67.510	55.740	**61.625**	8.32	0.153	0.125	**0.139**	0.02
NVP-7D								
Rat 2	47.810	40.090	**43.950**	5.46	0.085	0.071	**0.078**	0.01
Rat 3	57.730	51.670	**54.700**	4.29	0.103	0.092	**0.098**	0.01
Rat 5	53.870	46.160	**50.015**	5.45	0.096	0.082	**0.089**	0.01
NVP-14D								
Rat 2	54.170	59.530	**56.850**	3.79	0.109	0.120	**0.115**	0.01
Rat 3	50.820	58.140	**54.480**	5.18	0.104	0.120	**0.112**	0.01
Rat 5	52.710	48.330	**50.520**	3.10	0.106	0.097	**0.102**	0.01

Conc. = concentration; Abs. = absorption; SD = standard deviation; NVP = nevirapine; D = days

Table L-5: Protein concentrations in mg/ml of the NVP+CBZ group after 2, 7 and 14 days

Group (n = 3)	Conc. 1 (mg/ml)	Conc. 2 (mg/ml)	Mean	SD	Abs. 1 (nm)	Abs. 2 (nm)	Mean	SD
NVP+CBZ-2D								
Rat 2	64.350	60.840	**62.595**	2.48	0.133	0.126	**0.130**	0.00
Rat 3	75.550	72.540	**74.045**	2.13	0.150	0.144	**0.147**	0.00
Rat 5	54.020	54.020	**54.020**	0.00	0.107	0.107	**0.107**	0.00
NVP+CBZ-7D								
Rat 2	78.870	67.850	**73.360**	7.79	0.162	0.140	**0.151**	0.02
Rat 3	82.880	85.380	**84.130**	1.77	0.170	0.175	**0.173**	0.00
Rat 4	63.790	66.000	**64.895**	1.56	0.114	0.118	**0.116**	0.00
NVP+CBZ-14D								
Rat 1	52.710	56.610	**54.660**	2.76	0.106	0.114	**0.110**	0.01
Rat 2	71.220	67.330	**69.275**	2.75	0.144	0.136	**0.140**	0.01
Rat 5	57.220	65.920	**61.570**	6.15	0.118	0.137	**0.128**	0.01

Conc. = concentration; Abs. = absorption; SD = standard deviation; NVP = nevirapine; CBZ = carbamazepine; D = days

Appendix L-4: Microsomal protein concentrations of the PAR and PAR+CBZ groups from selected rat livers

Table L-6: Protein concentrations in mg/ml of the PAR group after 2, 7 and 14 days

Group (n = 3)	Conc. 1 (mg/ml)	Conc. 2 (mg/ml)	Mean	SD	Abs. 1 (nm)	Abs. 2 (nm)	Mean	SD
PAR-2D								
Rat 1	19.130	20.960	**20.045**	1.29	0.035	0.039	**0.037**	0.00
Rat 2	42.030	46.610	**44.320**	3.24	0.085	0.095	**0.090**	0.01
Rat 4	27.380	30.580	**28.980**	2.26	0.053	0.060	**0.057**	0.00
PAR-7D								
Rat 1	42.540	36.860	**39.700**	4.02	0.080	0.068	**0.074**	0.01
Rat 2	32.140	26.930	**29.535**	3.68	0.058	0.047	**0.053**	0.01
Rat 5	33.550	26.930	**30.240**	4.68	0.061	0.047	**0.054**	0.01
PAR-14D								
Rat 1	40.080	26.850	**33.465**	9.36	0.088	0.060	**0.074**	0.02
Rat 3	38.660	33.460	**36.060**	3.68	0.085	0.074	**0.080**	0.01
Rat 4	25.430	32.520	**28.975**	5.01	0.057	0.072	**0.065**	0.01

Conc. = concentration; Abs. = absorption; SD = standard deviation; PAR = paracetamol; D = days

Table L-7: Protein concentrations in mg/ml of the PAR+CBZ group at 2, 7 and 14 days

Group (n = 3)	Conc. 1 (mg/ml)	Conc. 2 (mg/ml)	Mean	SD	Abs. 1 (nm)	Abs. 2 (nm)	Mean	SD
PAR+CBZ-2D								
Rat 2	32.410	30.580	**31.495**	1.29	0.064	0.060	**0.062**	0.00
Rat 3	24.630	23.250	**23.940**	0.98	0.047	0.044	**0.046**	0.00
Rat 5	49.820	45.690	**47.755**	2.92	0.102	0.093	**0.098**	0.01
PAR+CBZ-7D								
Rat 3	45.380	45.850	**45.615**	0.33	0.086	0.087	**0.087**	0.00
Rat 4	53.420	57.680	**55.550**	3.01	0.103	0.112	**0.108**	0.01
Rat 5	24.570	28.350	**26.460**	2.67	0.042	0.050	**0.046**	0.01
PAR+CBZ-14D								
Rat 2	27.320	31.570	**29.445**	3.01	0.061	0.070	**0.066**	0.01
Rat 3	35.830	39.130	**37.480**	2.33	0.079	0.086	**0.083**	0.00
Rat 4	32.990	34.880	**33.935**	1.34	0.073	0.077	**0.075**	0.00

Conc. = concentration; Abs. = absorption; SD = standard deviation; PAR = paracetamol; CBZ = carbamazepine; D = days

APPENDIX M: CYP450 ACTIVITY IN VIVO

Appendix M-1: The effect of INH and INH+CBZ on CYP450 activity *in vivo*

Table M-1: CYP1A2 reaction rates in pmol/min*mg of the untreated, INH and INH+CBZ groups after 2, 7 and 14 days

Group (n = 3)	RF Conc. (pmol/ml)	RR (pmol/min*mg)	Mean RR/rat (pmol/min*mg)	SD	Mean RR/group (pmol/min*mg)	SD
Untreated						
Rat 2a	8.650	4.325	4.403	0.80	**4.403**	0.80
Rat 2b	8.959	4.480				
INH-2D						
Rat 1a	9.144	4.572	4.712	0.16	**4.998**	0.25
Rat 1b	9.703	4.852				
Rat 2a	10.355	5.178	5.109	0.21		
Rat 2b	10.083	5.041				
Rat 3a	10.076	5.038	5.172	0.18		
Rat 3b	10.613	5.307				
INH-7D						
Rat 1a	15.913	7.956	8.675	1.00	**8.252**	0.43
Rat 1b	18.789	9.394				
Rat 3a	18.230	9.115	8.255	1.13		
Rat 3b	14.790	7.395				
Rat 5a	15.886	7.943	7.825	0.43		
Rat 5b	15.416	7.708				
INH-14D						
Rat 1a	23.563	11.782	11.115	0.79	**10.115**	1.10
Rat 1b	20.897	10.448				
Rat 4a	18.344	9.172	10.293	1.37		
Rat 4b	22.827	11.413				
Rat 5a	15.720	7.860	8.936	1.52		
Rat 5b	20.022	10.011				
INH+CBZ-2D						
Rat 2a	9.612	4.806	4.948	0.20	**4.365**	0.70
Rat 2b	10.181	5.091				
Rat 3a	8.179	4.089	4.557	0.65		
Rat 3b	10.049	5.025				
Rat 4a	7.219	3.610	3.591	0.50		
Rat 4b	7.145	3.572				
INH+CBZ-7D						
Rat 2a	18.509	9.255	9.402	0.21	**9.777**	0.42
Rat 2b	19.100	9.550				
Rat 4a	17.640	8.820	9.703	1.16		
Rat 4b	21.171	10.585				
Rat 5a	20.447	10.224	10.226	0.00		
Rat 5b	20.455	10.228				
INH+CBZ-14D						
Rat 2a	18.931	9.465	10.464	1.25	**10.195**	0.93
Rat 2b	22.925	11.463				
Rat 3a	19.076	9.538	9.165	0.59		
Rat 3b	17.583	8.792				
Rat 4a	19.628	9.814	10.957	1.62		
Rat 4b	24.198	12.099				

RF = resorufin; Conc. = concentration; RR = reaction rate; SD = standard deviation; INH = isoniazid; CBZ = carbamazepine; D = days

Table M-2: CYP2E1 reaction rates in nmol/min*mg of the untreated, INH and INH+CBZ groups at 2, 7 and 14 days

Group (n = 3)	Ratio (Ar. 6-OH-CZN/ Ar. I.S)	Reaction rate (nmol/min*mg)	Mean RR/rat (nmol/min*mg)	SD	Mean RR/group (nmol/min*mg)	SD
Untreated						
Rat 2a	0.142	0.729	0.772	0.06	**0.772**	0.06
Rat 2b	0.155	0.816				
INH-2D						
Rat 1a	0.158	0.837	0.998	0.23	**1.083**	0.23
Rat 1b	0.206	1.160				
Rat 2a	0.163	0.870	0.907	0.05		
Rat 2b	0.174	0.944				
Rat 3a	0.179	0.978	1.345	0.52		
Rat 3b	0.288	1.712				
INH-7D						
Rat 1a	0.237	1.613	1.613	0.00	**1.303**	0.33
Rat 1b	0.237	1.613				
Rat 3a	0.166	1.206	0.954	0.36		
Rat 3b	0.082	0.703				
Rat 5a	0.125	0.960	1.341	0.54		
Rat 5b	0.252	1.722				
INH-14D						
Rat 1a	0.534	3.414	3.399	0.02	**2.404**	1.37
Rat 1b	0.529	3.384				
Rat 4a	0.533	3.408	2.976	0.61		
Rat 4b	0.389	2.544				
Rat 5a	0.124	0.838	0.838	0.00		
Rat 5b	0.124	0.838				
INH+CBZ-2D						
Rat 2a	0.298	1.779	1.779	0.00	**1.219**	0.50
Rat 2b	0.298	1.779				
Rat 3a	0.178	0.971	0.877	0.13		
Rat 3b	0.150	0.783				
Rat 4a	0.161	0.857	0.981	0.18		
Rat 4b	0.198	1.106				
INH+CBZ-7D						
Rat 2a	-*	-*	**-***	-*	**1.221**	0.24
Rat 2b	-*	-*				
Rat 4a	0.197	1.392	1.392	0.00		
Rat 4b	0.197	1.392				
Rat 5a	0.140	1.050	1.050	0.00		
Rat 5b	0.140	1.050				
INH+CBZ-14D						
Rat 2a	-*	-*	**-***	-*	**1.256**	0.27
Rat 2b	-*	-*				
Rat 3a	0.231	1.596	1.449	0.21		
Rat 3b	0.182	1.302				
Rat 4a	0.184	1.314	1.062	0.36		
Rat 4b	0.100	0.810				

6-OH-CZN = 6-hydroxychlorzoxazone; RR = reaction rate; SD = standard deviation; INH = isoniazid; CBZ = carbamazepine; D = days; * = reading out of range

Table M-3: CYP3A2 reaction rates in pmol/min*mg of the untreated, INH and INH+CBZ groups at 2, 7 and 14 days

Group (n = 3)	Ratio (Ar. 1-OH-MDZ/ Ar. I.S)	Reaction rate (pmol/min*mg)	Mean RR/rat (pmol/min*mg)	SD	Mean RR/group (pmol/min*mg)	SD
Untreated						
Rat 2a	0.153	89.500	84.625	6.89	**84.625**	6.89
Rat 2b	0.137	79.750				
INH-2D						
Rat 1a	0.126	73.000	73.000	0.00	**75.938**	6.55
Rat 1b	0.126	73.000				
Rat 2a	0.163	95.750	83.438	17.41		
Rat 2b	0.123	71.125				
Rat 3a	0.120	69.250	71.375	3.01		
Rat 3b	0.127	73.500				
INH-7D						
Rat 1a	0.143	109.125	102.063	9.99	**87.907**	20.02
Rat 1b	0.124	95.000				
Rat 3a	0.100	77.125	73.750	4.77		
Rat 3b	0.091	70.375				
Rat 5a	-*	-*	-*	-*		
Rat 5b	-*	-*				
INH-14D						
Rat 1a	0.092	71.125	78.188	9.99	**74.094**	5.79
Rat 1b	0.111	85.250				
Rat 4a	0.088	68.125	70.000	2.65		
Rat 4b	0.093	71.875				
Rat 5a	-*	-*	-*	-*		
Rat 5b	-*	-*				
INH+CBZ-2D						
Rat 2a	0.125	52.500	52.500	0.00	**71.042**	17.44
Rat 2b	0.125	52.500				
Rat 3a	0.127	73.500	73.500	0.00		
Rat 3b	0.127	73.500				
Rat 4a	0.149	87.125	87.125	0.00		
Rat 4b	0.149	87.125				
INH+CBZ-7D						
Rat 2a	0.137	104.625	96.813	11.05	**80.469**	23.11
Rat 2b	0.116	89.000				
Rat 4a	-*	-*	-*	-*		
Rat 4b	-*	-*				
Rat 5a	0.153	64.125	64.125	0.00		
Rat 5b	0.153	64.125				
INH+CBZ-14D						
Rat 2a	0.078	60.750	60.750	0.00	**61.104**	2.05
Rat 2b	0.078	60.750				
Rat 3a	0.076	59.250	59.250	0.00		
Rat 3b	0.076	59.250				
Rat 4a	0.075	58.500	63.313	6.81		
Rat 4b	0.088	68.125				

1-OH-MDZ = 1-hydroxymidazolam; RR = reaction rate; SD = standard deviation; INH = isoniazid; CBZ = carbamazepine; D = days; * = reading out of range

Appendix M-2: The effect of NVP and NVP+CBZ on CYP450 activity *in vivo*

Table M-4: CYP1A2 reaction rates in pmol/min*mg of the untreated, NVP and NVP+CBZ groups after 2, 7 and 14 days

Group (n = 3)	RF Conc. (pmol/ml)	RR (pmol/min*mg)	Mean RR/rat (pmol/min*mg)	SD	Mean RR/group (pmol/min*mg)	SD
Untreated						
Rat 2a	8.650	4.325	4.403	0.80	**4.403**	0.80
Rat 2b	8.959	4.480				
NVP-2D						
Rat 2a	24.655	12.327	11.904	0.75	**13.625**	1.50
Rat 2b	22.961	11.480				
Rat 3a	29.027	14.514	14.614	0.14		
Rat 3b	29.429	14.715				
Rat 4a	27.404	13.702	14.358	0.93		
Rat 4b	30.028	15.014				
NVP-7D						
Rat 2a	15.420	7.710	7.175	0.76	**7.347**	1.09
Rat 2b	13.281	6.641				
Rat 3a	17.421	8.711	8.514	0.28		
Rat 3b	16.634	8.317				
Rat 5a	14.900	7.450	6.353	1.29		
Rat 5b	10.512	5.256				
NVP-14D						
Rat 2a	8.286	4.143	4.074	0.33	**5.308**	1.64
Rat 2b	8.010	4.005				
Rat 3a	14.002	7.001	7.172	0.24		
Rat 3b	14.687	7.344				
Rat 5a	8.018	4.009	4.678	0.84		
Rat 5b	10.693	5.347				
NVP+CBZ-2D						
Rat 2a	24.962	12.481	13.004	0.61	**11.943**	1.35
Rat 2b	27.054	13.527				
Rat 3a	20.412	10.206	10.424	0.84		
Rat 3b	21.286	10.643				
Rat 5a	12.682	6.341	12.402	7.00		
Rat 5b	36.926	18.463				
NVP+CBZ-7D						
Rat 2a	16.303	8.151	8.032	0.91	**9.542**	1.43
Rat 2b	15.826	7.913				
Rat 3a	22.614	11.307	10.886	0.60		
Rat 3b	20.928	10.464				
Rat 4a	19.982	9.991	9.707	0.43		
Rat 4b	18.848	9.424				
NVP+CBZ-14D						
Rat 1a	18.714	9.357	8.760	0.84	**8.005**	1.08
Rat 1b	16.326	8.163				
Rat 2a	17.047	8.524	8.492	0.11		
Rat 2b	16.921	8.461				
Rat 5a	13.841	6.920	6.764	0.61		
Rat 5b	13.214	6.607				

RF = resorufin; Conc. = concentration; RR = reaction rate; SD = standard deviation; NVP = nevirapine; CBZ = carbamazepine; D = days

Table M-5: CYP2E1 reaction rates in nmol/min*mg of the untreated, NVP and NVP+CBZ groups at 2, 7 and 14 days

Group (n = 3)	Ratio (Ar. 6-OH-CZN/ Ar. I.S)	Reaction rate (nmol/min*mg)	Mean RR/rat (nmol/min*mg)	SD	Mean RR/group (nmol/min*mg)	SD
Untreated						
Rat 2a	0.142	0.729	0.772	0.06	**0.772**	0.06
Rat 2b	0.155	0.816				
NVP-2D						
Rat 2a	0.130	0.846	0.846	0.00	**0.867**	0.03
Rat 2b	0.130	0.846				
Rat 3a	-*	-*	-*	-*		
Rat 3b	-*	-*				
Rat 4a	0.136	0.887	0.887	0.00		
Rat 4b	0.136	0.887				
NVP-7D						
Rat 2a	-*	-*	-*	-*	**0.905**	0.00
Rat 2b	-*	-*				
Rat 3a	0.046	0.271	0.589	0.45		
Rat 3b	0.139	0.908				
Rat 5a	0.138	0.901	0.524	0.53		
Rat 5b	0.028	0.147				
NVP-14D						
Rat 2a	0.256	2.111	1.369	1.05	**1.149**	0.57
Rat 2b	0.098	0.628				
Rat 3a	0.182	1.417	1.571	0.22		
Rat 3b	0.215	1.726				
Rat 5a	0.085	0.506	0.506	0.00		
Rat 5b	0.085	0.506				
NVP+CBZ-2D						
Rat 2a	0.126	0.819	0.819	0.00	**0.814**	0.03
Rat 2b	0.126	0.819				
Rat 3a	0.130	0.846	0.846	0.00		
Rat 3b	0.130	0.846				
Rat 5a	0.120	0.777	0.777	0.00		
Rat 5b	0.120	0.777				
NVP+CBZ-7D						
Rat 2a	-*	-*	-*	-*	**0.908**	0.00
Rat 2b	-*	-*				
Rat 3a	0.139	0.908	0.908	0.00		
Rat 3b	0.139	0.908				
Rat 4a	-*	-*	-*	-*		
Rat 4b	-*	-*				
NVP+CBZ-14D						
Rat 1a	0.126	0.891	1.229	0.48	**0.925**	0.27
Rat 1b	0.198	1.567				
Rat 2a	0.108	0.722	0.722	0.00		
Rat 2b	0.108	0.722				
Rat 5a	0.119	0.825	0.825	0.00		
Rat 5b	0.119	0.825				

6-OH-CZN = 6-hydroxychlorzoxazone; RR = reaction rate; SD = standard deviation; NVP = nevirapine; CBZ = carbamazepine; D = days; * = reading out of range

Table M-6: CYP3A2 reaction rates in pmol/min*mg of the untreated, NVP and NVP+CBZ groups at 2, 7 and 14 days

Group (n = 3)	Ratio (Ar. 1-OH-MDZ/ Ar. I.S)	Reaction rate (pmol/min*mg)	Mean RR/rat (pmol/min*mg)	SD	Mean RR/group (pmol/min*mg)	SD
Untreated						
Rat 2a	0.153	89.500	84.625	6.89	**84.625**	6.89
Rat 2b	0.137	79.750				
NVP-2D						
Rat 2a	0.205	69.000	108.938	56.48	**107.167**	8.73
Rat 2b	0.358	148.875				
Rat 3a	0.300	118.500	114.875	5.13		
Rat 3b	0.286	111.250				
Rat 4a	0.256	95.625	97.688	2.92		
Rat 4b	0.264	99.750				
NVP-7D						
Rat 2a	0.258	96.625	90.625	8.49	**94.282**	5.17
Rat 2b	0.235	84.625				
Rat 3a	0.265	100.250	97.938	3.27		
Rat 3b	0.256	95.625				
Rat 5a	-*	-*	-*	-*		
Rat 5b	-*	-*				
NVP-14D						
Rat 2a	0.198	95.250	96.938	2.39	**105.792**	11.01
Rat 2b	0.206	98.625				
Rat 3a	0.251	118.125	118.125	0.00		
Rat 3b	0.251	118.125				
Rat 5a	0.221	105.125	102.313	3.98		
Rat 5b	0.208	99.500				
NVP+CBZ-2D						
Rat 2a	0.208	70.500	82.750	17.32	**86.771**	15.55
Rat 2b	0.255	95.000				
Rat 3a	0.257	107.250	103.938	4.68		
Rat 3b	0.241	100.625				
Rat 5a	0.214	73.625	73.625	0.00		
Rat 5b	0.214	73.625				
NVP+CBZ-7D						
Rat 2a	0.268	101.875	89.875	16.97	**82.188**	10.87
Rat 2b	0.222	77.875				
Rat 3a	-*	-*	-*	-*		
Rat 3b	-*	-*				
Rat 4a	0.198	82.750	74.500	11.67		
Rat 4b	0.158	66.250				
NVP+CBZ-14D						
Rat 1a	0.195	93.875	94.125	0.35	**94.688**	3.69
Rat 1b	0.196	94.375				
Rat 2a	0.191	92.125	91.313	1.15		
Rat 2b	0.187	90.500				
Rat 5a	0.206	98.625	98.625	0.00		
Rat 5b	0.206	98.625				

1-OH-MDZ = 1-hydroxymidazolam; RR = reaction rate; SD = standard deviation; NVP = nevirapine; CBZ = carbamazepine; D = days; * = reading out of range

Appendix M-3: The effect of PAR and PAR+CBZ on CYP450 activity *in vivo*

Table M-7: CYP1A2 reaction rates in pmol/min*mg of the untreated, PAR and PAR+CBZ groups after 2, 7 and 14 days

Group (n = 3)	RF Conc. (pmol/ml)	RR (pmol/min*mg)	Mean RR/rat (pmol/min*mg)	SD	Mean RR/group (pmol/min*mg)	SD
Untreated						
Rat 2a	8.650	4.325	4.403	0.80	**4.403**	0.80
Rat 2b	8.959	4.480				
PAR-2D						
Rat 1a	18.525	9.263	8.258	1.42	**7.080**	1.07
Rat 1b	14.506	7.253				
Rat 2a	13.159	6.580	6.166	1.02		
Rat 2b	11.505	5.752				
Rat 4a	13.411	6.706	6.813	0.98		
Rat 4b	13.841	6.920				
PAR-7D						
Rat 1a	14.549	7.275	9.155	2.26	**9.717**	0.58
Rat 1b	22.070	11.035				
Rat 2a	18.661	9.331	9.678	0.49		
Rat 2b	20.051	10.026				
Rat 5a	18.377	9.189	10.317	1.60		
Rat 5b	22.890	11.445				
PAR-14D						
Rat 1a	25.766	12.883	13.757	1.56	**12.633**	1.44
Rat 1b	29.264	14.632				
Rat 3a	22.015	11.008	11.015	0.42		
Rat 3b	22.047	11.023				
Rat 4a	28.630	14.315	13.127	1.39		
Rat 4b	23.879	11.939				
PAR+CBZ-2D						
Rat 2a	15.255	7.627	7.941	0.38	**7.983**	0.19
Rat 2b	16.508	8.254				
Rat 3a	15.247	7.624	7.817	0.27		
Rat 3b	16.019	8.010				
Rat 5a	15.270	7.635	8.191	0.79		
Rat 5b	17.492	8.746				
PAR+CBZ-7D						
Rat 3a	14.579	7.290	9.449	2.81	**9.363**	1.59
Rat 3b	23.219	11.609				
Rat 4a	21.268	10.634	10.904	0.38		
Rat 4b	22.347	11.174				
Rat 5a	15.580	7.790	7.736	0.08		
Rat 5b	15.365	7.683				
PAR+CBZ-14D						
Rat 2a	25.396	12.698	13.204	0.81	**11.793**	1.66
Rat 2b	27.420	13.710				
Rat 3a	21.204	10.602	12.211	1.86		
Rat 3b	27.641	13.820				
Rat 4a	20.620	10.310	9.964	0.49		
Rat 4b	19.234	9.617				

RF = resorufin; Conc. = concentration; RR = reaction rate; SD = standard deviation; PAR = paracetamol; CBZ = carbamazepine; D = days

Table M-8: CYP2E1 reaction rates in nmol/min*mg of the untreated, PAR and PAR+CBZ groups at 2, 7 and 14 days

Group (n = 3)	Ratio (Ar. 6-OH-CZN/ Ar. I.S)	Reaction rate (nmol/min*mg)	Mean RR/rat (nmol/min*mg)	SD	Mean RR/group (nmol/min*mg)	SD
Untreated						
Rat 2a	0.142	0.729	0.772	0.06	**0.772**	0.06
Rat 2b	0.155	0.816				
PAR-2D						
Rat 1a	0.279	2.327	2.247	0.11	**2.022**	0.32
Rat 1b	0.262	2.168				
Rat 2a	-*	-*	-*	-*		
Rat 2b	-*	-*				
Rat 4a	0.275	2.290	1.797	0.07		
Rat 4b	0.170	1.304				
PAR-7D						
Rat 1a	0.240	1.634	1.102	0.75	**1.234**	0.41
Rat 1b	0.085	0.570				
Rat 2a	0.199	1.352	1.383	0.04		
Rat 2b	0.208	1.414				
Rat 5a	0.225	1.531	1.218	0.44		
Rat 5b	0.134	0.906				
PAR-14D						
Rat 1a	0.111	0.749	0.718	0.04	**0.742**	0.02
Rat 1b	0.102	0.687				
Rat 3a	0.084	0.563	0.745	0.26		
Rat 3b	0.137	0.927				
Rat 4a	0.113	0.762	0.762	0.00		
Rat 4b	0.113	0.762				
PAR+CBZ-2D						
Rat 2a	0.157	1.182	1.182	0.00	**1.350**	0.24
Rat 2b	0.157	1.182				
Rat 3a	0.223	1.517	1.517	0.00		
Rat 3b	0.223	1.517				
Rat 5a	-*	-*	-*	-*		
Rat 5b	-*	-*				
PAR+CBZ-7D						
Rat 3a	0.130	0.879	1.298	0.59	**1.132**	0.19
Rat 3b	0.252	1.716				
Rat 4a	0.137	0.927	0.927	0.00		
Rat 4b	0.137	0.927				
Rat 5a	0.122	0.824	1.171	0.49		
Rat 5b	0.223	1.517				
PAR+CBZ-14D						
Rat 2a	0.094	0.632	0.632	0.00	**0.830**	0.17
Rat 2b	0.094	0.632				
Rat 3a	0.080	0.536	0.913	0.53		
Rat 3b	0.190	1.291				
Rat 4a	0.113	0.804	0.944	0.20		
Rat 4b	0.300	1.085				

6-OH-CZN = 6-hydroxychlorzoxazone; RR = reaction rate; SD = standard deviation; PAR = paracetamol; CBZ = carbamazepine; D = days; * = reading out of range

Table M-9: CYP3A2 reaction rates in pmol/min*mg of the untreated, PAR and PAR+CBZ groups at 2, 7 and 14 days

Group (n = 3)	Ratio (Ar. 1-OH-MDZ/ Ar. I.S)	Reaction rate (pmol/min*mg)	Mean RR/rat (pmol/min*mg)	SD	Mean RR/group (pmol/min*mg)	SD
Untreated						
Rat 2a	0.153	89.500	84.625	6.89	**84.625**	6.89
Rat 2b	0.137	79.750				
PAR-2D						
Rat 1a	0.188	90.875	87.875	4.24	**83.604**	6.45
Rat 1b	0.174	84.875				
Rat 2a	0.161	79.250	76.188	4.33		
Rat 2b	0.147	73.125				
Rat 4a	0.200	96.000	86.750	13.08		
Rat 4b	0.157	77.500				
PAR-7D						
Rat 1a	0.155	66.875	66.250	0.88	**68.396**	7.45
Rat 1b	0.152	65.625				
Rat 2a	0.147	63.500	62.250	1.77		
Rat 2b	0.141	61.000				
Rat 5a	0.188	80.625	76.688	5.57		
Rat 5b	0.169	72.750				
PAR-14D						
Rat 1a	0.162	69.750	69.125	0.88	**72.229**	5.16
Rat 1b	0.159	68.500				
Rat 3a	0.169	72.750	78.188	7.69		
Rat 3b	0.195	83.625				
Rat 4a	0.155	66.875	69.375	3.54		
Rat 4b	0.167	71.875				
PAR+CBZ-2D						
Rat 2a	0.151	74.875	72.313	3.62	**72.709**	0.44
Rat 2b	0.139	69.750				
Rat 3a	0.168	70.375	72.625	3.18		
Rat 3b	0.179	74.875				
Rat 5a	0.135	68.000	73.188	7.34		
Rat 5b	0.159	78.375				
PAR+CBZ-7D						
Rat 3a	0.202	86.500	82.125	6.19	**80.292**	9.76
Rat 3b	0.181	77.750				
Rat 4a	0.176	75.625	89.000	18.92		
Rat 4b	0.240	102.375				
Rat 5a	0.162	69.750	69.750	0.00		
Rat 5b	0.162	69.750				
PAR+CBZ-14D						
Rat 2a	0.187	80.250	75.438	6.81	**68.479**	7.45
Rat 2b	0.164	70.625				
Rat 3a	0.150	64.750	69.375	6.54		
Rat 3b	0.172	74.000				
Rat 4a	0.164	70.625	60.625	14.14		
Rat 4b	0.116	50.625				

1-OH-MDZ = 1-hydroxymidazolam; RR = reaction rate; SD = standard deviation; PAR = paracetamol; CBZ = carbamazepine; D = days

APPENDIX N: PUBLICATIONS

Appendix N-1: Conference abstracts

Poster presentations

Z. Bekker and A. Walubo. **Simultaneous determination of isoniazid, nevirapine and paracetamol in plasma by high performance liquid chromatography.** 17th World Congress of Basic and Clinical Pharmacology (WorldPharma 2014), Cape Town, South Africa, 13 – 18 July 2014.

Z. Bekker and A. Walubo. **Immune changes during prolonged nevirapine administration in rats.** 17th World Congress of Basic and Clinical Pharmacology (WorldPharma 2014), Cape Town, South Africa, 13 – 18 July 2014.

Z. Bekker and A. Walubo. **Nevirapine influenced red blood cell production and development during prolonged administration in rats.** 17th World Congress of Basic and Clinical Pharmacology (WorldPharma 2014), Cape Town, South Africa, 13 – 18 July 2014.

Oral presentations

Z. Bekker and A. Walubo. **Simultaneous determination of isoniazid, nevirapine and paracetamol in plasma by high performance liquid chromatography.** Faculty Forum 2013, Faculty of Health Sciences of the University of the Free State, Bloemfontein, South Africa, 29 – 30 August 2013.

Z. Bekker and A. Walubo. **Immune changes during prolonged nevirapine administration in rats.** Faculty Forum 2014, Faculty of Health Sciences of the University of the Free State, Bloemfontein, South Africa, 28 – 29 August 2014.

Z. Bekker and A. Walubo. **Nevirapine influenced red blood cell production and development during prolonged administration in rats.** Faculty Forum 2014, Faculty of Health Sciences of the University of the Free State, Bloemfontein, South Africa, 28 – 29 August 2014.

Simultaneous determination of isoniazid, nevirapine and paracetamol in plasma by high performance liquid chromatography

Z Bekker and A Walubo
Department of Pharmacology, UFS

Background:
Whereas isoniazid, nevirapine and paracetamol are known to cause drug-induced hepatotoxicity, patients with human immune deficiency syndrome with tuberculosis (AIDS/TB) who are on nevirapine and isoniazid are often prescribed paracetamol for pain. As concurrent use of the three drugs increases the potential for development of hepatotoxicity, plasma drug monitoring in these patients would be appropriate. Therefore, a high performance liquid chromatography (HPLC) assay was developed for the simultaneous determination of isoniazid, nevirapine and paracetamol.

Methods:
To 100µl of human plasma, isoniazid, nevirapine and paracetamol were added to appropriate concentrations, followed by sulfapyridine (internal standard). After protein precipitation with zinc sulphate and methanol, the sample was centrifuged, the supernatant purified by solid phase extraction, and 50µl of the eluent was injected into the HPLC. The compounds were separated on a C18 analytical column using a mobile phase of 0.06% trifluoroacetic acid (A) and acetonitrile (B) with gradient programmer at a flow rate of 1ml/min for 13 minutes. Detection was by UV at 260nm and the retention times for isoniazid, paracetamol, sulfapyridine and nevirapine were 3.1, 9.9, 10.6 and 11.6 minutes, respectively.

Results:
The average 5 days' calibration curve was linear for isoniazid ($y = 0.029x + 0.025$; $r = 0.9977$), nevirapine ($y = 0.043x + 0.127$; $r = 0.9984$) and paracetamol ($y = 0.097x + 0.070$; $r = 0.9998$) with a CV% of less than 20%. Accuracy at low, medium and high concentrations was 102%, 98% and 101% (isoniazid), 94%, 96% and 100% (nevirapine), and 99%, 97% and 99% (paracetamol), respectively. Isoniazid was least stable at ambient temperature, 4ºC and -20ºC, while paracetamol proved to be most stable at all temperatures. The method was also used successfully to monitor isoniazid, nevirapine and paracetamol in the plasma of treated rats.

Conclusion:
An accurate HPLC method for simultaneous determination of isoniazid, nevirapine and paracetamol in plasma was successfully developed.

Immune changes during prolonged nevirapine administration in rats

Z Bekker and A Walubo
Department of Pharmacology, UFS

Background:

Nevirapine (NVP) is an antiretroviral agent used for prophylaxis and treatment of the human immune deficiency virus (HIV) infection. Unfortunately, NVP is associated with hypersensitivity reactions and hepatotoxicity. Hepatotoxicity occurs within six weeks of treatment onset in HIV+ patients with CD4 counts >250 cells/mm^3. It is hypothesized that NVP stimulates a cell-mediated immune response by an unknown mechanism, resulting in hepatotoxicity. Therefore, immune changes after prolonged administration of NVP in rats were investigated.

Methods:

The study was part of an investigation on drug-induced hepatotoxicity and was approved by the UFS Animal Ethics Committee. Two groups of 25 male Sprague-Dawley rats (200 – 250g), each, received daily saline (S) or NVP (200mg/kg). Five animals were sacrificed after 2, 7, 14, 28 and 42 days of dosing, and blood was tested for full blood count and CD4 count; pro-inflammatory and anti-inflammatory cytokines, interleukin-2 (IL-2) and interleukin-10 (IL-10), respectively; and antibodies, immunoglobulin G (IgG) and immunoglobulin M (IgM), at each time frame.

Results:

By day 42 of treatment, the NVP group showed increased lymphocyte and CD4 counts ($x10^9$/L), while for S both parameters decreased. IL-2 and IL-10 (pg/ml) decreased in the NVP group and remained constant for S. IgM (mg/ml) was sharply increased for NVP, but for S it declined. Both groups showed elevated IgG (mg/ml). The lymphocyte count on day 42 was 3.23±0.3 for S versus 3.52±0.6 for NVP; the CD4 count was 1.47±0.1 for S versus 1.23±0.2 for NVP; the IL-2 level for S was 74.39±5.7 versus 61.92±4.5 for NVP; the IL-10 level was 30.98±3.7 for S versus 28.98±1.0 for NVP; the IgG level was 17.11±0.3 for S versus 16.72±2.2 for NVP; and the IgM level was 0.046±0.01 for S versus 0.089±0.04 for NVP.

Conclusion:

These results indicate that NVP did not induce such a strong cell-mediated immune response as expected when compared to the control. Although the root of NVP-induced hepatotoxicity is yet unknown, involvement of the immune system in one way or another should not be excluded.

Nevirapine influenced red blood cell production and development during prolonged administration in rats

Z Bekker and A Walubo
Department of Pharmacology, UFS

Background:

Nevirapine (NVP) is an antiretroviral agent used for prophylaxis and treatment of the human immune deficiency virus (HIV) infection. Although NVP is known to cause hypersensitivity reactions and hepatotoxicity, as well as eosinophilia and granulocytopenia as part of the hypersensitivity reactions, it has not been associated with red blood cell (RBC) abnormalities. Here is reported an incidental finding of RBC abnormalities after prolonged administration of NVP in a rat model.

Methods:

The study was part of an investigation on drug-induced hepatotoxicity and was approved by the Animal Ethics Committee of the UFS. Two groups of 25 male Sprague-Dawley rats (200 – 250g) each received daily saline (S) or NVP (200mg/kg). Five animals were sacrificed after 2, 7, 14, 28 and 42 days of dosing, and blood was sent for full blood count at each time frame.

Results:

By day 42 of treatment, all groups exhibited significantly ($p<0.05$) elevated RBC count ($x10^{12}$/L), a decreased mean corpuscular volume (MCV), and increased mean corpuscular haemoglobin (MCH) and mean corpuscular haemoglobin concentration (MCHC). The RBC count on day 42 was 6.93±0.8 for the S group versus 7.73±0.3 for NVP; the MCV (fL) was 53.9±1.0 for S versus 52.5±2.3 for NVP; the MCH (pg) was 19.3±0.4 for S versus 18.8±0.5 for NVP; and the MCHC (g/dL) was 35.8±0.2 for S versus 35.8±0.8 for NVP. Haemoglobin, haematocrit and platelets were not changed. Similar studies with isoniazid and paracetamol, respectively, did not exhibit such changes in RBC indices.

Conclusion:

These results imply that NVP influenced RBC production and development during prolonged administration by an unknown mechanism, but the clinical implications of this observation are not yet known.

Appendix N-2: Manuscripts in preparation

1. **Bekker Z**, Walubo A and du Plessis JB. **Simultaneous Determination of Isoniazid, Nevirapine and Paracetamol in Plasma by High Performance Liquid Chromatography.** Journal of Chromatography B. Submitted October 2014.
2. **Bekker Z** and Walubo A. **The Effect of Prolonged Administration of Isoniazid on the Immune System and the Cytochrome P450 Response.** Drug Disposition and Metabolism. Submitted November 2014.
3. **Bekker Z** and Walubo A. **The effect of Prolonged Nevirapine Administration on the Immune System and CYP3A2 Activity in a Rat Model.** Drug Disposition and Metabolism. Submitted November 2014.

SUMMARY

Key terms: isoniazid, nevirapine, paracetamol, prolonged administration, liver injury, immune response, cytochrome P450, HPLC.

Drug-induced liver injury is commonly seen during isoniazid (INH) and nevirapine (NVP) treatment, and after paracetamol (PAR) overdose. It was postulated that after metabolic activation of these drugs, reactive metabolites are formed which attack cellular proteins, thereby creating antigenic metabolite-protein adducts. Subsequently, the immune system is stimulated to eliminate hepatocytes expressing these adducts, and overt hepatotoxicity ensues. Therefore the cytochrome P450 (CYP450) and immune response to prolonged administration of INH, NVP and PAR were investigated here.

First, a high performance liquid chromatography (HPLC) method for the simultaneous determination of INH, NVP and PAR in plasma was developed. Sample preparation involved protein precipitation with zinc sulphate and methanol, followed by solid phase extraction. The mobile phase was 0.06% trifluoroacetic acid (A) and acetonitrile (B), run by a gradient programmer over a C_{18} (4.60 x 250mm) 5µ analytical column at 1ml/min, while the eluent was detected by UV at 260nm. INH, PAR, internal standard and NVP had retention times of 3.1, 9.9, 10.6 and 11.6 minutes, respectively. The respective average 5 day calibration curves of INH, NVP and PAR delivered linear regression equations and correlation coefficients of $y = 0.029x + 0.025$ ($r^2 = 0.9954$), $y = 0.043x + 0.127$ ($r^2 = 0.9968$) and $y = 0.097x + 0.070$ ($r^2 = 0.9997$). The method was used successfully to monitor INH, NVP and PAR in treated rats.

The CYP450 and immune response to prolonged administration of INH, NVP and PAR was investigated using an SD rat model, and the animal experiment divided into three phases. In phase I, rats were orally administered saline (S), INH (20mg/kg), NVP (200mg/kg) or PAR (500mg/kg), while for phase II, rats received S, INH, NVP or PAR combined with an immune stimulant, levamisole (LMS; 2.5mg/kg), and lastly, during phase III, rats received S, INH, NVP or PAR along with a CYP450 inducer, carbamazepine (CBZ; 60mg/kg). Five rats per group were sacrificed after 2, 7, 14,

28 and 42 days. Blood was analysed for full blood count, CD4 and CD8 count, liver function, renal function, IL-2, IL-10, IgG, IgM and drug concentrations. A liver section was sent for histopathology testing, and the activity of rat CYP1A2, CYP2E1 and CYP3A2 measured.

During administration of the test drugs alone, both INH and NVP triggered an early Th1 immune response that was associated with liver injury, and counteracted by a later Th2 immune response which was associated with healing. Overall, the liver injury correlated with low concentrations of NVP, but high INH concentrations. Furthermore, INH increased CYP2E1 activity, while NVP increased that of CYP3A2.

When LMS (immune stimulant) was co-administered, INH liver injury was exacerbated, while for NVP it was the same. Again, INH and NVP provoked a Th1 response (injury) that was counteracted by a Th2 response (healing). Here, the liver injury was also associated with low NVP concentrations, and high INH concentrations.

During CBZ (CYP450 inducer) co-administration, INH and NVP caused the same immune response, which resulted in improvement of the liver injury. Again, the liver injury was associated with low NVP concentrations, and high INH concentrations. Here, INH increased CYP2E1 activity and NVP increased CYP3A2 activity, but not to the same extent as the test drugs alone.

PAR did not exhibit a distinct pattern of immune response to associate it with the liver injury, most probably because the concentrations were too low for generation of toxic metabolites.

In conclusion, the pattern of immune response to prolonged administration of INH and NVP shows that the immune system is involved in the drug-induced liver injury, probably as a protective buffer to prevent further drug toxicity.

OPSOMMING

Sleutelterme: isoniasied, nevirapien, parasetamol, langtermyn toediening, lewerskade, immuunrespons, sitochroom P450, hoëdrukvloeistof-chromatografie.

Middel-geïnduseerde lewerskade kom algemeen voor tydens isoniasied (INH) en nevirapien (NVP) behandeling en na parasetamol (PAR) oordosering. Dit is voorgestel dat ná metaboliese aktivering van die middels, reaktiewe metaboliete gevorm word wat sellulêre proteïene aanval en daardeur antigeniese metaboliet-proteïen neweprodukte vorm. Gevolglik word die immuunsisteem gestimuleer om hepatosiete te verwyder wat uitdrukking gee aan dié neweprodukte en dit lei tot waarneembare hepatotoksisiteit. Dus is die sitochroom P450 (CYP450) en immuunrespons van langtermyn toediening van INH, NVP en PAR hier ondersoek.

Eerstens is `n hoëdrukvloeistof-chromatografie (HPLC) metode vir die gelyktydige bepaling van INH, NVP en PAR in plasma ontwikkel. Monstervoorbereiding het behels proteïenbesinking met sinksulfaat en metanol, gevolg deur vaste fase ekstraksie. Die mobiele fase het bestaan uit 0.06% trifloroasetaatsuur (A) en asetonitriel (B) en is gechromatografeer met `n gradiënt programmeerder deur `n C_{18} (4.60 x 250mm) 5µ analitiese kolom teen 1ml/min, terwyl die eluaat bepaal is deur UV teen 260nm. INH, PAR, die interne standard en NVP het onderskeidelike retensietye van 3.1, 9.9, 10.6 en 11.6 minute gehad. Die onderskeie, gemiddelde 5 dag kalibrasiekromme van INH, NVP en PAR het liniêre regressievergelykings en korrelasie-koëffisiënte van $y = 0.029x + 0.025$ ($r^2 = 0.9954$), $y = 0.043x + 0.127$ ($r^2 = 0.9968$) en $y = 0.097x + 0.070$ ($r^2 = 0.9997$) gehad. Die metode is suksesvol gebruik om INH, NVP en PAR in behandelde rotte te bepaal.

Die CYP450 en immuunrespons van langtermyn toediening van INH, NVP en PAR is bestudeer in `n SD rotmodel en die diereëksperiment is verdeel in drie fases. In fase I is die rotte oraal gedoseer met soutoplossing (S), INH (20mg/kg), NVP (200mg/kg) of PAR (500mg/kg). In fase II is die rotte doseer met S, INH, NVP of PAR in kombinasie met `n immuunstimulant, levamisool (LMS; 2.5mg/kg) en tydens fase III het rotte S, INH, NVP of PAR ontvang saam met `n CYP450-induseerder, karbamasepien (CBZ; 60mg/kg). Vyf rotte per groep is geslag na 2, 7, 14, 28 en 42

dae. Bloed is getoets vir volbloedtelling, CD4 en CD8 tellings, lewerfunksie, nierfunksie, IL-2, IL-10, IgG, IgM en middelkonsentrasies. `n Lewermonster is gestuur vir histopatologiese ondersoek en die aktiwiteit van rot CYP1A2, CYP2E1 en CYP3A2 bepaal.

Tydens toediening van die toetsmiddels op hul eie, het beide INH en NVP `n vroeë Th1 immuunrespons ontlok wat verbind kon word met lewerskade. Die reaksie is teengewerk deur `n latere Th2 immuunrespons wat verbind kon word met herstel. In die geheel het die lewerskade gekorreleer met lae konsentrasies van NVP, maar hoë INH-konsentrasies. Verder het INH CYP2E1 aktiwiteit verhoog, terwyl NVP dié van CYP3A2 verhoog het.

Waar LMS (immuunstimulant) gelyktydig toegedien is, is INH-lewerskade vererger, maar vir NVP was die lewerskade onveranderd. Weereens het INH en NVP `n Th1 respons (lewerskade) ontlok wat teengewerk is deur `n Th2 respons (herstel). Hier kon die lewerskade ook verbind word met lae NVP-konsentrasies en hoë INH-konsentrasies.

Tydens die gelyktydige toediening van CBZ (CYP450-induseerder), het INH en NVP dieselfde immuunrespons veroorsaak wat gelei het tot verbetering van die lewerskade. Weereens kon die lewerskade verbind word met lae NVP-konsentrasies en hoë INH-konsentrasies. Hier het INH CYP2E1 aktiwiteit verhoog en NVP het CYP3A2 aktiwiteit verhoog, maar nie tot die volle omvang soos met die toetsmiddels op hulle eie nie.

PAR het nie `n onderskeibare immuunresponspatroon ten toon gestel wat met die lewerskade verbind kon word nie, heel waarskynlik omdat die konsentrasies te laag was om toksiese metaboliete te vorm.

Gevolglik het die immuunresponspatroon van langtermyn toediening van INH en NVP gewys dat die immuunsisteem betrokke is by middel-geïnduseerde lewerskade, waarskynlik as `n beskermende buffer om verdere middeltoksisiteit te voorkom.