The correlation between raised liver enzyme levels and high concentrations of rifampicin and its metabolites: 25-desacetyl rifampicin and 3-formyl rifampicin

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Declaration

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Abstract

Tuberculosis is widespread in Southern Africa. In the Western Cape alone in 1998 there were 22 942 cases of tuberculosis and 16.8% of them were HIV positive (Department of Health, 1998). It was projected that in the year 2001 there would be 36 922 tuberculosis cases, 36.5% of whom would be HIV positive (Weyer and Fourie, 1996).

As a result of the prevalence of the disease, antituberculosis drugs are widely used in this area and it is important to ensure that they are safe. Rifampicin has been shown to cause hepatitis in approximately 1.1% of patients (Dickinson *et al*, 1981). The mechanism by which rifampicin causes hepatitis is unknown. Based on the fact that isoniazid metabolites induce hepatotoxicity, it was hypothesised that the metabolites of rifampicin, might too be hepatotoxic. The aim of this project was to determine whether there was a correlation between raised liver enzyme levels and high concentrations of rifampicin and its metabolites.

Rifampicin has two main metabolites: 25-desacetyl rifampicin and 3-formyl rifampicin. Two high performance liquid chromatography (HPLC) methods were used. One method had already been developed to detect rifampicin and 25-desacetyl rifampicin (Zent and Smith, 1995) and another method was developed to detect 3-formyl rifampicin. These methods were validated for specificity, reproducibility, recovery and sensitivity. A clinical trial was conducted at D.P. Marais Hospital, Tokai. In the study 68 patients were screened for raised liver enzyme levels of 1-2 times the normal upper range. Out of the these patients 9 control patients, who had normal enzyme levels were then matched with the test group, which consisted of 12 patients with raised liver enzyme levels. Blood was taken over a period of 24 hours and then analysed using the HPLC methods.

The concentration of 3-formyl rifampicin was below the limit of detection in the serum of the patients. This is consistent with the literature, which states that 3-formyl rifampicin is produced in the urine and is found in minute concentrations in the serum,

if at all (Ishii and Hiroyasu, 1988 and Lecaillon *et al*, 1978). Rifampicin and 25-desacetyl rifampicin were detected in the serum of the patients.

The albumin, body mass index (BMI), haemaglobin (Hb), international normalising ratio (INR), mean corpuscular volume (MCV) and platelet levels of the test group were plotted against those of the control group. No significant difference could be seen between the two groups, indicating that none of the above parameters can serve as reliable markers of mild liver injury.

There was no significant difference between the maximum concentration (Cmax) or half-life ($t_{1/2}$) of rifampicin and 25-desacetyl rifampicin in patients with and without raised liver enzyme levels. However the area under the curve (AUC) of rifampicin and the AUC of the combination of rifampicin + 25-desacetyl rifampicin were significantly higher in patients with raised liver enzyme levels than in patients with normal liver enzyme levels. This trend could also be observed for the AUC of 25-desacetyl rifampicin, however the difference between the test and control groups was not significant. There was no significant difference between the Cmax, $t_{1/2}$ or AUC ratio of rifampicin:25-desacetyl rifampicin between the test and control groups.

The ratio of the drug concentration to the metabolite is an indication of the ability of a patient to metabolise rifampicin. Since the ratio was the same for the test and the control groups, it can be assumed that both groups were able to metabolise the drug equally well, and the observed deranged liver enzyme levels were not severe enough to impair rifampicin metabolism. Rather the presence of higher concentrations of rifampicin and 25-desacetyl rifampicin led to raised liver enzyme levels in the test subjects. It was therefore shown, that rifampicin and the combined effect of rifampicin + 25-desacetyl rifampicin may have a causal relationship with raised liver enzyme levels. However because the cohort of patients was very small, it is necessary to conduct further studies using a larger number of subjects.

The results of this study indicate that it would be beneficial to run routine tests to determine the rifampicin levels in patients' serum with a view to modifying dosage in those patients in which the rifampicin levels are too high. This however may not be feasible due to the high costs involved given the large number of TB patients in the Western Cape and in South Africa at large.

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Chapter 1

Introduction

Part A: Tuberculosis and antituberculosis therapy

In 1993 the World Health Organisation (WHO) declared tuberculosis a global emergency and estimated that approximately 90 million people would be infected with tuberculosis and 30 million would die of the disease in the 1990's (WHO, 1996). In South Africa tuberculosis has already reached epidemic proportions, and in the Western Cape the high prevalence of alcoholism and Human Immunodeficiency Virus (HIV) make the study of tuberculosis and the metabolism of antimicrobial drugs in liver disease patients very relevant. The nutritional status of the patient is also an important factor in the development of hepatotoxicity, and in South Africa poverty among a high proportion of the population increases the risk. Standard therapy antituberculosis drugs: isoniazid, rifampicin and pyrazinamide, have been widely implicated in the cause of drug-induced liver disease. There is an incidence of approximately 1% overt and 10-20% subclinical hepatitis associated with antituberculosis therapy (Dickinson *et al*, 1981). The incidence of hepatotoxicity resulting from rifampicin therapy alone is estimated at 1.1% (Steele, 1991).

1. Etiologic agent

The bacterium responsible for tuberculosis is a facultative intracellular parasite called *Mycobacterium tuberculosis*. It invades host macrophages by binding to specific receptors such as complement receptor 4. The receptors become tyrosine phosphorylated and are internalised, resulting in the entry of the bacterium into the macrophages. *M. tuberculosis* grows and multiplies by degrading host macromolecules and by utilising host growth factors. The bacterium has an outer capsule that provides passive defence against the invasion of degradative enzymes and bactericidal peptides. It damages the host mitochondria by inhibiting respiration and phosphorylation. *M. tuberculosis* also contains haemolysins, which allow it to break out of the macrophage and to multiply extracellularly (Daffe and Ettiene, 1999). The bacterium has a high cell wall content of high-molecular-

weight lipids which give it an innate resistance to the immune system. *M.tuberculosis* grows slowly, having a 15-20 hour generation time (Mandell *et al*, 1995).

2. Epidemiology

M. tuberculosis is transmitted from an individual with infectious pulmonary tuberculosis to other people by droplet nuclei, which are aerosolised by coughing. The smallest particles may remain suspended in the air for several hours and may gain direct access into the terminal air passages. The risk of acquiring *M. tuberculosis* infection is determined by exogenous factors such as intimacy and duration of contact, extent of infectiousness of the carrier and the environment of the contact. The risk of actually developing the disease, however is determined by endogenous factors such as the individual's susceptibility to the disease and the strength of their immune system. Clinical illness directly following infection is classified as primary tuberculosis and is common among young children. Usually when *M. tuberculosis* is acquired in adulthood, the immune system will initially control it and the dormant bacilli can persist for years before being reactivated to cause secondary tuberculosis. Reinfection of a previously infected individual in areas with high prevalence of *M. tuberculosis* is common (Raviglione and O'Brien, 1998).

3. Pathogenesis

Droplet nuclei, containing microorganisms are inhaled and approximately 10% of the bacilli reach the alveoli where they are ingested by macrophages. The macrophages may either succeed in stopping bacterial multiplication through the release of cytokines and proteolytic enzymes or the bacilli may multiply uncontrolled, and eventually burst the macrophage. Non-activated monocytes from the blood stream ingest the released bacilli. Two to four weeks after infection two more types of immunological responses develop. The first is a delayed-type hypersensitivity response, which destroys non-active macrophages containing bacilli resulting in tissue damage. The second is a macrophage-activating response, which is cell-mediated resulting in active macrophages that are capable of killing mycobacteria. The balance between these two responses determines the form of tuberculosis that will develop. When large numbers of activated macrophages

accumulate at the site of primary lesion, granulomatous lesions (tubercles) are formed (Raviglione and O'Brien, 1998). Tubercles consist of a central area of caseation, surrounded by epithelioid cells and Langhan's giant cells with multiple nuclei. Subsequently the caseated areas heal completely and become calcified. Approximately 20% of these calcified lesions contain dormant bacilli (Rich, 1952), which are capable of being reactivated by depression of the host defence system (Kumar and Clark, 1998). In the early stages of infection, bacilli are usually transported by macrophages to the lymph nodes, from which they are disseminated widely to many organs and tissues. Most of the resulting lesions tend to heal however, in individuals with poor immunity, hematogenous dissemination may result in fatal miliary tuberculosis or in tuberculosis meningitis (Raviglione and O'Brien, 1998).

4. Clinical manifestations

M. tuberculosis can be classified as pulmonary and extrapulmonary. Until the spread of HIV, 80% of the cases used to present as pulmonary tuberculosis, however now, up to two thirds of HIV infected patients can present with extrapulmonary tuberculosis or a combination of both (Raviglione and O'Brien, 1998). In South Africa extrapulmonary tuberculosis was also found to be more common in HIV-positive patients (Wilkinson and Moore, 1996). Primary tuberculosis is symptomless in most individuals apart from a slight cough and wheeze. Cellular immunity develops within 3-8 weeks after the initial reaction and can be detected by the tuberculin skin test. At this stage the classical pathology of granulomatous lesions can be seen. Enlargement of lymph nodes, compressing the bronchi can give rise to collapse of segments or lobes of the lung, however the collapse disappears as the primary complex heals. Enlargement of the lymph nodes can also give rise to hyperinflation, consolidation and consolidation with the appearance of an expansile pneumonia (Miller, 1982). Primary tuberculosis usually occurs in children soon after infection and is characterised by enlargement of the lymph nodes and the resulting lesions (Lincoln and Sewell, 1963). Post-primary tuberculosis occurs after the first few weeks of primary infection but often can occur years later due to reactivation or reinfection usually as a result of diabetes, malnutrition, immunosuppression or drugs such as cytotoxins and steroids. Post-primary tuberculosis usually occurs in adults and is characterised by malaise,

loss of weight, fever, cough and night sweats, apical lung lesions and frequent cavitation (Davies PDO, 1998). Sputum may be mucoid, purulent or blood-stained. Many patients have a dull ache in the chest and suffer from recurring colds. A pleural effusion can be seen as a presenting sign, however there are often no physical signs in the chest. The chest X-ray usually shows patchy or nodular shadows in the upper zone, loss of volume and fibrosis with or without cavitation. The diagnosis will often be suggested by X-rays but should be confirmed by positive identification of the organism in sputum (Kumar and Clark, 1998). When there is acute diffuse dissemination of tubercle bacilli in the blood stream, extrapulmonary tuberculosis may develop. The sites most commonly involved in tuberculosis are the lymph nodes, pleura, genitourinary tract, bones and joints, meninges, and peritoneum. However virtually all organs can be affected in HIV-positive individuals (Raviglione and O'Brien, 1998).

5. Antituberculosis drug therapy

The three cornerstone drugs administered in the treatment of tuberculosis are rifampicin, pyrazinamide and isoniazid. Ethambutol and streptomycin are also often administered. There are two phases of the 'short course chemotherapy'. During the intensive phase of treatment, which lasts 2 months, rifampicin, isoniazid, pyrazinamide and ethambutol are given in a combination tablet containing concentrations of 120mg, 60mg, 300mg and 200mg respectively. Patients below 50kg receive 4 tablets and patients over 50kg receive 5 tablets. All drugs are given daily, 5 days a week. The continuation phase lasts a further 4 months and consists of rifampicin and isoniazid given either in doses of 400mg and 300mg respectively for patients below 50kg, or 600mg rifampicin and 400mg isoniazid for patients above 50kg (Gibbon CJ, 1997). The mechanisms of action and the pharmacokinetics of the three main drugs are described below.

5.1 Rifampicin

5.1.1 Mechanism of Action

Rifampicin is a semisynthetic drug derived from rifamycin B, which is produced by *Streptomyces mediterranei*. The rifamycin drugs bind to the β-subunit of bacterial DNA-dependent RNA polymerase to prevent initiation of transcription. In this way protein synthesis is inhibited. Electron microscopy shows that the cytoplasm of *Mycobacterium tuberculosis* treated with rifampicin is less compact and the ribosomes become coarse granules, while the structure of the mesosome, involved in cell growth, is lost (Konno *et al*, 1973). Resistance to rifampicin is the result of a mutation of the *rpoB* gene that codes for the β-subunit of RNA polymerase (Chopra and Brennan, 1998). Rifampicin is both bactericidal and bacteriostatic (Proust, 1971).

5.1.2 Absorption and bioavailability of rifampicin

Rifampicin has a recommended therapeutic range of 8-24μg/ml (Peloquin, 1996). It is absorbed more effectively at low gastric pH and on an empty stomach and under these conditions absorption is rapid and complete (Riess *et al*, 1969). Food has been demonstrated to cause a 1-2 hour delay in the time taken to reach the maximum concentration (Tmax) and a reduction in the maximum drug concentration (Cmax) and area under the concentration-time curve (AUC) of rifampicin (Kenny and Strates, 1981 and Zent and Smith, 1995). Rifampicin does not influence the serum concentrations of isoniazid, however there have been conflicting reports on whether isoniazid affects the levels of rifampicin. Para-aminosalicylic acid was found to delay the T_{max} of rifampicin from 2 to 4 hours, reduce its C_{max} from 8 to 3.8μg/ml and decrease the AUC by approximately 50% (Holdiness, 1984). In a bioavailability study conducted in 45 patients variations in particle size, excipients or manufacturing processes of the experimental preparations yielded marked differences in bioavailability. It was concluded that because a wide inter-individual variability in serum levels is reported in the literature, patients who do not absorb rifampicin well, might receive ineffective treatment if there are other contributing factors such as administration with food or poor drug formulations (Buniva *et al*, 1983). Peak concentrations of 10μg/ml are observed 2.1 hours after a standard dose of 600mg (Riess *et al*, 1969). Increase in the dose does not result in a proportional increase in concentration and deviation from linearity has been observed in the AUC from 0-12 hours. This is because with dose of the order of 300 to 450mg, the excretory capacity of

the liver for the antibiotic is saturated. As a consequence, increasing the dose of antibiotic results in a more than proportional increase in serum concentrations (Acocella, 1978). The biological half-life of rifampicin is between 2.3 and 5 hours on initiation of therapy, however rifampicin is a potent enzyme inducer and stimulates its own metabolism, so its half-life decreases to between 2 and 3 hours after repeated administration (Douglas and Mcleod, 1999).

5.1.3 Distribution of rifampicin

Rifampicin is estimated to be 80% protein bound. It binds to albumin, γ-globulin and fibrinogen (Acocella, 1978) however the γ-globulins appear to be the major binding proteins (Kenny and Strates, 1981). Binding is reversible and very weak. Rifampicin is widely distributed in the body fluids and tissues. The ratio of blood:tissue concentration of rifampicin in bile, liver, gallbladder wall, ureter and ovarian cyst is lower than 1. A ratio of 1 is found in spleen tissue, appendix, skin, muscle, rib and mammary tumour. In lung, stomach wall, fat and breast milk a ratio greater than 1 is found. Very high concentrations of rifampicin are found in sputum and cavitary fluid of patients with tuberculosis (Acocella, 1978). At physiological pH only 25% of the compound is ionised, but the molecule as a whole is lipid soluble (Kenny and Strates, 1981).

5.1.4 Metabolism of rifampicin

Rifampicin undergoes desacetylation at the C-25 position resulting in 25-O-desacetyl rifampicin (Holdiness, 1984). Desacetylation takes place in the hepatocyte and results in a more polar compound, which facilitates its excretion in bile (Acocella, 1978). 25-desacetyl rifampicin accounts for 80% of the microbiological activity in human bile and its rate of transfer into bile is 10 to 20 times greater than that of the parent compound. The desacetylating enzymes are thought to be located in the smooth endoplasmic reticulum of the hepatocytes (Holdiness, 1984). Increases in hepatic cytochrome P-450, β-glucuronidase, paranitrophenolglucuronyltransferase, β-N-acetylglucuronidase, β-N-acetylglucoseaminidase, and corticoid hydroxylase indicate their involvement in rifampicin metabolism (Kenny and Strates, 1981). In aqueous solutions with lower pH values,

rifampicin hydrolyses to yield 3-formyl rifampicin and 4-methylpiperazine (Weber *et al*, 1983). In the urine 3-formyl rifampicin spontaneously forms and can account for 10% of the antibacterial activity found there (Acocella, 1978). The 3-formyl rifampicin metabolite could not be detected in the plasma of a healthy volunteer and a tuberculosis patient treated with rifampicin (Ishii and Hiroyasu, 1988).

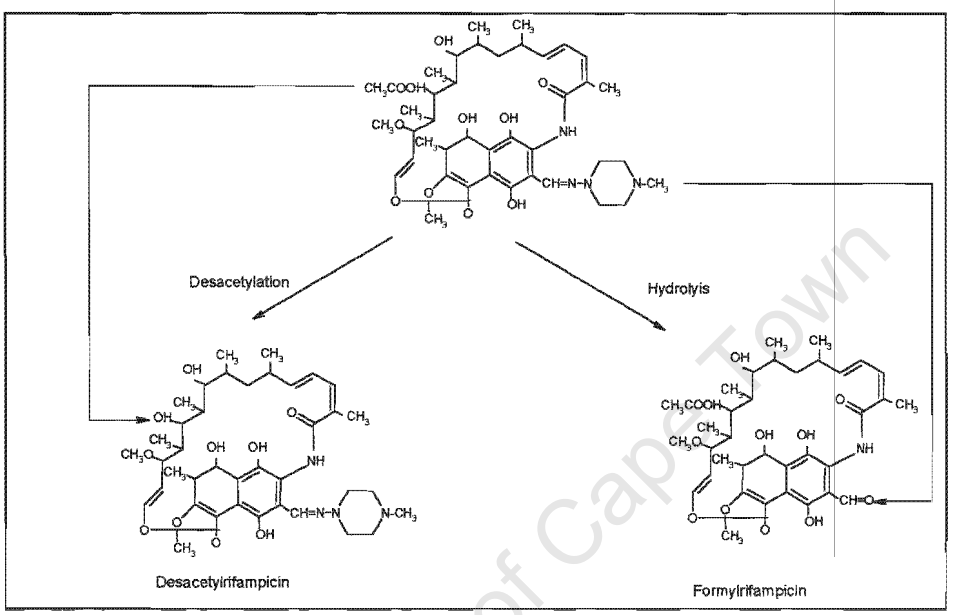


Figure 1: Metabolic products of rifampicin (Acocella, 1979)

5.1.5 Excretion of rifampicin

Both biliary and urinary excretion of rifampicin occur in man. The hepatic excretory capacity for rifampicin is suggested to be saturable since an increase in dose from 150mg to 600mg does not increase the rifampicin bile concentration (Acocella *et al*, 1978) Biliary excretion of rifampicin is saturated at doses of 300 to 450mg (Acocella, 1978). Bile extracts analysed by thin layer chromatography after oral administration of rifampin were found to contain 50-79% of the drug as its 25-desacetyl derivative 3-4 hours postadministration and 88% after 5hours (Furesz, 1970). In serum after 5 hours 6-11% of the total dose is found as 25-desacetyl rifampicin (Holdiness, 1984). Absorption of orally administered 25-desacetyl rifampicin from the gastrointestinal tract is poor. After oral administration of 300mg rifampicin, however, levels of 4μg/ml and 0.8μg/ml 25-desacetyl rifampicin were found in the portal and peripheral venous circulation, respectively, indicating some enterohepatic circulation. After a single dose of rifampicin, the drug is

rapidly excreted in urine with a peak concentration occuring at 6 hours. On repeated administration, the levels of serum rifampicin decrease, as do the levels of urinary rifampicin, which follow the serum level pattern (Acocella, 1978).

5.2 Pyrazinamide

5.2.1 Mechanism of action of pyrazinamide

Although the exact mode of action of pyrazinamide is not known, it is thought to be a prodrug, converted by the bacterial enzyme pyrazinamidase to the active pyrazinoic acid metabolite, the target of which is not known. *M. tuberculosis* has developed resistance to pyrazinamide through single mutations in the *pncA* gene encoding pyrazinamidase (Mestdach *et al*, 1999). Pyrazinamide is bactericidal to mycobacteria multiplying intracellularly at low pH (Douglas and Mcleod, 1999).

5.2.2 Absorption and bioavailability of pyrazinamide

Pyrazinamide is well absorbed from the gastrointestinal tract and a 500mg oral dose produces peak plasma concentrations of approximately 38.7μg/ml after 1 hour (Lacroix *et al*, 1989). Absorption is virtually complete (Ellard, 1969). The cumulative urinary excretion of pyrazinamide and its metabolites represents 73% of the ingested dose, which demonstrates the high bioavailability of this substance (Lacroix *et al*, 1989).

5.2.3 Distribution of pyrazinamide

PZA is rapidly distributed within 1-2 hours following administration (Holdiness, 1984) in most tissues and fluids of the body with a distribution volume of 0.6-0.7L/kg (Gibbons, 1997). The drug was not found in spleen, brain, bone marrow or skeletal muscle of rabbits after a single oral dose

5.2.4 Metabolism of pyrazinamide

Pyrazinamide is metabolised by the action of the hepatic microsomal pyrazinamide deamidase into 2-pyrazinoic acid, which is then hydroxylated to 5-hydroxypyrazinoic acid by xanthine oxidase (Ellard and Haslam, 1976, Mehmedagic *et al*, 1997) but it has not been shown that 5-hydroxypyrazinamide is produced exclusively by this enzyme (Yamamoto T et al, 1987). Pyrazinamide is also converted to pyrazinuric acid (Lacroix *et al*, 1989).

5.2.5 Excretion of pyrazinamide

Pyrazinamide is mainly eliminated by glomerular filtration. The plasma half-life of pyrazinamide is 9-10 hours (Douglas and Mcleod, 1999). Renal clearance of pyrazinamide is low at 1.9ml/min (Lacroix *et al*, 1989). Four to 14% of the pyrazinamide absorbed, is excreted unchanged in the urine (Gibbons, 1997). After 48 hours 40% of all the pyrazinoic acid is excreted (Holdiness, 1984). The rate of pyrazinoic acid excretion in the urine increases rapidly and is maximal at 2 hours (Holdiness, 1984).

5.3 Isoniazid

5.3.1 Mechanism of action of isoniazid

Isonicotinic acid hydrazide (isoniazid) is a nicotinamide derivative. The active compound is isonicotinic acid and both the pyridine ring and the hydrazine group are necessary for its biological activity. Although isoniazid is bactericidal against actively dividing organisms, it is only bacteriostatic in the presence of semidormant organisms and therefore has less sterilising activity than rifampicin or pyrazinamide (Douglas and Mcleod, 1999). Isoniazid inhibits protein and nucleic acid synthesis. Mycolic acid, found in the cell walls of mycobacteria, is targeted, making isoniazid specific to mycobacteria. Isoniazid is transformed into isonicotinic acid by the catalase-peroxidase system. Isonicotinic acid has a similar structure to nicotinic acid, a precursor to nicotinamide adenine dinucleotide (NAD). Isonicotinic acid is used to produce iso-NAD instead of NAD. Iso-NAD is then incorporated as a non-functional coenzyme of multiple enzyme systems, leading to the death of *M. tuberculosis*. Isoniazid resistant mycobacteria lose their catalase activity,

preventing the conversion of isoniazid to isonicotinic acid. Another mechanism of resistance may be reduced oxygen-dependent active uptake of the drug by *M. tuberculosis* (Douglas and Mcleod, 1999).

5.3.2 Absorption and bioavailability of isoniazid

Limited absorption area in the gastrointestinal tract and any changes in gastric emptying (Weber and Hein, 1979) or food contact (Zent and Smith, 1995) may alter absorption Isoniazid is absorbed much better on an empty stomach, as it is highly reactive with antacids and food components, which reduce its bioavailability. The peak serum concentrations are higher and the area under the curve (AUC) is greater when administered to patients in the fasting state. The formulation of the tablet does not seem to be a significant factor in the variation of bioavailability of isoniazid (Holdiness, 1984).

5.3.3 Distribution of isoniazid

Isoniazid is well distributed and has been detected in pleural effusions, faeces, saliva, placenta, breast milk, peripheral nerves and red blood cells (Weber and Hein, 1979). It has been suggested that the lung and skin may serve as storage depots of the drug (Holdiness, 1984). The apparent distribution volumes of acetylisoniazid and isonicotinic acid are similar to that calculated for the total body water (Ellard and Gammon, 1975).

5.3.4 Metabolism of isoniazid

Acetylation is essential in isoniazid metabolism. It is mediated by a non-microsomal liver enzyme called N-acetyltransferase. There are three acetylator phenotypes: fast, slow and intermediate. The difference in the activity of the three states is determined by a genetic mutation in the gene responsible for controlling the rate of synthesis of N-acetyltransferase (Douglas and Mcleod, 1999). Rapid acetylation results in an elimination half-life that is 3-5 times faster than that of slow acetylation (Ellard and Gammon, 1975). Both slow and fast acetylator types have an equivalent response to treatment with standard antituberculosis

regimes when the drug is continually given daily for a prolonged period of time (Douglas and Mcleod, 1999). The major metabolites of isoniazid are depicted in the figure below.

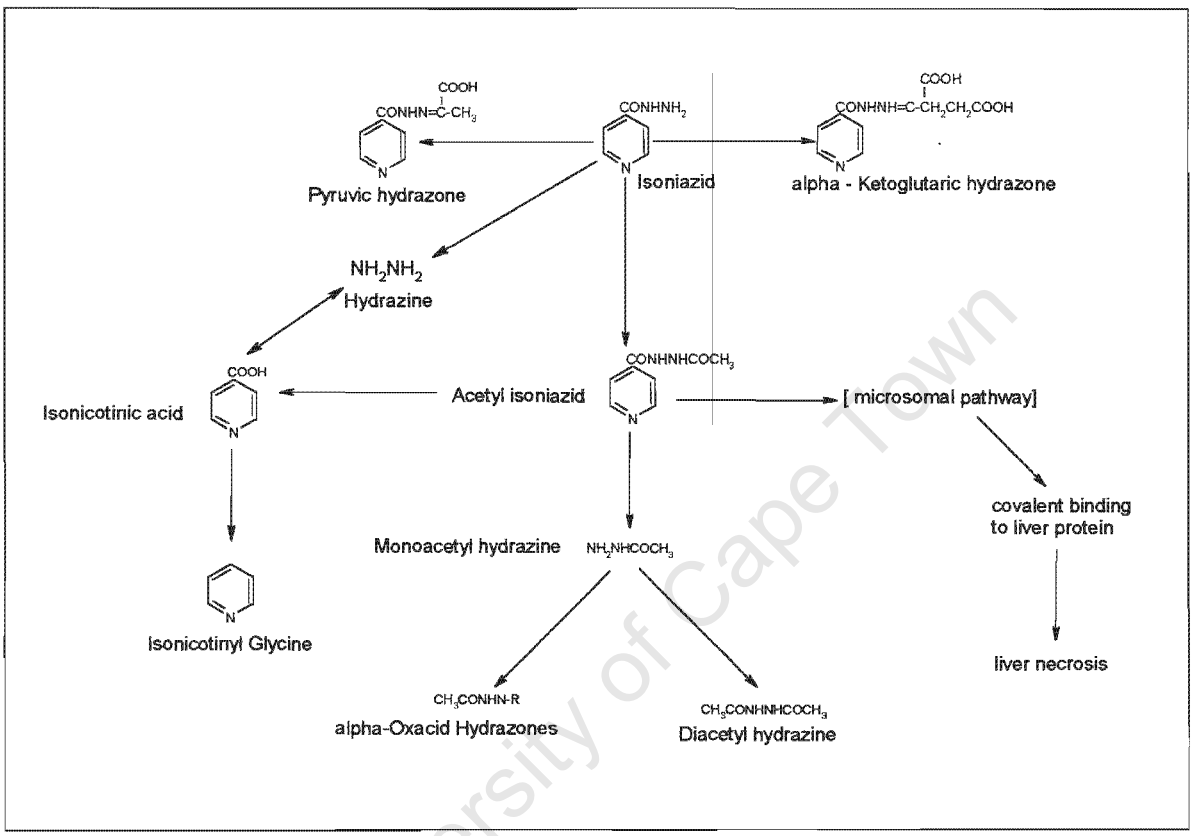


Figure 2: Metabolism of isoniazid (modified from Weber and Hein, 1979, Timbrell *et al*, 1980, Ellard and Gammon, 1976)

The half-life of the active metabolite, isonicotinic acid, after oral administration is 0.7 hours and is similar to that of intravenous administration. Isonicotinic acid can be obtained from isoniazid by direct hydrolysis and in slow acetylators, approximately 30% of the isonicotinic acid and isonicotinylglycine excreted after administration of isoniazid, is formed by this route whereas in rapid acetylators the proportion is less than 10% (Ellard and Gammon, 1975).

5.3.5 Excretion of isoniazid

The majority of a dose of isoniazid is excreted in the urine within 24 hours, all in the form of metabolites (Holdiness, 1984). The terminal metabolites of isoniazid are isonicotinylglycine, which is excreted, and diacetylhydrazine. The rate constants for the elimination of both these substances is very similar. The plasma renal clearance of acetylisoniazid is similar to the glomerular filtration rate, however it has been stated that simultaneous active secretion and reabsorption cannot be excluded (Ellard and Gammon, 1976).

Part B: The liver and its functions

The liver is the largest organ in the body. It constitutes approximately 2.5% of the body weight of a grown man and it performs over 500 vital functions. The hepatocyte is the main functional unit of the liver. The liver's main functions are uptake of substrates from the intestine and their storage; metabolism and distribution to blood and bile; synthesis, degradation and detoxification (Arias *et al*, 1988).

1. Synthesis and degradation

Although the body muscle mass constitutes the greatest total amount of protein, the liver has the highest rate of synthesis per gram of tissue (Podolsky and Isselbacher, 1998A). Plasma contains 60-80g/L of protein, mainly in the form of albumin, globulin and fibrinogen (Kumar and Clark, 1998). Albumin is quantitatively the most important protein and is produced at the rate of 12g per day, representing half of all exported protein. The average normal half-life of serum albumin is 17-20 days (Podolsky and Isselbacher, 1998A). The main function of albumin is to maintain the intravascular oncotic pressure and to transport water-insoluble substances such as bilirubin, hormones, fatty acids and drugs. Reduced synthesis of albumin over prolonged periods of time produces hypoalbuminaemia and is seen in chronic liver disease and malnutrition. Albumin is also a negative acute phase reactant. The liver also synthesises all coagulation factors: fibrinogen, prothrombin factors and components of the complement system. In the liver amino acids are degraded by transamination and oxidative deamination to produce ammonia, which is then converted to urea and excreted by the kidneys. This is a major pathway for the elimination of nitrogenous waste (Kumar and Clark, 1998).

2. Carbohydrate metabolism

Glucose supplies the metabolic needs of the central nervous system and the red blood cells, and is also oxidised to CO_2 and H_2O with the generation of adenosine triphosphate (ATP) by various tissues including the liver. The liver can be seen as a distribution centre for the coordination of energy metabolism. Following the ingestion of a meal that contains carbohydrate, of each 100g glucose contained in it, for instance, man will store approximately 25% of the carbon in his liver as glycogen. Most of the remainder will be delivered to peripheral muscle for storage, some will be converted by the liver to fatty acids, esterified and transported as very low density lipoproteins from the liver to peripheral adipose tissue for storage as fat. Amino acids, pyruvate and lactate will be used to synthesise glycogen, which will then be delivered to tissues to be used in protein synthesis. Hepatic glycogen is not only a storage form of glucose but is also formed by gluconeogenesis, whose product is routed towards glycogen when there is a sufficient exogenous supply of glucose. Thus, the liver switches between its roles as a source and a sink of glucose (Radziuk, 1990).

3. Immunological function

The liver acts as a 'sieve' for the bacteria and other antigens carried to it via the portal tract from the gastrointestinal tract. These antigens are degraded by Kupffer cells. Kupffer cells are macrophages attached to the endothelium and are activated by factors such as infection. The antigens are degraded without the production of antibodies and in this way, adverse immunological reactions are circumvented. The reticuloendothelial system is also involved in tissue repair, T and B lymphocyte interaction and cytotoxic activity in disease processes (Kumar and Clark, 1998).

4. Lipid metabolism

The liver metabolises lipoproteins, which are protein-lipid complexes formed so that insoluble fats can be transported in the plasma. Phospholipids such as lecithin are synthesised in the liver. The liver produces both very-low-density lipoproteins (VLDL) and high-density lipoproteins (HDL) (Kumar and Clark, 1998). Under normal conditions fatty acids are mainly of dietary origin or derived from adipose tissue but can be produced in the liver from acetate. They will then be converted enzymatically to trigliceride, esterified with cholesterol and incorporated into phospholipids or oxidised to CO_2 or ketone bodies (Podolsky and Isselbacher, 1998A). The liver synthesises cholesterol from acetyl-CoA some of which is then esterified with fatty acids by the lecithin-cholesterol acyltransferase (LCAT) enzyme for which HDLs are the substrate (Kumar and Clark, 1998).

5. Formation of bile

Bile consists of water, electrolytes and conjugated bilirubin and its formation requires the uptake of bile acids and other organic and inorganic ions across the sinusoidal membranes. This is an energy-dependent process. Na^+ and water follow the passage of the salts into the biliary canaliculus by passive diffusion. The average total bile flow is 600ml per day. After a meal, the gallbladder contracts and the sphincter of Oddi relaxes so that bile enters the duodenum. An adequate bile flow is dependant on bile salts being returned to the liver by enterohepatic circulation. Bile acids are synthesised in hepatocytes from cholesterol. The two primary bile acids - cholic acid and chenodeoxycholic acid are conjugated with glycine or taurine, a process which increases their solubility. Bile acids solubilise lipids through the formation of micelles (Kumar and Clark, 1998).

6. Detoxification

The liver is the main organ in which detoxification of foreign substances takes place. Glutathione metabolism is an important detoxification pathway. Glutathione neutralises electrophilic substances, which would otherwise cause hepatic necrosis. Other pathways of detoxification are discussed in more detail on pg. 27: Drug metabolism.

Part C: Liver disease

Liver disease can be categorised into 3 main groups: acute hepatitis, chronic hepatitis and hepatic cirrhosis (Hift and Trey, 1995). Hepatitis is diagnosed when a biopsy reveals inflammatory activity within the hepatic parenchyma. Acute hepatitis refers to evidence of the presence of liver cell injury. This is most readily identified by serum transaminase elevations and by the degree of hepatocellular necrosis and inflammation on biopsy. Clinical features of acute hepatitis include nausea, vomiting, fever, jaundice, leukocytosis and abdominal pain. Hepatomegaly with a tender liver due to distention of the hepatic capsule by fatty infiltration is also common. Chronicity refers to the continuing or recurrent disease and abnormal liver enzymes (Isselbacher and Podolsky, 1998). Hepatic cirrhosis describes a liver showing fibrosis, nodule formation and hepatocyte regeneration. Up to 40% of patients are asymptomatic (Hift and Trey, 1995).

1. Acute hepatitis

Acute hepatitis is a nonspecific clinical syndrome of variable severity that results from inflammation and necrosis of the hepatic parenchyma. Usually it is characterised by elevated serum aminotransferase levels. Jaundice, nausea, vomiting and pain may or may not be present. Most cases resolve spontaneously within 6 months, however a few are fatal and others progress to chronic liver disease (Gholson and Bacon, 1993). Hepatotropic viruses (A, B, C, D and E), drugs and toxins are the main causes of acute hepatitis (Dienstag and Isselbacher, 1998). Drug-induced hepatitis will be discussed below on pg. 26.

1.1 Acute viral hepatitis

Acute viral hepatitis occurs after an incubation period that varies according to the type of virus. The prodromal symptoms of acute viral hepatitis are systemic, quite variable and nonspecific. The onset of jaundice is preceded by symptoms of fatigue, malaise, vomiting, nausea, arthralgias, myalgias, headache, photophobia, pharyngitis and a cough. With the onset of jaundice the prodromal symptoms usually diminish. The liver becomes enlarged and tender and may be associated with right upper quadrant pain

and discomfort. During the recovery phase, constitutional symptoms disappear but usually some liver enlargement and abnormalities in liver biochemical tests are still evident. The duration of the posticteric phase ranges from 2-12 weeks. Complete clinical and biochemical recovery should be expected 1-2 months after all uncomplicated cases of hepatitis A and E and 3-4 months after the onset of jaundice in most cases of hepatitis B and C (Dienstag and Isselbacher, 1998). There is no proven therapy for acute viral hepatitis and therefore most therapeutic efforts are invested in prevention (Gholson and Bacon, 1993).

1.1.1 Hepatitis A virus

Hepatitis A virus is a ribonucleic (RNA) virus with a hexagonal capsid composed of 3 major structural polypeptides that make up the hepatitis A antigen (Gholson and Bacon, 1993). Most people in developing countries have hepatitis A antibodies by the age of 20. Hepatitis A is spread by the faecal-oral route through person-to-person contact. Poverty and bad sanitation are associated with hepatitis A. Progression to chronic liver disease does not occur with this type of virus. Recent infection can be diagnosed by hepatitis A virus IgM antibodies, which remain present in the serum 45-60 days after the onset of symptoms. Anti-hepatitis A IgG is seen during the early phase of the illness but persists for many years and indicates immunity (Rob*son et al*, 1995).

1.1.2 Hepatitis B and D viruses

Hepatitis B virus is also called the Dane particle. It is a deoxyribonucleic (DNA) virus. The inner core of the virus contains the hepatitis B core antigen (HbcAg). The HBeAg is a soluble component of the core antigen and is detected in the blood of patients infected with hepatitis B (Robson *et al*, 1995). The virus itself is not cytopathic. Instead cytotoxic T-cells and natural killer cells mediate hepatocyte damage. An additional factor important in the development of hepatitis B virus is integration of the viral DNA into the host's genome (Gholson and Bacon, 1993). Raised ALT and AST levels together with serological hepatitis markers are used to diagnose hepatitis B. Hepatitis B surface antigen (HBsAg) is present for 3 months after which anti-HBsAg appears and remains for a prolonged period of time. Anti-HBc IgM appears early in the disease and disappears after 6 months. The disease is usually cured within 6 months

with only 5-10% of adults developing chronic hepatitis. Chronic hepatitis B virus carriers are defined as people in whom HbsAg persists for longer than 6 months and HbeAg for longer than 3 months. Hepatitis B is acquired through blood products and sexual transmission (Robson *et al*, 1995).

Hepatitis D virus or delta agent is an incomplete protein-coated RNA virus (Gholson and Bacon, 1993) that can only exist in patients who are HBsAg-positive, as it is dependent on the hepatitis B virus for replication. It is directly cytopathic and can present as a superinfection or co-infection. Superinfection leads to chronic infection while co-infection can cause fulminant hepatitis (Robinson, 1995). Hepatitis D is very uncommon in South Africa (Dusheiko *et al*, 1989).

1.1.3 Hepatitis C virus

Hepatitis C virus is a single stranded, lipid-coated RNA virus that is the major cause of post-transfusion hepatitis. Post-transfusion hepatitis infects approximately 0.5% transfused patients and over 80% of these cases have serologic evidence of hepatitis C virus (Gholson and Bacon, 1995). It is transmitted through blood and blood products. It is usually asymptomatic but hepatitis C may progress to chronic liver disease. Hepatitis C virus has a strong association with hepatic carcinoma. The virus can be diagnosed through fluctuating ALT levels and antibodies against hepatitis C serve as a screening marker. The results can be confirmed with polymerase chain reaction (PCR) (Robson *et al*, 1995).

1.1.4 Hepatitis E virus

Hepatitis E virus resembles the hepatitis A virus but is serologically distinct from it. It is a non-enveloped RNA virus. Hepatitis E is an acute illness, which is self-limiting. It occurs mainly in developing countries. Hepatitis E is spread by contaminated water and food. Infectious virions pass from the liver to the intestine via the bile, thereby infecting the stool (Gholson and Bacon, 1993). Hepatitis E viral antigen is expressed in the cytoplasm of hepatocytes. Specific anti-hepatitis E viral antibodies are found in acute serum (Robson *et al*, 1995). Chronic liver disease resulting from hepatitis E infection has not been described. The acute illness is characterised by modest

elevations in bilirubin and aminotransferases. Mortality rates of 10% have been described among pregnant women (Gholson and Bacon, 1993).

2. Chronic Hepatitis

Chronic hepatitis is defined as a chronic inflammatory reaction in the liver continuing without improvement for at least 6 months. Subjects may display a range from no clinical symptoms to incapacitating exhaustion. Features of symptomatic portal hypertension such as ascites and bleeding oesophageal varices, are associated with the development of cirrhosis. Biochemical tests show a variably elevated serum bilirubin level. Serum transaminase values are usually increased and the γ-globulin concentration is also elevated (Sherlock and Dooley, 1993). Chronic hepatitis is characterised histologically by interface necrosis with inflammatory infiltrates consisting mainly of mononuclear cells (Robson and Neuberger, 1995). There are three main etiological agents associated with chronic hepatitis. These are chronic hepatitis B infection, chronic hepatitis C infection and autoimmune diseases (Sherlock and Dooley, 1993).

2.1 Chronic viral hepatitis

Chronic viral hepatitis only occurs with hepatitis C, B and D. Progression from acute viral hepatitis to chronic viral hepatitis depends on a combination of continuing viral replication in the liver and the immunological status of the patient (Sherlock and Dooley, 1993). It also varies with age. Infection at birth is associated with a 90% chance of developing chronic viral hepatitis while infection in young adulthood only carries with it a 10% chance of progression to chronicity (Dienstag and Isselbacher, 1998A). The virus is not directly cytopathic and lysis of infected hepatocytes depends on the immune response of the host. Those developing chronic hepatitis show a poor cell-mediated response to the virus. If the response is particularly poor, the virus may continue to proliferate without causing any damage to the liver. Such a patient would be an apparently healthy carrier. The liver of this type of patient will show large amounts of HBsAg in the absence of hepatocellular necrosis, but the response is insufficient to clear the virus and a chronic hepatitis results (Sherlock and Dooley, 1993). Patients who have chronic hepatitis B virus may also be infected with chronic hepatitis D, however at a rate no higher than the rate of chronicity of hepatitis B. Although hepatitis D infection increases the severity of acute hepatitis B infection, it

does not increase the probability of progression to chronic hepatitis B. However once a person already infected with chronic hepatitis B is also infected with hepatitis D, the liver disease is expected to worsen. A distinguishing feature of chronic hepatitis D is the presence of anti liver-kidney microsomes (anti-LKM3) (Dienstag and Isselbacher, 1998A).

2.2 Chronic autoimmune hepatitis

Chronic autoimmune hepatitis is a condition that predominates in young people. Half of the patients present between the ages of 10 and 20 and three quarters are female (Sherlock and Dooley, 1993). It is characterised by continuing hepatocellular necrosis and inflammation, usually with fibrosis, which tends to progress to cirrhosis and liver failure. This type of chronic hepatitis may have a 6-month mortality of as high as 40% (Dienstag and Isselbacher, 1998A). Serum γ-globulin levels are extremely elevated and tissue antibodies, smooth muscle antibodies and mitochondrial antibodies are found in most patients (Sherlock and Dooley, 1993). This disease is the result of a cell-mediated immunologic attack directed against liver cells. Predisposition to autoimmmune hepatitis is probably inherited while the liver specificity of this injury is triggered by environmental factors such as chemical or viral agents (Dienstag and Isselbacher, 1998).

3. Cirrhosis

In cirrhosis normal liver architecture is altered by nodular regeneration and fibrosis. Cirrhosis can be categorised under three main classes: biliary cirrhosis, postnecrotic cirrhosis and portal cirrhosis. Portal cirrhosis includes all other forms of cirrhosis with alcohol being the main cause (Gholson and Bacon, 1993B). Biliary cirrhosis results from injury to or prolonged obstruction of either the intrahepatic or extrahepatic biliary system. It is associated with impaired biliary excretion, destruction of hepatic parenchyma and progressive cirrhosis (Podolsky and Isselbacher, 1998).

4. Alcoholic liver disease

Alcoholism is very prevalent in the Western Cape and is a common cause of hepatitis. It is therefore a confounding factor in this study. The three principal alcohol-induced hepatic lesions are alcoholic fatty liver (steatosis), alcoholic hepatitis and alcoholic cirrhosis (Podolsky and Isselbacher, 1998). Approximately 10-15% of alcoholics will develop cirrhosis in their lifetime. The risk is dependent on the duration of alcohol abuse and the quantity of consumption and genetic predisposition. In men consumption of >80g/day and in women >40g/day alcohol predisposes to cirrhosis (Hift and Trey, 1995).

4.1 Metabolism of alcohol

Ethanol diffuses easily through membranes because it is a small polar substance. It is absorbed primarily in the small intestine. After absorption, ethanol is carried to the liver via the portal circulation before entering the systemic circulation. Almost all ethanol metabolism occurs in the liver although 2-10% of absorbed ethanol is eliminated through the kidney and the lungs (Lieber, 1980). There are four different pathways by which alcohol is metabolised. They occur in the cytoplasm, the endoplasmic reticulum, the peroxisomes and the mitochondria (Arias *et al*, 1988).

4.1.1 Cytoplasm

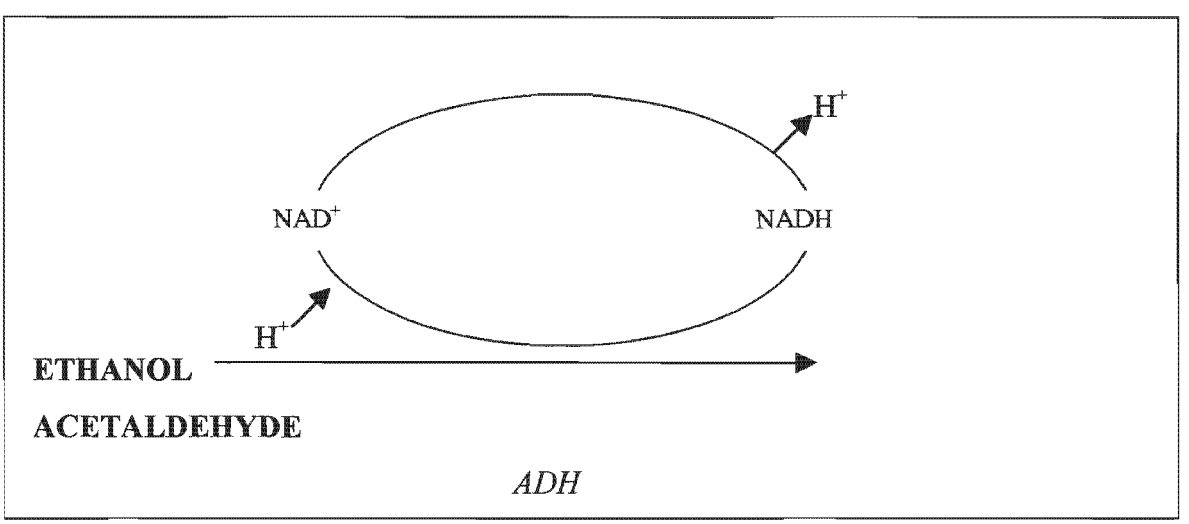


Figure 3: Metabolism of ethanol in the cytoplasm

Alcohol dehydrogenase (ADH) is a non-specific enzyme that metabolises a range of substances, some of which have toxic products. The accumulation of acetaldehyde has deleterious effects such as increased membrane permeability and protein assembly, disorganisation of microtubular structures, disturbed export protein secretion, enhanced display of altered cell surface antigens and inhibition of enzymes responsible for the metabolism of drugs and toxins. Acetaldehyde is metabolised by aldehyde dehydrogenase (ALDH). Chronic alcohol consumption leads to increased synthesis and decreased metabolism of acetaldehyde (Hift and Trey, 1995)

4.1.2 Endoplasmic Reticulum

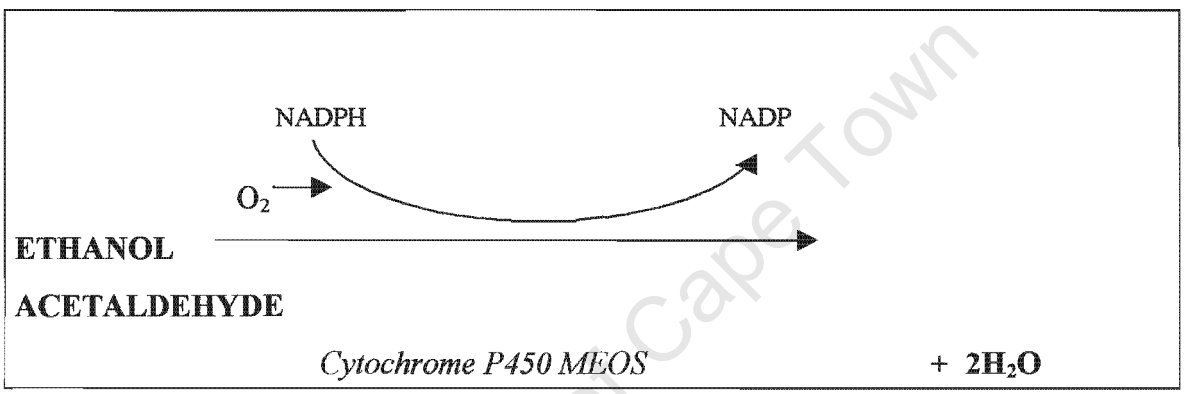


Figure 4: Metabolism of ethanol in the endoplasmic reticulum

The microsomal ethanol oxidising system (MEOS) is a subfraction of the P450 family. Chronic intake of alcohol at high concentrations leads to the induction of the MEOS after a period of a week or more of exposure (Lieber, 1984 and Alderman *et al*, 1984). Drugs like rifampicin will be more quickly metabolised, increasing the tolerance of the patient to the drug. Induction of enzyme activity may result in increased breakdown of the drug to toxic products putting the alcoholic at greater risk of drug toxicity. The alcoholic will also be more sensitive to hepatotoxic effects of other drugs metabolised by the liver, particularly those with affinity for ethanol related P450 eg. isoniazid. Once the ingestion of alcohol ceases, P450 activity returns to normal within a week (Hift and Trey, 1995).

4.1.3 Peroxisome

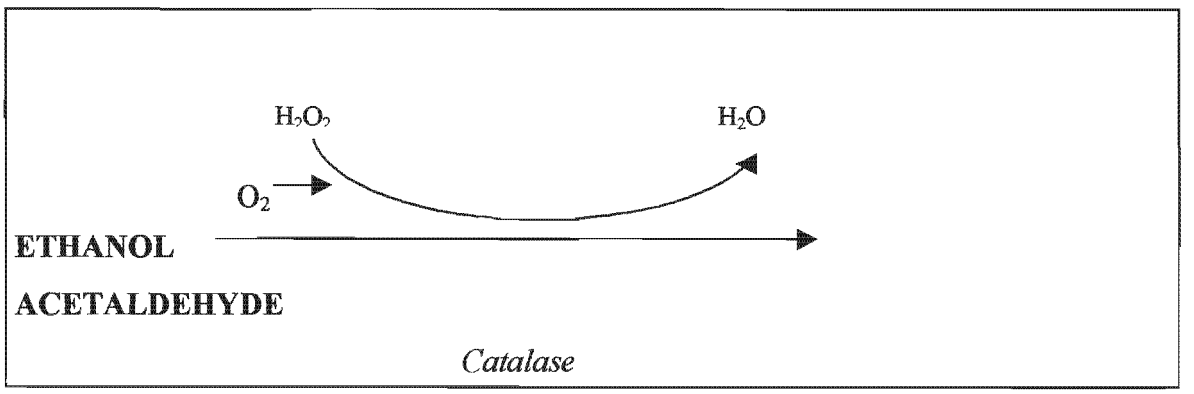


Figure 5: Metabolism of ethanol in the peroxisome

The peroxisome catalase system contributes minimally to ethanol metabolism.

4.1.4 Mitochondria

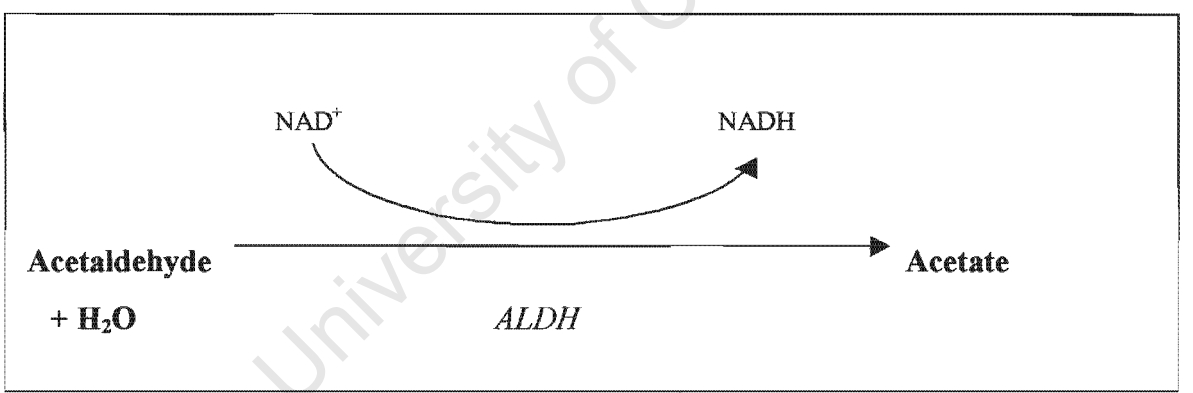


Figure 6: Metabolism of ethanol in the mitochondrion

Aldehyde dehydrogenase is responsible for the majority of acetaldehyde oxidation *in vivo* (Lindros, 1978). Increased production of acetaldehyde reflects increased oxidation of ethanol by ADH and MEOS and decreased catabolism of acetaldehyde. This occurs due to decreased ALDH activity (Lieber, 1984B).

4.2 Mechanism of alcohol-induced liver disease

While ethanol and even more importantly its metabolites appear to be primarily responsible for the toxic effects of alcohol, other alcohols, aldehydes and organic molecules in alcoholic beverages may contribute to tissue toxicity (Arias *et al*, 1988)

The main mechanism of alcohol toxicity is through the production of NADH and acetaldehyde. NADH is a reducing agent and its excess production in the metabolism of ethanol by ADH results in a marked shift in the cytosolic redox potential. Ethanol oxidation by the ADH-mediated reaction is thought to be the rate-limiting step in the metabolism and elimination of ethanol (Lieber, 1984). To regenerate NAD, large quantities of protons have to be managed by the body. Pyruvate is converted to lactate but because the citric acid cycle requires pyruvate there is a depletion of glycogen due to decreased gluconeogenesis. This leads to hypoglycemia. In the long term the protons are accommodated by the kidneys. This leads to reduced uric acid secretion, hyperuricemia and gout. Protein synthesis is impaired and lipid peroxidation is increased. This damages cell walls. Increased proton concentrations may also alter the balance between synthesis, export and breakdown of hepatic lipid, thus contributing to a fatty liver (Hift and Trey, 1995). Chronic alcoholics tolerate more alcohol due to a central nervous system adaptation (Lieber (1984A) however this is deleterious to the body as more harmful by-products are generated leading to further damage. The high levels of aldehyde associated with chronic alcohol intake appear to alter mitochondrial function and specifically ALDH activity, and may reduce the activity of various shuttles involved in the placement of reducing equivalents (Lindros, 1978).

4.3 Clinical syndromes of alcoholic liver disease

Alcoholic fatty liver is very common and can be observed after only 1 week of high alcohol exposure. Alcohol causes increased lypolysis of peripheral adipose tissue with increased delivery to the liver and inhibition of fatty acid oxidation. Hepatic synthesis is also increased using acetate from alcohol metabolism (Hift and Trey, 1995). After prolonged alcohol consumption chronic alcoholic hepatitis and eventually cirrhosis will develop along with all the characteristic clinical features of these diseases.

4.4 Hematological manifestations of alcoholic liver disease

Alcohol adversely effects the normal functioning of enzymes. It has a toxic effect on blood and bone marrow. It decreases folate absorption, inhibits its metabolism and may increase its excretion. Alcohol will allow for greater delivery and absorption of iron because the blood vessels dilate and the blood flow is greater. Macrocytosis is a good indicator of alcoholic liver disease and slowly responds to alcohol withdrawal (Hift and

Trey, 1995). Alcohol blocks conversion of pyridoxine to pyridoxal phosphate resulting in pyridoxine deficiency. Alcohol impairs the ability of granulocytes to combat infection. There is often lymphopenia with reduced T-cell numbers and impaired blastogenesis (production of immature cells) *in vitro*. Platelet production is decreased. Thrombocytopenia, which occurs due to the toxic effect of alcohol in megakaryocytes will improve when alcohol is withdrawn (Robson and Chisholm, 1995).

4.5 The interaction of ethanol with drugs

One major mechanism by which ethanol is thought to influence drug metabolism is through its effect on hepatic microsomes. Chronic ethanol consumption leads to the induction of the ethanol-specific cytochrome P450 – MEOS (Lieber, 1984) resulting in a greater substrate affinity (Lieber, 1984B). The administration of ethanol to humans results in the accelerated clearance of meprobamate and pentobarbital. Aminopyrine, tolbutamide, propanol, warfarin, phenytoin and isoniazid have increased metabolism in the presence of chronic ethanol ingestion and thus the therapeutic doses of those drugs may differ between alcoholics and non-alcoholics (Lieber, 1984). Metabolic upregulation can last several weeks following the discontinuation of chronic alcohol use and therefore drug metabolism can remain altered even in abstaining alcoholics. However these effects occur primarily in the setting of chronic alcoholism. With acute alcohol ingestion an inhibitory effect on drug metabolism may be noted due to competition for the mutually shared microsomal pathway (Lieber, 1984B). The induction can also alter the metabolism of hepatotoxins eg. Isoniazid, carbon tetrachlorides, bromobenzene and paracetamol (Arias *et al*, 1988).

5. Drug-Induced liver disease

Drug-induced liver injury is one of the important causes of acute and chronic liver disease among adults. Although drug-induced hepatotoxicity is generally reversible, if the drug is not discontinued, it may be fatal. The liver is very susceptible to toxicity mainly due to the fact that it is exposed to high concentrations of drugs on a continual basis. This is because it is the main organ in which drug metabolism occurs and because orally ingested compounds are delivered via the portal vein from the gut to the liver (Feinberg, 1981). Usually the drug metabolites are the toxic agents and therefore drug metabolism plays a major role in drug-induced hepatotoxicity.

Additional factors increase the likelihood and severity of hepatic drug reactions. Advanced age tends to increase susceptibility to hepatotoxicity although the reason for this is not entirely clear. In neonate rats the drug-metabolising enzyme systems have not yet been developed and therefore less toxic metabolites will be formed. The tendency for females to develop hepatotoxicity to a larger degree than males has also been observed. Nutritional status is another contributing factor. It is widely accepted that malnutrition in general and protein deficiency in particular, enhance the adverse effects of all hepatotoxic agents. This is due to the depletion of tissue glutathione as it is involved in detoxification of some metabolites. Protein deprivation leads to depressed activity of the mixed-function oxidase system and of the cytochrome P-450 content of the smooth endoplasmic reticulum. This causes a decrease in drug metabolism and thus a decrease in metabolite concentration in the body. However protein deficiency also leads to decreased detoxification by glutathione, so even though the overall concentration of metabolite is lower, the metabolite is more toxic because it is not being disposed of. Generally the metabolite is more toxic than the parent drug and hepatotoxicity is increased with protein deficiency. The fatty liver that results from protein and choline deficiency can contribute to hepatotoxicity by permitting greater affinity for and more prolonged storage of lipid-soluble toxins. (Zimmerman, 1978).

5.1 Drug metabolism

The mixed-function oxidase enzyme system is responsible for the metabolism of foreign compounds. It is an integral part of the smooth endoplasmic reticulum and is attached to the lipid bilayers of its membranes (Zimmerman, 1978). The drug-metabolising enzymes are highly non-specific (Brodie et al, 1958). Most drugs are lipophilic and circulate in the blood bound to plasma proteins. In the hepatocyte they are converted to metabolites of greater polarity that are more easily excretable. Biotransformation is achieved in two phases. The addition of a polar group through oxidation, reduction or hydrolysis is referred to as the non-synthetic phase and is carried out by a system of microsomal enzymes referred to as mixed-function oxidases. The conjugation reactions are referred to as the synthetic phase (Williams, 1959). Phase I non-synthetic reaction can be summarised as follows:

$$NADPH + O_2 + Drug \longrightarrow NADP^+ + H_2O + Oxidised\ Drug$$

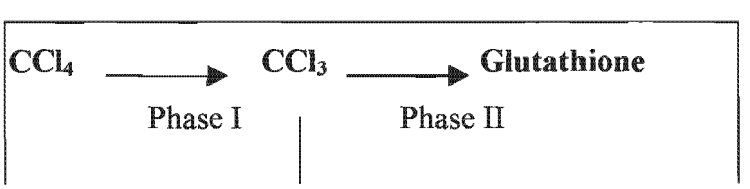
The terminal oxidase of the mixed function oxidase system is a carbon monoxide-sensitive cytochrome that absorbs light at 450nm and is hence called cytochrome P450. It reacts with NADPH, activates O_2 and transfers it to the drug (Gillette JR, 1966 and Feinberg, 1981). Phase I reactions usually result in reactive, toxic metabolites, the accumulation of which, causes liver damage. The liver prevents the reactive intermediate products of phase I from causing damage by detoxifying the metabolites through phase II synthetic conjugations. In phase II reactions a glucuronate, sulphate or glycine group is added to the polar group of the intermediate metabolite, resulting in a water-soluble compound that can be excreted via the kidneys or bile (Hift and Trey, 1995). Occasonially the phase II products are more electrophilic than the phase I intermediate and are more toxic (Feinberg, 1981).

5.2 Mechanisms of drug-induced liver damage

There are two main types of adverse drug reactions. Type A drug reactions are a result of the intrinsic toxicity of the drug and are dose-dependent. Type B drug reactions are dose-independent, idiosyncratic and unpredictable (Anderson, 1981).

5.2.1 Type A drug-induced liver injury

Type A reactions usually result from the known pharmacological effect of the drug. Usually these reactions are less serious and rapidly resolve on stopping the drug (Kumar and Clark, 1998). There are two types of intrinsic hepatotoxins: direct and indirect. Direct hepatotoxins are agents, which by a direct attack on the hepatocyte, disrupt it, destroy its organelles and thus destroy the structural basis of cell function. Carbon tetrachloride (CCl_4) is an example of a direct hepatotoxin (Zimmerman, 1975). When CCl_4 is overdosed the concentration of its toxic metabolite, CCl_3, increases and necrosis ensues (Hift and Trey, 1995).



Vitamin E scavenging

Figure 7: Mechanism of carbon tetrachloride toxicity

Indirect hepatotoxins are antimetabolites and related compounds, which produce hepatic injury by the diversion or competitive inhibition of essential metabolites or by other forms of interference with specific metabolic or secretory processes of the hepatocyte. Indirect hepatotoxins can be cytotoxic or cholestatic. Cholestatic hepatotoxins produce jaundice or hepatic dysfunction by selective interference with mechanisms for excretions of substances into the bile or uptake from the blood (Zimmerman, 1975). Paracetamol is an indirect cytotoxic hepatotoxin and when given in high concentrations, the toxic phase I metabolite N-acetyl-p-benzoquinone imine (NAPQI) accumulates and arylates or oxidises sulphydryl groups on critical cellular enzymes with resultant injury (Hift and Trey, 1995).

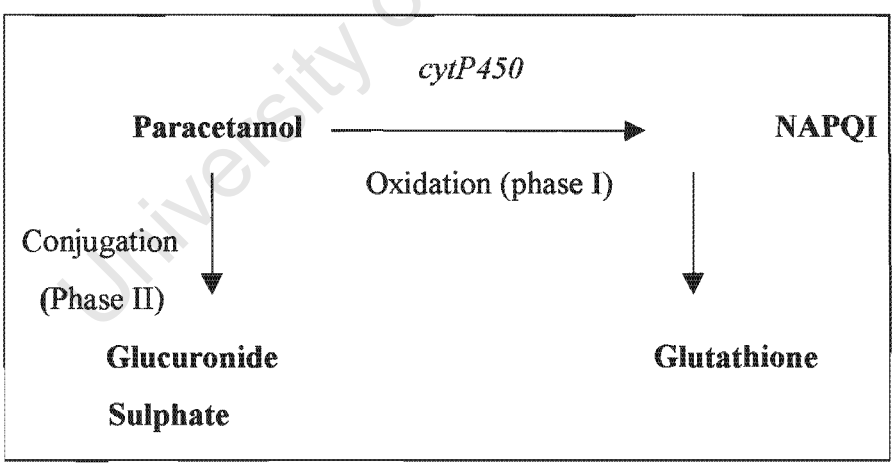


Figure 8: Mechanism of paracetamol toxicity

5.2.2 Type B drug-induced liver injury

Liver injury caused by therapeutic agents is generally dose-independent, unpredictable and often related to variations in the way individual patients metabolise drugs or dispose of their active metabolites (Feinberg, 1981). Genetic polymorphisms of

cytochrome P450 enzymes may predispose a patient to hepatotoxicity if toxic metabolite production is increased, if detoxification of these reactive species is limited or if there is a delayed response to rechallenge. The metabolism of isoniazid by the N-acetyl transferase enzyme is a good example of genetic variability, which may effect hepatotoxicity (Hift *et al*, 1995).

Idiosyncratic reactions can be immunologically mediated. Prior to biotransformation, parent drugs are largely poorly immunogenic. In order to become immunogenic these small organic molecules have to form haptenmacromolecular complexes by making stable bonds with circulating or tissue proteins. The reactive metabolites are more likely to form these complexes. Cellular proteins that have already been altered by damage caused by the reactive species are more likely to be immunogenic when bound to the metabolite. Drug-induced antigens are formed in the hepatocyte but must be exposed to the membrane to elicit an immune response. This occurs when hepatocytes are damaged or if the antigens are expressed on the hepatocyte membrane. Therefore a patient with pre-existing liver disease is more predisposed to drug toxicity. Susceptibility to drug toxicity can vary according to genetic traits. Theoretically patients that produce immunogenic metabolites or patients who are more capable of making haptenmacromolecular complexes, will be more susceptible to drug toxicity. Not only reactive drug metabolites can be immunogenic. If the drug metabolites alter endogenous macromolecules these compounds may also become immunogenic. Dose-independent hypersensitivity will result after a shorter period of exposure to the drug than dose-dependant hypersensitivity reactions (Hift *et al*, 1995). The onset of idiosyncratic drug-induced liver disease is rapid and can be life-threatening. The transaminases are elevated to extremely high levels (Kumar and Clark, 1998).

5.3 Drug-Induced Cholestasis

Cholestasis is an idiosyncratic reaction that often mimics obstructive biliary disease. In the formation and secretion of bile, solutes, bile acids and water from sinusoidal blood, pass through the cytosol of the hepatocyte to the bile canalicular lumen. The Na^+/K^+ pumps in the hepatocytes provide the energy for the uptake and secretion of bile acids in the hepatocyte. Specific protein carriers in the sinusoidal and canalicular membranes transport bile acid from plasma to bile. Bile acid flow is dependent on osmotic

pressure, bile acid molecules and passive diffusion. If any of these mechanisms are upset bile flow will stop or cholestasis will occur (Hift *et al,* 1995).

There are 3 types of syndromes of drug-induced cholestasis. The first is non-inflammatory or 'bland' isolated intrahepatic cholestasis, in which centrilobular accumulation of bile occurs within the hepatocytes and bile canaliculi. The second is cholestatic hepatitis, characterised by a combination of cholestasis and features of inflammation such as hepatocellular degeneration and a mixed mononuclear cell and eosonophilic infiltrate in portal tracts and the lobular parenchyma. Chlorpromazine and erythromycin have been known to cause cholestatic hepatitis. The final syndrome of drug-induced cholestasis is bile duct damage (ductal or cholangiodestructive injury). It resembles primary biliary cirrhosis in its clinical, histological and laboratory features. Psychotropic agents such as carbamazepine which are subject to genetic polymorphisms that influence their metabolism via different pathways of oxidation, sulphoxidation and hydroxylation. Aberations in drug metabolism may lead to the generation of toxic metabolites that trigger an autoimmune reaction resulting in chronic bile duct injury (Hift *et al*, 1995).

5.4 Factors accounting for variations in drug response

Variability in handling drugs usually arises from differences in rates of drug biotransformation in hepatic microsomes whereas variability in type or degree of response may be attributed to receptor sites on which the drugs act. Responsiveness to many drugs is directly related to their concentrations in plasma (Vesell, 1972). There are a number of factors that are critical in the variability of drug plasma levels. The most obvious factor is drug dose and formulation. If the dose and formulation in two patients is different, the accumulation of drug in the plasma will also be different. Absorption is another important factor. The amount of orally ingested drug reaching the circulation is referred to as 'bioavailability'. Liver disease may impair absorption and the 'first-pass effect' (when a drug is metabolised in the liver before it even reaches the circulation) can account for interindividual variation in plasma drug levels. Once the drug is absorbed in the systemic circulation, the concentration of drug is determined by its distribution within the body. This can be affected by the extent of drug-protein binding. If less drug is bound, more free drug will be available to enter into the tissue. Only free drug can cross the tissue membrane barrier. The total drug

concentration in plasma will decrease as a result. Variability in drug elimination can also cause a difference in drug response. Individuals with liver disease may have diminished drug clearance, which could result in an increase in plasma drug concentration (Nies and Gal, 1981). Genetic factors and drug interactions also play an important role in the interindividual variation of drug metabolism (Hift *et al*, 1995).

5.5 Antituberculosis drugs as the cause of liver disease

Antituberculosis drugs have been shown to account for 50% of the severe drug-induced liver injury seen in Southern Africa (Hift *et al*, 1995). The antituberculosis drugs cause acute hepatitis which is dose-independent. Isoniazid is known to cause liver injury through the production of toxic metabolites. The mechanisms by which rifampicin and pyrazinamide cause liver toxicity, are unclear.

5.5.1 Isoniazid-induced hepatotoxicity

Isoniazid is one of the main culprits of drug-induced hepatotoxicity (Cohen *et al*, 1983; Dickinson *et al*, 1981; Ellard *et al*, 1978; Mitchell *et al*, 1976). It causes elevation of serum AST levels more than twice the normal in 10-20% of persons taking the drug. These abnormalities are usually transient and represent mild focal hepatitis. The average duration of isoniazid monotherapy prior to the onset of jaundice is 3 months and almost all clinical hepatitis occurs within 6 months (Melikian, 1993). Overt hepatitis arises in about 1% of cases (Black, *et al*, 1975). The risk of hepatitis increases from 2.8 per 1000 subjects under the age of 35 to 7.7 per 1000 subjects over 55 years of age (Riska, 1976). Clinical features of isoniazid induced hepatic injury resemble those of acute viral hepatitis and alcoholic hepatitis (Black *et al*, 1975). Biopsy of the liver reveals diffuse degeneration and necrosis. Liver sections of fatal cases show massive or submassive necrosis (Zimmerman, 1975).

Although immunologically mediated liver injury is possible, isoniazid toxicity is mainly caused by toxic metabolites (Sherlock and Dooley, 1993). Isoniazid and acetylisoniazid are hydrolysed to monoacetylhydrazine. Monoacetylhydrazine can be metabolised by 3 routes: excreted as a hydrazone, acetylated to diacetylhydrazine or N-hydroxylated by the cytochrome P-450 system into a highly electrophilic acyl radical (Holdiness, 1984). The first two are detoxification pathways whereas the third product is associated with

hepatotoxicity (Mitchell *et al*, 1976 and Lauterberg *et al*, 1985). The radicals cannot be quenched especially by elderly people (Leibovitz and Siegal, 1980 and Girling, 1978) and they bind to liver proteins and cause liver necrosis in both man and rats (Mitchell *et al*, 1976; Timbrell *et al*, 1980; Lauterburg *et al*, 1985). Hydrazine is another toxic metabolite of isoniazid, which has been implicated in drug-induced hepatotoxicity (Woo *et al*, 1992). The mechanisms by which hydrazine causes hepatotoxicity, are different to the mechanism by which monoacetylhydrazine and acetylisoniazid cause hepatotoxicity. This is evident from the different type of hepatocellular damage that results by the two routes. Hydrazine is produced in smaller quantities than acetylisoniazid and monoacetylhydrazine and is thus less harmful (Walubo *et al*, 1998). Enzyme inducers were shown to protect against hydrazine toxicity as it was metabolised more quickly to the non-toxic pyruvic hydrazone metabolites (Jenner and Timbrel, 1994). Rifampicin combined with isoniazid have been shown to increase the hepatotoxicity of isoniazid. This is thought to be the result of induction of the cytochrome P450 isoenzymes CYP3A (induced by rifampicin) and CYP2E1 (induced by isoniazid). These findings indicate that the CYP3A and CYP2E1 isoenzymes are involved in the metabolism of isoniazid to its toxic metabolites and that isoniazid induces its own metabolism (Nicod *et al*, 1997).

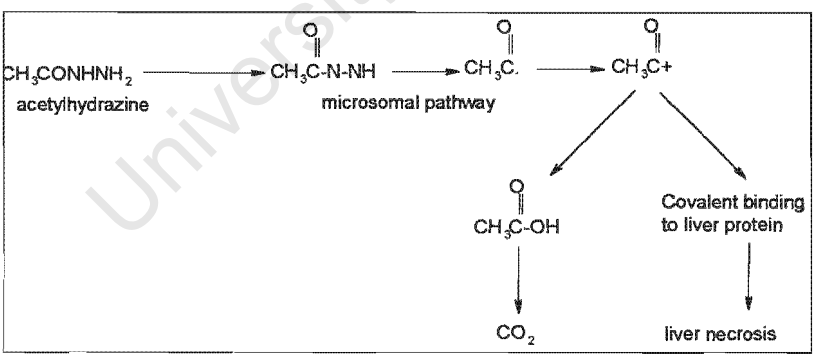


Figure 9: Mechanism of liver damage by monoacetylhydrazine (Timbrell *et al*, 1980)

It is unclear whether acetylator status affects isoniazid toxicity. Mitchell *et al* proposed that fast acetylators would be more prone to isoniazid-induced liver disease. Fast acetylators metabolise 46% of the isoniazid as opposed to the slow acetylators who only acetylate 30.5%. Fast acetylators are therefore exposed to higher concentrations of monoacetylhydrazine than slow acetylators (Mitchell *et al*, 1975). However this does not take into account that monoacetylhydrazine is acetylated to diacetylhydrazine

(a non-toxic intermediate) by the same enzyme pathway that acetylates isoniazid. Therefore although monoacetylhydrazine is being formed quicker in fast acetylators, it is also being detoxified quicker and therefore slow and fast acetylators are exposed to the same amount of toxic intermediate after a 300mg dose (Ellard and Haslam, 1976). There is an opinion, however, that slow acetylators are likely to be exposed to higher concentrations of monoacetylhydrazine because the half-life of monoacetylhydrazine is 5 times greater that of isoniazid. Also if high doses of isoniazid are given such as 10mg/kg, then saturation of acetylation may occur (Lauterberg *et al*, 1985, Ohno *et al*, 2000 and Yew, 2001). However a clinical trial done on 3000 Indian patients showed that there was no correlation between isoniazid hepatotoxicity and acetylator phenotype (Gurumurthy *et al*, 1984).

The combination of isoniazid with an enzyme inducer such as rifampicin increases the risk of isoniazid hepatotoxicity (Steele *et al*, 1991). Anaesthetic drugs and alcohol may also enhance isoniazid toxicity. Para-amino salicylate, on the other hand, is an enzyme retarder and this may account for the relative safety of the para-amino salicylate combination formerly used in the treatment of tuberculosis (Sherlock and Dooley, 1993).

The major route of isoniazid elimination is through hepatic metabolism, therefore dosage modification in patients with severe hepatic failure may be indicated. Isoniazid's half-life has been observed to increase in patients with severe liver injury therefore accumulation of toxic metabolites would be a risk factor for these patients (Melikian, 1993).

5.5.2 Rifampicin-induced hepatotoxicity

Rifampicin alone only rarely causes hepatitis. Lesions are characterised by hepatocellular changes with centrilobular necrosis, which may be associated with cholestasis. The mechanism of this injury is unknown but is unpredictable and not dose-related (Westphal *et al*, 1994). Rifampicin induces elevation of alanine aminotransferase and alkaline phosphotase activities (Gangadharam, 1986) and causes transient hyperbilrubinemia (Segre, 1972). Hyperbilrubinemia is caused by inhibition (Cohn, 1969) of both uptake and excretion of bilirubin in a dose-related manner giving rise to elevated plasma levels of both conjugated and unconjugated bilirubin (Capelle *et*

al, 1972). Bilirubin levels have become elevated transiently approximately 3 hours after a single dose but return to normal after 24 hours (Steele, 1991). The total exposure of the patient to the drug appears to play an important role in the development of hepatotoxicity (Parthasarathy, 1986).

Studies have shown that hepatitis is more frequent and severe in patients receiving both isoniazid and rifampicin than in those receiving isoniazid alone. This phenomenon is more marked in children than in adults. From the pooled results of a number of studies, the incidence of hepatotoxicity resulting from rifampicin therapy alone was estimated at 1.1% as opposed to 2.5% seen in isoniazid plus rifampicin combination therapy (Steele, 1991). In one study elevated transaminase levels were observed in more than 35% of the patients taking isoniazid and rifampicin together compared to 10% in those taking isoniazid alone (Lees *et al*, 1971). This was also shown in two studies done in Madras and Bangalore. The difference in the incidence of hepatotoxicity may be due to variation in nutritional status, geographical location, drug formulation and age. Hepatitis was confined to patients in the initial phase of antituberculosis therapy, in which they received isoniazid and rifampicin. In the second stage when rifampicin was replaced with ethambutol, the incidence of hepatitis was rare (Parthasarathy *et al*, 1986). It is possible that the increased toxicity seen in patients taking isoniazid plus rifampicin is simply additive, however the hypothesis that the effect might be synergistic was entertained because of the potent inducing effect that rifampicin has on the hepatic P-450 mixed oxidase drug detoxifying pathway. The smooth endoplasmic reticulum, the main site of drug metabolism in the hepatocyte, was observed to proliferate from the second day of rifampicin treatment in almost all hepatocytes. This information correlates well with the finding that rifampicin exhibits a decreased half-life during chronic treatment, showing that it undergoes self-induction (Segre, 1972). Isoniazid is metabolised to acetylisoniazid and monoacetylhydrazine by the P450 system and increased enzyme induction supposedly would lead to the increased production of toxic acyl radicals (Steele, 1991). Whereas the presence of rifampicin would increase the toxicity of acetylisoniazid and monoacetylhydrazine, which need to be further metabolised to become harmful, it would decrease the toxicity of hydrazine, which becomes less toxic once it is metabolised to hydrazones. However hydrazine, being the less significant metabolite of isoniazid with respect to liver toxicity, the overall effect of administering rifampicin in conjunction with isoniazid would be one of increased hepatotoxicity. The reported incidence of hepatotoxic

reactions during rifampicin and isoniazid therapy is much higher in developing countries than it is in the developed world. This is attributed to rampant malnutrition, widespread parasitism, indiscriminate use of drugs, chronic infections, a genetic predisposition and the higher rate of misdiagnosed viral hepatitis, which cannot be distinguished clinically, biochemically or histologically from drug-induced hepatitis (Kumar *et al*, 1991).

Because hepatic metabolism accounts for 85-95% elimination of rifampicin, severe hepatic failure or biliary obstruction may result in accumulation of rifampicin in the serum. Dosage adjustment may be necessary to avoid drug accumulation and toxicity (Melikian, 1993).

5.5.3 Pyrazinamide-induced hepatotoxicity

Pyrazinamide-induced hepatotoxicity is hepatocellular in type, is not associated with hypersensitivity manifestations and has been shown to be dose related. At a 40-50mg/kg daily dosage, pyrazinamide causes acute hepatitis, sometimes fatal. Since this dosage has been halved, hepatic complications have been substantially reduced and are generally mild (Danan *et al*, 1981; Parthasarathy *et al*, 1986 and Citron *et al*, 1980). In a study conducted on alcoholic patients pyrazinamide in therapeutic concentrations was shown not to cause hepatitis (Pilheu, 1981). One study conducted at the Brooklyn Chest Hospital in Cape Town, South Africa, showed conflicting results. It was found that of the cases of drug-induced liver disease, 25% were caused by pyrazinamide, 21.4% were caused by the combination of isoniazid and pyrazinamide and 10.7% were caused by the combinations of pyrazinamide and rifampicin (Cohen *et al*, 1983). This, however is an isolated finding and the majority of the literature points to the observation that pyrazinamide is only toxic at high doses that are not used in the routine regimens of antituberculosis therapy. The mechanism by which pyrazinamide causes hepatotoxicity is unknown.

Part D: Evaluation of liver disease

The wide range of liver functions and the disorders which can accompany them, preclude the use of a single test as a reliable marker for liver disease. Instead, a number of tests should be used, taking into account the total clinical context of the patient (Podolsky and Isselbacher, 1998B).

1. Biochemical Evaluation of Liver Disease

The presence of certain liver enzymes can be used to differentiate between hepatocellular injury and biliary tract dysfunction or obstruction (Podolsky and Isselbacher, 1998B). The serum aminotransferases are primarily nonspecific markers of necrosis and inflammation while alkaline phosphatase and γ-glutamyl transpeptidase are nonspecific indicators of cholestasis (Gholson and Bacon, 1993B).

1.1 Necroinflammatory markers

Aspartate aminotransferase (AST) and alanine transferase (ALT) catalyse the transfer of the γ-amino groups of aspartate and alanine respectively to the γ-keto group of ketoglutarate, leading to the formation of oxaloacetic acid and pyruvic acid (Podolsky and Isselbacher, 1998B). They are commonly measured to screen for hepatocellular dysfunction. Although an elevation of AST may reflect damage to several organs, ALT is more specific to hepatocellular injury (Gholson and Bacon, 1993B). In the hepatocytes ALT is found exclusively in the cytosol while AST can be found both in the cytosol and the mitochondrion. Elevation of AST and ALT levels is found in all forms of liver disease (Podolsky and Isselbacher, 1998B) however absolute and relative values are somewhat useful in differential diagnosis. Liver diseases that result in serum aminotransferase levels exceeding 1000IU/L include viral hepatitis, ischaemic injury, drug-induced hepatitis, flares of autoimmune hepatic disease and early acute common bile duct obstruction. Alcoholic hepatitis results in mild elevation (<400IU/L) with an AST to ALT ratio usually >2 (Gholson and Bacon, 1993B) this is due to pyridoxal 5-phosphate deficiency (Sherlock and Dooley, 1993).

1.2 Cholestatic markers

Alkaline phosphatase (ALP) is a membrane-associated protein. Increased levels of ALP are found in diseases of most organs in which this enzyme occurs. In liver disease high levels are found in cholestasis due to increased mRNA translation stimulated by retained bile. High molecular weight forms of ALP appear in the serum of patients with cholestasis. This may be due to complexes of ALP with lipoprotein x and fragments of plasma membrane that have been released into the circulation (Kirsch *et al*, 1995). Slight to moderate increases in ALP such as 1-2 times the upper limit of normal, occur in patients with parenchymal liver disorders such as hepatitis and cirrhosis. Increases of 10 times the upper limit of normal occur more consistently with extrahepatic biliary tract obstruction or with intrahepatic biliary cirrhosis (Podolsky and Isselbacher, 1998B).

γ-Glutamyl Transpeptidase (γGT) catalyses the transfer of γ-glutamyl groups from peptides such as glutathione to other amino acids. It also catalyses the conversion of xenobiotic glutathione conjugates to mercapturic acid. γGT levels are higher in males than in females and increase with age (Kirsch *et al*, 1995). Serum γGT values parallel the levels of ALP in cholestasis and may be used to confirm that a raised serum ALP is of hepatic-biliary origin. An isolated rise in serum γGT is seen in patients with alcohol abuse, even without liver disease, perhaps because of microsomal enzyme induction (Sherlock and Dooley, 1993).

1.3 Nonspecific enzyme markers

Lactate Dehydrogenase (LDH) is widely distributed in human tissue (Kirsch *et al*, 1995) and for that reason is not a sensitive marker of liver disease. Moderate LDH elevations are common in acute viral hepatitis, cirrhosis and metastatic carcinoma to the liver. Biliary tract disease also may produce slight elevations. Marked elevation of LDH in association with abnormal results of other tests of liver function may reflect a hematologic malignancy such as lymphoma (Podolsky and Isselbacher, 1998B)

1.4 Synthetic function markers

Albumin is quantitatively the most important plasma protein synthesised in the liver. It has a fairly long half-life of 17-20 days with less than 5% turnover daily. There is a substantial reserve of hepatic albumin synthesis, thus adequate synthesis may continue until there is extensive hepatocellular injury. For these reasons it is not a good indicator of acute or mild liver injury. Serum albumin levels are influenced by a variety of nonhepatic factors most notably nutritional status, infection and hormonal factors (Podolsky and Isselbacher, 1998B). Serum concentrations of albumin represent the net result of synthesis, distribution (between the intra and extravascular compartments) and degradation (Kirsch *et al*, 1995). In patients with chronic active hepatitis there is a general increase in all immunoglobulins while the concentration of albumin may be decreased. Cirrhosis is usually accompanied by increasing retention of fluid and a gradual decrease in the concentration of albumin (Fleck, 1990). In acute liver disease, serum albumin levels are usually not affected (Gholson and Bacon, 1993B).

Individuals with liver disease tend to have high levels of IgG and IgA because the bowel-derived antigens bypass the liver directly into the systemic circulation (postsystemic shunt) and there is an increase in antigens due to impaired Kupffer cells. The antigens also bypass the liver causing increased production of antibodies (Kirsch *et al*, 1995).

Abnormalities in serum lipids and lipoproteins are sensitive but nonspecific indicators of liver diseases. Acute parenchymal liver disease is commonly associated with increased plasma triglycerides, decreased cholesterol esters and abnormal lipoproteins. Less marked abnormalities are found in patients with chronic hepatitis. Either intra or extrahepatic cholestasis may lead to an increase in unesterified cholesterol and in serum phospholipids (Podolsky and Isselbacher, 1998B).

Urea synthesis may be reduced in cirrhosis resulting in hyperammonemia and increased amino acid concentrations with a decreased plasma urea (Kirsch *et al*, 1995).

1.5 Indices of hepatocyte uptake and transport

Bilirubin accumulation in the bloodstream to levels above 34-43 μmol/L causes yellow pigmentation of the plasma leading to discoloration of the tissues, and is referred to as jaundice. Approximately 80% of circulating bilirubin is derived from senescent red blood cells. When circulating erythrocytes are destroyed at the end of their lifespan, oxygenation of the haem moiety dissociated from the haemoglobin generates biliverdin. Biliverdin is then metabolised to bilirubin (Kaplan and Isselbacher, 1998). The conversion of haem to bilirubin occurs in the spleen, but once in the plasma, the bilirubin is bound tightly but non-covalently to albumin and is referred to as unconjugated bilirubin. This makes the bilirubin more soluble in plasma. The albumin is recycled back into the plasma and the free bilirubin enters the hepatocyte. Inside the hepatocyte, the bilirubin is conjugated by UDP-glucuronyl transferase into bilirubin diglucuronide or monoglucuronide and is called conjugated bilirubin (Kirsch *et al*, 1995). In normal circumstances only conjugated bilirubin is excreted into bile. Bilirubin excretion is an energy-dependant process limited to the canalicular membrane and is the rate-limiting step in hepatic metabolism of this pigment. Impaired excretion leads to decreased bilirubin concentrations in the bile and concomitant efflux of conjugated bilirubin through the sinusoidal membrane of the hepatocyte into the bloodstream (Kaplan and Isselbacher, 1998). Liver disease and biliary obstruction are the most common disorders associated with jaundice. When the liver cell is damaged, as in viral hepatitis, there is often impairment in all three major hepatic phases of bilirubin metabolism, namely uptake, metabolism and excretion. With complete failure of bilirubin excretion into the intestine (ie. complete biliary obstruction) serum bilirubin levels are extremely high.. High concentrations of unconjugated bilirubin indicate haemolysis, impaired uptake (eg. Gilbert's Syndrome) or impaired conjugation (eg. Gilbert's / Crigler Najjar Syndrome- in which there is no UDP-glucuronyl transferase) (Isselbacher, 1998). Bile acids are the major organic anions excreted by the liver into the gall bladder. Although their pool size and flux is much larger than that of bilirubin, it is difficult to measure bile acid secretion.

2. Hematological Evaluation of Liver Disease

The liver is the major site of hemopoiesis (production of blood cells) in the foetus. The liver synthesises proteins that are involved in haemostasis. It metabolises and stores nutrients such as iron, folate and vitamin B12, which are crucial for blood cell proliferation and maturation. Liver disease is therefore associated with disturbances of red blood cells, white blood cells and platelets (Robson and Chisholm, 1995).

2.1 Blood volume

Plasma volume is frequently increased in patients with cirrhosis, especially with ascites and also with long-standing obstructive jaundice or with hepatitis. However in some patients decreased blood volume may account for low peripheral haemoglobin or erythrocyte level (Sherlock and Dooley, 1995). This is due to overt or occult blood loss.

2.2 Erythrocyte changes

Anaemia is often associated with chronic liver disease. This is due to a number of factors. Increased plasma volume causes dilution of the blood. Gastrointestinal hemorrhage causes blood loss. Impaired bone marrow production results from folate, iron or pyridoxine deficiency and increased consumption by the spleen leads to hypersplenism and erythrocyte membrane lipid abnormalities. Serious anaemia often goes hand in hand with alcoholism, nutritional deficiency and blood loss. The erythrocyte shape, size, volume and haemoglobin content are useful aids in diagnosis (Robson and Chisholm, 1995). Hypochromic erythrocytes often reflect gastrointestinal bleeding. In portal hypertension anaemia follows gastro-oesophageal bleeding, which is enhanced by thrombocytopenia and disturbed blood coagulation. In liver disease the average size of the erythrocytes is usually normocytic. This results from a combination of the microcytosis of chronic blood loss and the macrocytosis inherent in people with liver disease (Sherlock and Dooley, 1995). Severe macrocytosis may occur with alcohol abuse and with folate and vitamin B12 deficiency (Robson and Chisholm, 1995). Hypochromic microcytic cells denote iron

deficiency. Target cells are red blood cells with low concentrations of haemaglobin that are centralised in the cell, resulting in a red blood cell that appears to look like a target. They are often seen in cholestasis as a consequence of bile salt fluxes (Robson and Chisholm, 1995).

2.3 White cell abnormalities

Leukopenia (neutropenia and lymphopenia) is a common feature of chronic liver disease. Alcohol impairs the ability of granulocytes to combat bacterial infection. Hypersplenism results in leukopenia. High white blood cell counts due to release of colony stimulating factors from damaged hepatic parenchymal cells and inflammatory cytokines are also found in acute alcoholic hepatitis. Neutropenia may be seen in viral hepatitis (Robson and Chisholm, 1995). Leukopenia may also occur in cholangitis, malignant disease and viral hepatitis (Sherlock and Dooley, 1993).

2.4 Platelet changes

Abnormalities in platelet numbers, structure and function are common in patients with all forms of liver disease. Reduced platelet numbers is due to increased splenic sequestration resulting from hypersplenism. Platelet-associated antibodies are found in patients with chronic active hepatitis (Sherlock and Dooley, 1993). In alcoholics, thrombocytopenia, thought to be due to a direct toxic effect of alcohol or its metabolites on megakaryocytes, occurs and responds to withdrawal of alcohol, often with transient rebound thrombocytosis (Robson and Chisholm, 1995).

2.5 Coagulation

The liver is the primary site of synthesis of almost all the coagulation proteins. It also synthesises protease inhibitors, which modulate the coagulation cascade, clears activated clotting factors from the blood and produces vitamin K-dependant proteins. Hepatic necrosis is followed by the activation of haemostasis and the defective clearance of activated factors. This leads to disseminated intravascular coagulation and bleeding.

Because the half-life of the clotting factors is so short, reduced concentrations of clotting factors can follow soon after hepatic necrosis (Sherlock and Dooley, 1993). Patients with hepatocellular dysfunction and associated cholestasis are deficient in vitamin K as a result of fat malabsorption because bile acids aren't released into the intestine (Robson and Chisholm, 1995). Prothrombin time before and after 10mg vitamin K intramuscularly is the most satisfactory test for coagulation defect in patients with hepato-biliary disease associated with vitamin K deficiency (Sherlock and Dooley, 1993). International normalising ratio (INR) is also a sensitive marker of synthetic function.

Chapter 2

Aims and Objectives

It is well established that rifampicin plays a role in the development of hepatotoxicity in a small proportion of the population (Steele, 1991). The mechanism of rifampicin hepatotoxicity is not known, however. The metabolic products of a drug are often more toxic than the drug itself. This is clearly the case with isoniazid. It was therefore postulated that the mechanism by which rifampicin may cause hepatotoxicity is through its metabolites. In order for a metabolite to exhibit its deleterious effect, it is necessary for it to be present at the site of its toxic action. An indication of its presence at this site is the presence of the metabolite in the serum of the patient. If it can be shown that there is a difference in the concentration of rifampicin and its metabolites in the serum of patients with and without liver enzyme abnormalities, this could be the first step in determining the mechanism of rifampicin hepatototoxicity. To this end the following aims and objectives were compiled.

1. Aim

To determine whether there is a correlation between raised liver enzyme levels and the concentration of rifampicin and its metabolites 3-formyl rifampicin and 25-desacetyl rifampicin in the serum of patients infected with tuberculosis.

2. Objectives

2.1 To develop an HPLC assay for the detection of the metabolite of rifampicin: 3-formyl rifampicin in serum and to validate the HPLC method for the detection of rifampicin and 25-desacetyl rifampicin that has already been developed (Zent and Smith, 1995).

2.2 To establish whether there is a correlation between raised liver enzyme levels in tuberculosis patients and the concentrations of rifampicin, 25-desacetyl rifampicin and 3-formyl rifampicin in their serum.

2.3 To determine whether there is a difference in the metabolite levels of tuberculosis patients with and without HIV. Although this objective is not directly related to the overall aim of the study, HIV is an important and prevalent disease in South Africa and it was thought that these results would be relevant in the study's setting. It must, however be noted that only 2 of the patients were HIV positive. This is also true for the following objective.

2.4 To determine whether there is a difference in frequency of raised liver enzyme levels of patients with and without HIV.

2.5 To determine whether there are any markers of general health which correlate with raised liver enzyme levels.

Chapter 3

Materials and Methods

In this chapter the development of the high performance liquid chromatography (HPLC) assays for rifampicin, 25-desacetyl rifampicin and 3-formyl rifampicin in serum will be described. In addition, the clinical trial procedure and the statistical methods used to analyse the data will be described.

1. Assay development

Rifampicin and 25-desacetylrifampicin were extracted from plasma and detected on the HPLC using a method that was developed in the department of Pharmacology at the University of Cape Town (Zent and Smith 1995). The same extraction method was used for 3-formyl rifampicin as for rifampicin and 25-desacetyl rifampicin. A slight modification was made to the above-mentioned HPLC method in order to detect 3-formyl rifampicin. Although it is known that 3-formyl rifampicin is produced in the urine and very small quantities have been found in serum, it was decided to develop the method in order to verify these results.

1.1 HPLC detection method

1.1.1 Instrumentation and materials

The following instrumentation was used in the HPLC method: a Waters 712 WISP injector, a Waters 600 pump, a Waters 484 detector set at 270nm, a Waters 600E integrator and a C8 Higgins column (150 x 4.6mm) packed with silica beads with a diameter of 5µm each. The solvent system consisted of acetonitrile (Merck), triflouroacetic acid (Merck) and millipore filtered water. The rifampicin metabolites, 25-desacetyl rifampicin and 3-formyl rifampicin, were kindly donated by Gordon Ellard (12 Queen's Walk, Ealing, London, W5 1TP, fax: (0181) 998 1034). ifampicin was bought from Aldrich and plasma was obtained from the blood bank at Groote Schuur Hospital.

1.1.2 Mobile phase

The mobile phase for the rifampicin and 25-desacetyl rifampicin method consisted of an isocratic solution of 0.1% TFA and 100% acetonitrile in a 2:3 ratio (Zent and Smith, 1995). The 3-formyl rifampicin metabolite did not elute with the original mobile phase used by Zent and Smith. In order to decrease its retention time on the column, the acetonitrile concentration in the mobile phase was increased and the final solution used was a mixture of 0.1% TFA and 100% acetonitrile in a 1:4 ratio. The flow rate of the mobile phase was 1.5ml/min. For each run 60µl of sample was injected.

1.1.3 Standard curves

Half-serial dilutions were made starting at 10µg/ml of rifampicin, 25-desacetyl rifampicin and 3-formyl rifampicin in acetonitrile. The samples were injected onto the HPLC. The same was done in plasma, however the drug and its metabolites were extracted from the plasma prior to injection on the HPLC.

1.1.4 Sensitivity of the HPLC method

The sensitivity of the method was determined by measuring the final concentration at which rifampicin and its metabolites could be detected.

1.1.5 Specificity of the HPLC method

Streptomycin and co-trimoxazole were dissolved in acetonitrile at therapeutic concentrations of 10µg/ml and 30µg/ml respectively and injected onto the HPLC to determine whether they could be detected at 270nm.

1.2 Extraction

An Analytichem International Vacelut system with a pump set at 15kPa was used. The samples were centrifuged prior to extraction to remove particulate matter. The supernatant was then used in the extraction. C18 solid phase extraction columns were washed twice with 1ml methanol and 1ml water. They were then conditioned with 0.5mM KH_2PO4 at pH 4.5. A volume of 500µl of the plasma sample was injected on

to the column. The plasma was left on the column for 10 minutes and then pulled through to waste. The columns were washed with 1ml KH_2PO4 buffer at pH 4.5 after which rifampicin and its metabolites were eluted in a total of 500µl methanol. Methanol elution took place in 3 phases: 200µl methanol was loaded onto the column and left for 2 minutes before it was eluted. Another 200µl methanol was added to the column and left for 2 minutes before it was eluted. After this 100µl methanol was injected and left for 1 minute before it was eluted. Finally 500µl acetonitrile was injected onto the column and immediately eluted (Zent and Smith, 1995).

1.2.1 Specificity of the extraction method

The two drugs that were tested for interference in the extraction and the detection methods were streptomycin and co-trimoxazole. These drugs were dissolved in plasma at therapeutic concentrations of 10µg/ml and 30µg/ml respectively and underwent the extraction procedure in the presence and absence of rifampicin and its metabolites. They were then run on the HPLC.

1.2.2 Reproducibility of the extraction method

Thirteen extraction columns were used. The columns and the extraction method were validated for reproducibility by performing repeated extractions of the same concentrations (10µg/ml) of rifampicin, 25-desacetyl rifampicin and 3-formyl rifampicin from plasma.

1.2.3 Recovery of drugs from plasma

The percentage recovery of the drugs from the plasma was determined by using the following equation where I = initial concentration of drug in plasma, F = final concentration in plasma after extraction: F/I x 100

1.3 Stability of rifampicin and its metabolites

Plasma was spiked with 10µg/ml rifampicin and its metabolites and stored at –80°C. The samples were then analysed at 1 week, 2 weeks, 1 month and 2 months to see whether the drug and its metabolites were stable.

2. Clinical Trial

2.1 Study Site

The study was conducted at D.P. Marais Hospital in Tokai Cape Town. D.P. Marais is a government-owned hospital dedicated solely to the treatment of tuberculosis. Most patients are admitted due to perceived poor compliance, disease complications or poor response to treatment. The population is mainly from the lower income bracket. Most patients have histories of chronic alcohol abuse, however all subjects claimed to have stopped drinking prior to admission. Alcohol is forbidden in the hospital and there are severe disciplinary consequences such as expulsion if patients are caught drinking.

2.2 Inclusion criteria for test group

2.2.1 Subjects were on a drug regimen containing pyrazinamide, rifampicin and isoniazid for 1-2 months.

2.2.2 Subjects gave informed consent

2.2.3 Subjects were 18 years or older

2.2.4 Subjects had ALT and/or AST and/or γGT and/or AP levels that were above the normal range, but less than 2 times the upper limit.

The patients who fulfilled the above inclusion criteria and participated in the trial were referred to as test subjects.

2.3 Inclusion criteria for control group

2.3.1 The criteria for the control group were the same as for the test group, however the control group had liver enzyme levels in the normal range.

2.3.2 Controls Subjects were paired as closely as possible with the test patients on the basis of the following criteria: gender, age, race and period for which the medication was taken.

The patients who fulfilled the above inclusion criteria and participated in the clinical trial were referred to as control subjects.

2.4 Exclusion criteria

2.4.1 Subjects who were critically ill and could not undergo multiple blood sampling were excluded from the trial.

2.4.2 Subjects who tested positive for hepatitis B antigen or hepatitis C antibody were also excluded from the trial.

2.5 Sample size

A total of 68 patients were screened in order to obtain at least 10 test subjects. In the test group there were 12 subjects and in the control group there were 9 subjects, who were matched as closely as possible for age, gender, race. Although the sample size was not big enough to provide statistical significance, the trial was intended as a pilot study. The estimation of the number of patients that needed to be screened was based on a trial that was run previously in our department on a similar population in Brewelskloof Hospital in Worcester. In that trial, out of 100 patients studied, 6 had raised liver enzyme levels. The proportion of patients with raised liver enzymes at D.P Marais Hospital was slightly higher, therefore it was not necessary to screen the initially intended, 100 patients.

2.6 Informed consent

Each patient signed a written consent form in English, Afrikaans or Xhosa containing detailed information regarding the procedure of the study and the fact that it would not benefit him/her personally (Appendices 1-6). Participation was voluntary and patients were not discriminated against for not participating. Consent was obtained for HIV testing, and thorough pre- and post-test counselling were given in the patients' mother tongue.

2.7 Screening Procedure

Patients were identified by the inclusion criteria and gave written, informed consent before the following parameters were documented: age, gender, race, weight, past medical history, concomitant illnesses (especially liver disease) or allergies, concomitant medication or treatment, current tuberculosis treatment including drug

doses and dosing interval, consumption of alcohol in the past and in the present, and use of any recreational drugs.

The following departments at Groote Schuur Hospital conducted various tests.
Virology: HIV, Hepatitis B surface antigen, Hepatitis C Antibody.
Haematology: full blood count, differential and INR.
Chemical Pathology: urea, creatinine, total protein, albumin, total bilirubin, conjugated bilirubin, ALT AST, LDH, AP and γ-GT. The liver enzyme levels were only tested once for each patient.

In order to determine whether there were any factors related to health, which correlated with liver enzyme levels, all the subjects' (n = 21) liver enzyme levels were plotted against albumin, total protein, body mass index (BMI), mean corpuscular volume (MCV), international normalising ratio (INR), platelet count and haemoglobin (Hb).

2.8 Pharmacokinetic profiles

2.8.1 Pharmacokinetic sampling was conducted no later than two weeks from the date on which the subject was screened.

2.8.2 The antituberculosis drugs were administered according to the standard doses for the patient concerned. Drug ingestion was observed by an investigator or an investigator's assistant, and the exact time of drug administration was recorded. Patients ingested their drugs with 100ml water after breakfast in keeping with their daily routine.

2.8.3 Blood samples were taken as close as possible to the following times:
At least 45 minutes prior to drug administration, ½ hour, 1hour, 1½ hours, 2 hours, 2½ hours, 3 hours, 4 hours, 6 hours, 8 hours and 24 hours after drug administration. The exact time of blood taking was recorded.

2.8.4 The blood was collected in heparinised tubes and spun immediately in a centrifuge at 1820rpm for 5 minutes. The plasma was aliquoted into eppendorf tubes. The eppendorfs were frozen on dry ice and stored in

cryoboxes. At the end of the first trial day, the samples were transferred to a -80°C freezer until they were used for HPLC determination.

2.9 Insurance and liability

Students and staff of UCT are insured against liability claims incurred during research. The subjects were insured under the Professional Indemnity Policy that has a limit of R10 Million. The cover extends to include clinical trials performed by students in the name of the University of Cape Town.

2.10 Ethical considerations

The Research Ethics Committee of the University of Cape Town's medical faculty approved the protocol of the study (Appendix 7). Ethics approval was also obtained independently from the South African National Tuberculosis Association (SANTA) as they have jurisdiction over D.P. Marais Hospital (Appendix 8).

3. Statistics

The statistical computer packages Graphpad Prism Version 2.1 (Graphpad Software Incorporated) and WinNonlin Standard Edition Version 1.5 (Scientific Consulting Incorporated) were used in the analysis of the data. Prism was used to compare the AUC, $t_{1/2}$ and Cmax of control groups with those of the test groups using a nonparametric unpaired t-test (Mann Whitney U). The paired students t-test was used to validate the reproducibility of the extraction columns and the stability of the drug and its metabolites. WinNonlin was used in the determination of the area under the curve (AUC), half-life ($t_{1/2}$) and maximum concentration (Cmax) of rifampicin and 25-desacetyl rifampicin. Concentrations that were below the limit of detection were recorded as half of the lowest detectable concentration.

The AUC was computed to the time of the last observation and determined using the following equation:

$$AUC\Big|_{t_1}^{t_2} = \delta t \times \frac{C_1 + C_2}{2}$$

The terminal half-life ($t_{1/2}$) was determined using the following equation:

$$t_{1/2} = \frac{\ln(2)}{\lambda z}$$

λz is a first order rate constant associated with the terminal (log-linear) portion of the curve. This is estimated via linear regression of log concentration vs. time. The number of points used to calculate λz, was determined by the number of points that produced the largest square of the correlation coefficient (r^2).

The pharmacokinetic profiles of the subjects were plotted using Prism. The exact time of blood collection was used to plot the profiles. Where there were inexplicable drops in drug or metabolite concentrations in the middle of the pharmacokinetic profile, the assay on these points was repeated.

P values that were below 0.05 were deemed to be significant.

Chapter 4

Results

This chapter describes the development and validation of the high performance liquid chromatography and extraction methods used in this project. The analysis of the data and the results from the clinical trial, are also described. The full details of the methodology used to obtain these results are presented in chapter 6: materials and methods.

1. High performance liquid chromatography detection method

Two high performance liquid chromatography (HPLC) methods were used, one for the detection of rifampicin and 25-desacetyl rifampicin and one for the detection of 3-formyl rifampicin. The rifampicin and 25-desacetyl rifampicin method had already been developed (Zent and Smith, 1995) while the 3-formyl rifampicin method was developed in this study as a modification on the former method.

1.1 Development of 3-formyl rifampicin HPLC method

The rifampicin and 25-desacetyl rifampicin HPLC method used a mobile phase consisting of a 2:3 ratio of 0.1%TFA in water:acetonitrile. Under these conditions, the retention time of 25-desacetyl rifampicin was 2.25 ± 0.048 minutes and the retention time of rifampicin was 2.97 ± 0.081 minutes, however 3-formyl rifampicin did not elute. The mobile phase was changed to a 1:4 0.1%TFA in water:acetonitrile ratio in order to shorten the retention time of 3-formyl rifampicin. The retention time of 3-formyl rifampicin under the new conditions was 3.46 ± 0.018 minutes. Plates 1 and 2 show chromatographs of solutions of rifampicin, 25-desacetyl rifampicin and 3-formyl rifampicin in acetonitrile run at 2:3 0.1% TFA in water:acetonitrile (plate 1) and 1:4 0.1%TFA in water:acetonitrile (plate 2).

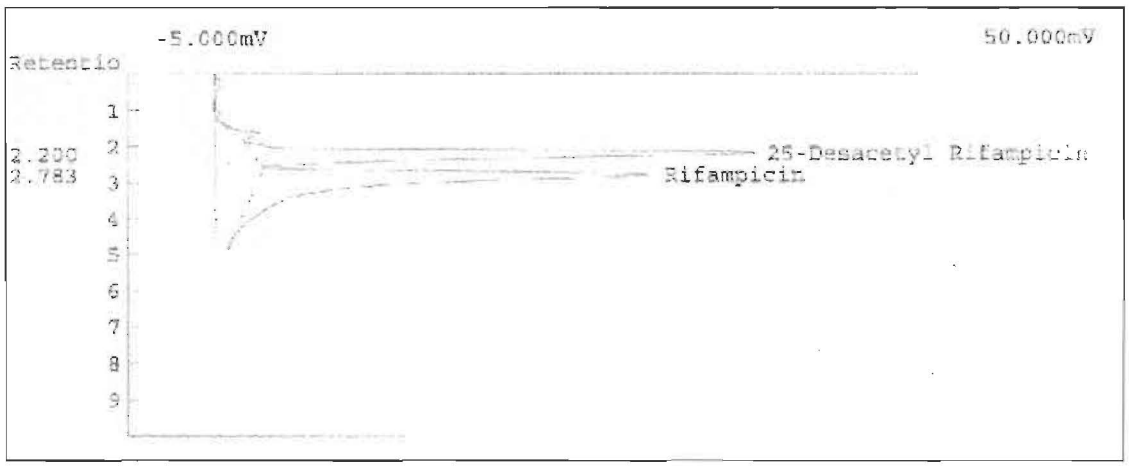


Plate 1: Chromatograph of rifampicin, 25-desacetyl rifampicin and 3-formyl rifampicin in acetonitrile run at 2:3 0.1%TFA in water:acetonitrile

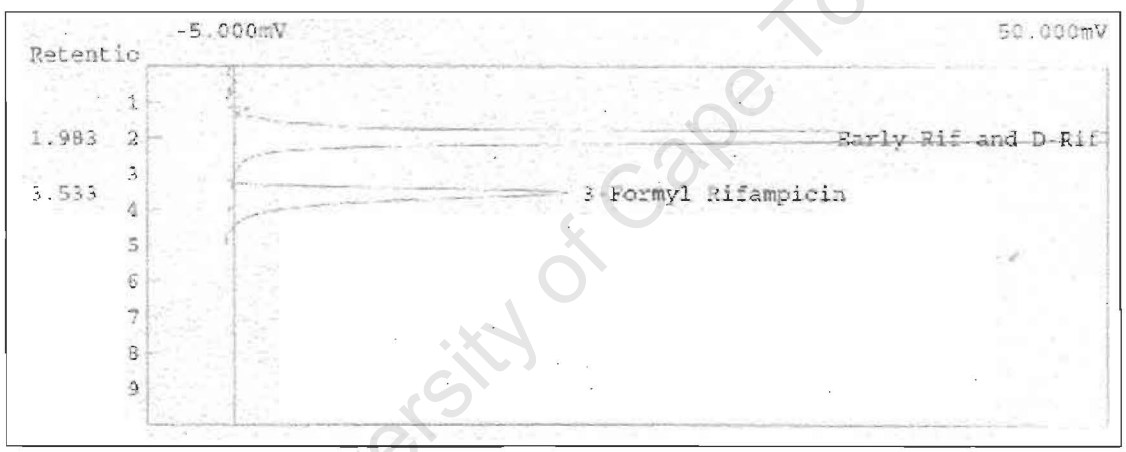


Plate 2: Chromatograph of rifampicin, 25-desacetyl rifampicin and 3-formyl rifampicin in acetonitrile run at 1:4 0.1%TFA in water:acetonitrile

1.2 Standard curves in acetonitrile

The two HPLC assays were validated by analysing spiked solutions of rifampicin, 25-desacetyl rifampicin and 3-formyl rifampicin in acetonitrile. Half serial dilutions were performed from a stock solution of 10μg/ml. Figures 1-3 show the standard curves of rifampicin (CV = 84.47%, $r^2 = 0.96$), 25-desacetyl rifampicin (CV = 167.58%, $r^2 = 0.91$) and 3-formyl rifampicin (CV = 134.21%, $r^2 = 0.99$) in acetonitrile. The curves were repeated 15 times for the rifampicin and 25-desacetyl rifampicin assay and 11 times for the 3-formyl rifampicin assay. The error bars indicate SD.

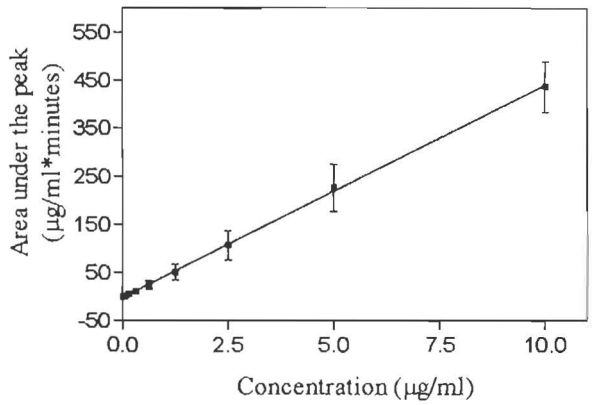


Figure 1: Standard curve of rifampicin in acetonitrile (n = 15)

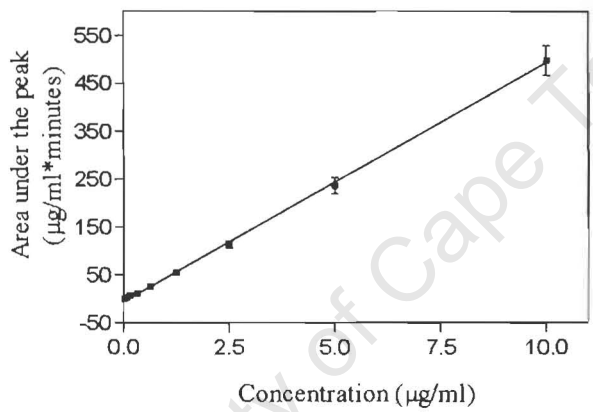


Figure 2: Standard curve of 25-desacetyl rifampicin in acetonitrile (n = 15)

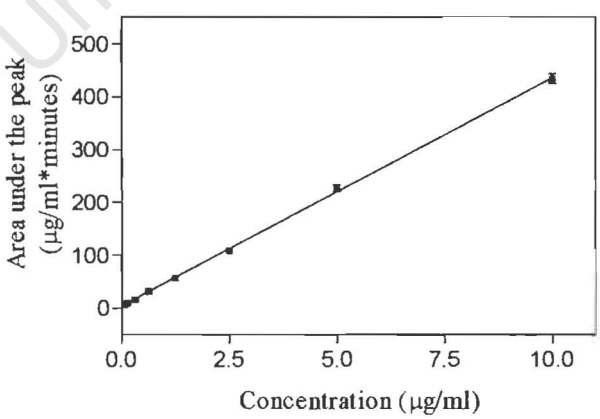


Figure 3: Standard curve of 3-formyl rifampicin in acetonitrile (n = 11)

2. Extraction method

A single method was used to extract rifampicin and its metabolites from plasma (Zent and Smith, 1995). Plate 3 is a chromatograph of a blank plasma sample. Plates 4 and 5 are HPLC chromatographs of a plasma sample containing rifampicin, 25-desacetyl rifampicin and 3-formyl rifampicin, having undergone the extraction procedure and been run on the rifampicin / 25-desacetyl rifampicin HPLC method (plate 4) and the 3-formyl rifampicin method (plate 5). In the 3-formyl rifampicin assay, the rifampicin and 25-desacetyl rifampicin elute as a single early peak as part of the frontal peak. This was verified by running a sample only containing 3-formyl rifampicin in which the rifampicin and 25-desacetyl rifampicin "frontal peak" did not appear.

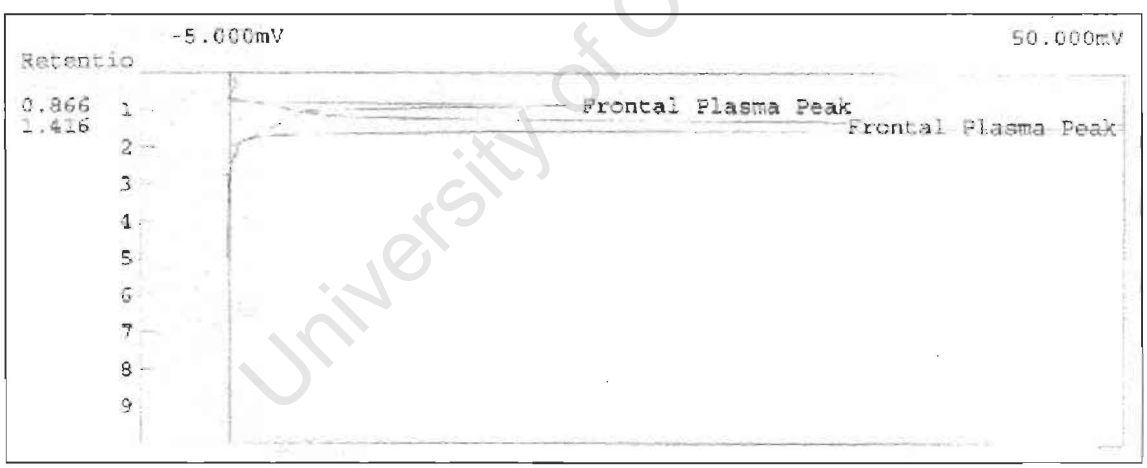


Plate 3: Chromatograph of a blank plasma sample, extracted and run at 2:3 0.1%TFA in water:acetonitrile

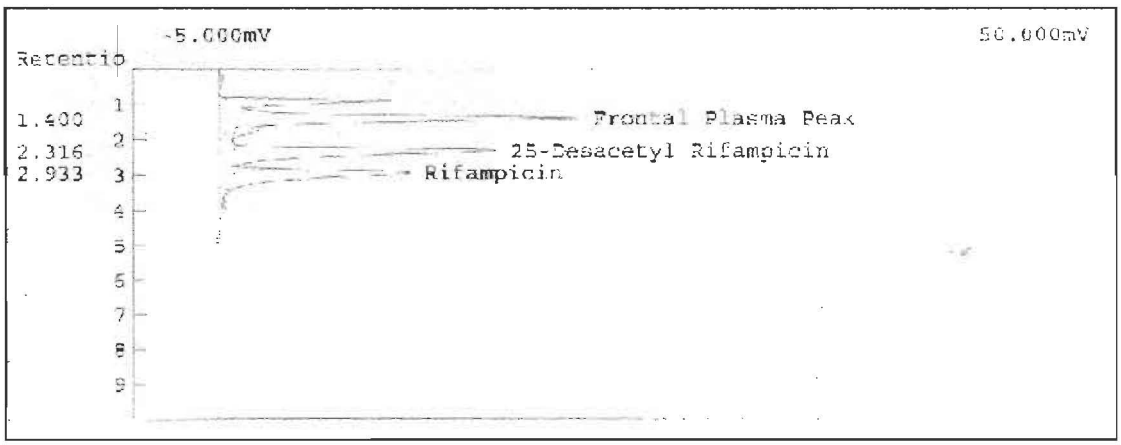


Plate 4: Chromatograph of an extracted plasma solution containing rifampicin, 25-desacetyl rifampicin and 3-formyl rifampicin in plasma, run at 2:3 0.1%TFA in water:acetonitrile

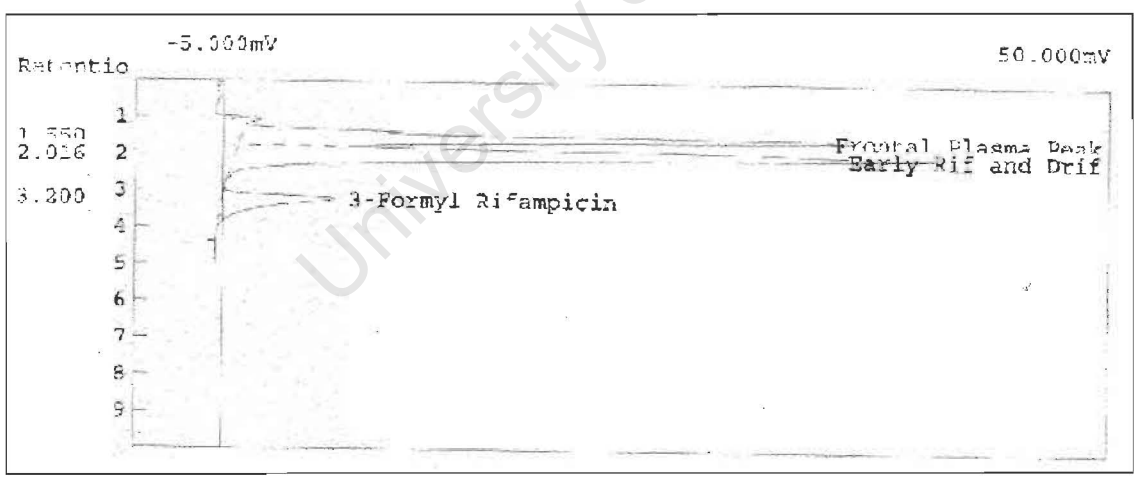


Plate 5: Chromatograph of an extracted plasma solution containing rifampicin, 25-desacetyl rifampicin and 3-formyl rifampicin in plasma, run at 1:4 0.1%TFA in water:acetonitrile

2.1 Standard curves in plasma

Half serial dilutions were done from plasma solutions consisting of 10μg/ml rifampicin, 10μg/ml 25-desacetyl rifampicin and 10μg/ml 3-formyl rifampicin. The solutions of the different concentrations were then extracted and analysed on the HPLC machine. Figures 4–6 show the standard curves of rifampicin (CV = 144.53%, r^2 = 0.96), 25-desacetyl rifampicin (CV = 159.73%, r^2 = 0.92) and 3-formyl rifampicin (CV = 117.69%, r^2 = 0.95) in plasma. The rifampicin and 25-desacetyl rifampicin assay was repeated 9 times and the 3-formyl rifampicin assay was repeated 10 times. Error bars indicate SD.

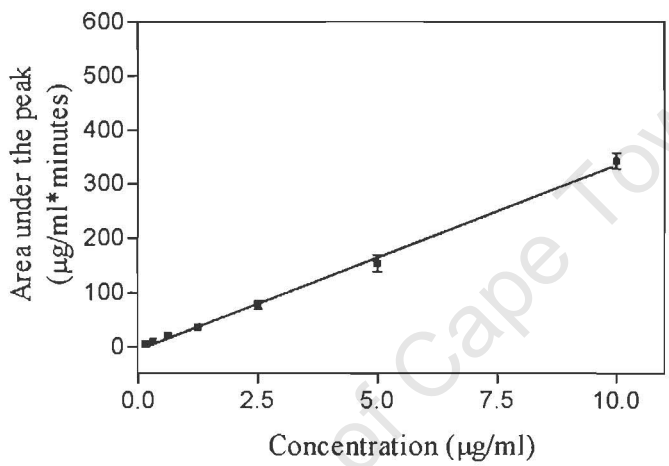


Figure 4: Standard curve of rifampicin in plasma (n = 9)

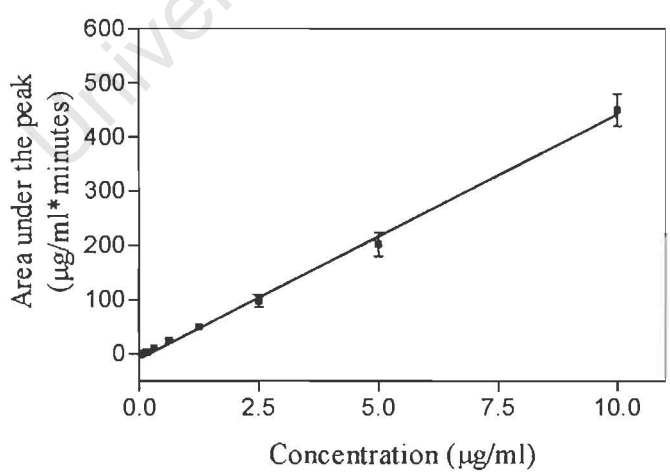


Figure 5: Standard curve of 25-desacetyl rifampicin in plasma (n = 9)

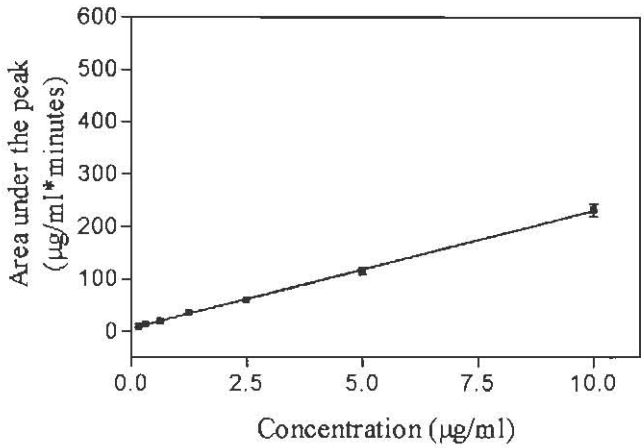


Figure 6: Standard curve of 3-formyl rifampicin in plasma (n =10)

3. Validation of the HPLC method

3.1 Sensitivity of the HPLC assay

The limit of detection for rifampicin, 25-desacetyl rifampicin and 3-formyl rifampicin were 0.078µg/ml, 0.078µg/ml and 0.156µg/ml respectively.

3.2 Specificity of the HPLC assay

Most patients received a combination drug consisting of rifampicin, isoniazid, ethambutol and pyrazinamide. Some patients received concomitant medication of streptomycin and co-trimoxazole (a combination of sulphamethoxazole and trimethoprim). The Zent and Smith method was tested for its specificity to rifampicin in the presence of isoniazid and pyrazinamide, (Zent and Smith, 1995). Ethambutol does not absorb in the ultra violet range and therefore could not pose any interference with the detection method. It was therefore not tested for interference. Two drugs were tested for interference with the HPLC methods: streptomycin and co-trimoxazole. Plate 6 shows a chromatograph of co-trimoxazole in acetonitrile run at 2:3 0.1%TFA in water:acetonitrile. Under these conditions co-trimoxazole has a retention time of 1.48 minutes. The retention time of co-trimoxazole when run at 1:4 0.1%TFA in water:acetonitrile was even shorter at 1.42 minutes. Co-trimoxazole, therefore would not have posed an interference to the HPLC assay even if it could have been extracted with the rifampicin and its metabolites in the extraction procedure. Plate 7 shows a chromatograph of streptomycin run at 2:3

streptomycin run at 2:3 0.1%TFA in water:acetonitrile. Streptomycin could not be detected by this method or by the 3-formyl rifampicin method of 1:4 0.1%TFA in water:acetonitrile.

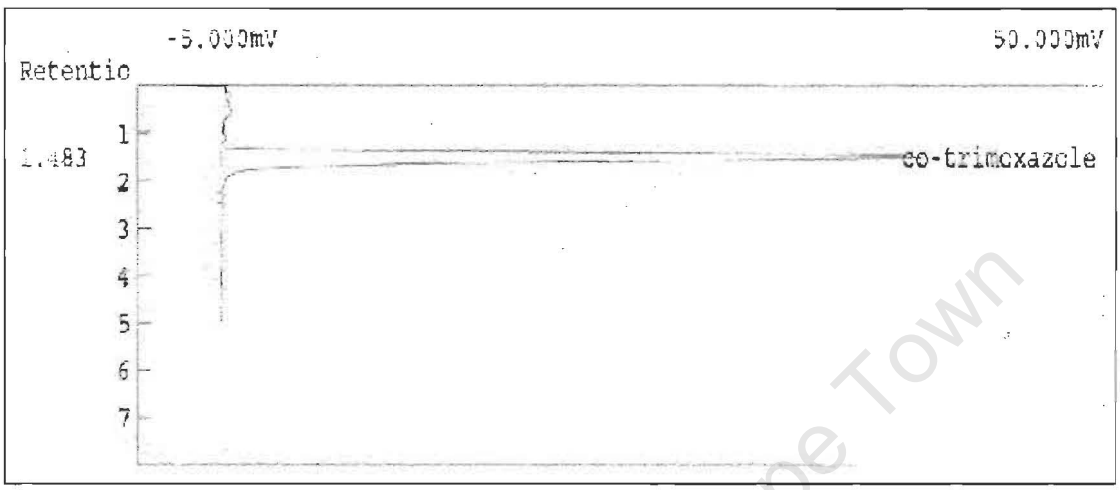


Plate 6: Chromatograph of co-trimoxazole in acetonitrile run at 2:3 0.1%TFA in water:acetonitrile

Plate 7: Chromatograph of streptomycin in acetonitrile run at 2:3 0.1%TFA in water:acetonitrile

4. Validation of the extraction method

4.1 Reproducibility of the extraction columns

Thirteen extraction columns were used. Figures 7-9 below show the concentration yield after the extraction of a plasma solution containing 10μg/ml rifampicin, 10μg/ml 25-desacetyl rifampicin and 10μg/ml 3-formyl rifampicin. There was no significant difference in the yields from any of the columns as can be noted from the P values, which are greater than 0.05. Error bars indicate SD.

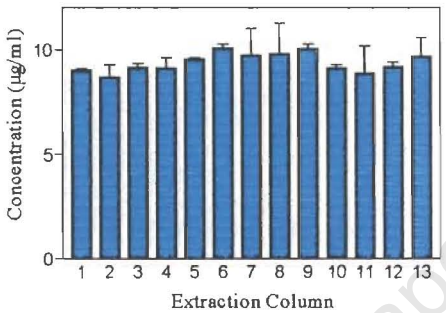


Figure 7: Reproducibility: 10μg/ml rifampicin (P = 0.7)

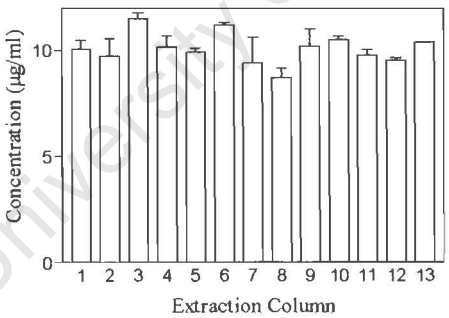


Figure 8: Reproducibility: 10μg/ml 25-desacetyl rifampicin (P = 0.3)

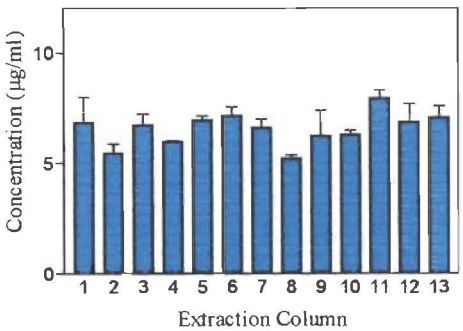


Figure 9: Reproducibility: 10μg/ml 3-formyl rifampicin (P = 0.7)

4.2 Recovery of the extraction method

Figures 10-12 show the recoveries of rifampicin, 25-desacetyl rifampicin and 3-formyl rifampicin from plasma at a range of concentrations. The mean % ± SEM recovery was 75.47 ± 3.77% for rifampicin, 79.65 ± 2.83% for 25-desacetyl rifampicin and 59.34 ± 3.39% for 3-formyl rifampicin.

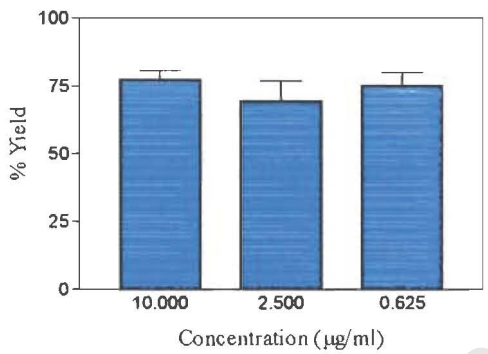


Figure 10: Percentage yield of rifampicin after extraction

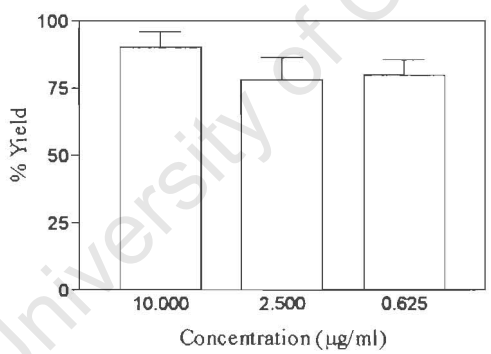


Figure 11: Percentage yield of 25-desacetyl rifampicin after extraction

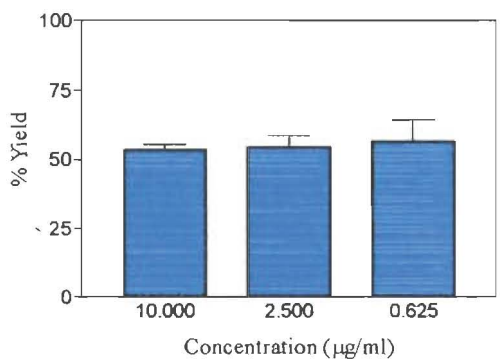


Figure 12: Percentage yield of 3-formyl rifampicin after extraction

4.2 Specificity of the extraction method

Two drugs were tested for interference with the extraction method: streptomycin and co-trimoxazole. Neither drug could be detected on the HPLC methods after the drugs had undergone extraction (plates 8 and 9). The presence of streptomycin and co-trimoxazole did not interfere with the recovery of rifampicin or its metabolites. The comparison of the percent yield of rifampicin from a plasma sample with and without co-trimoxazole and streptomycin gave a P value of 0.85. The same comparison of 25-desacetyl rifampicin and 3-formyl rifampicin gave P values of 0.20 and 0.11 respectively.

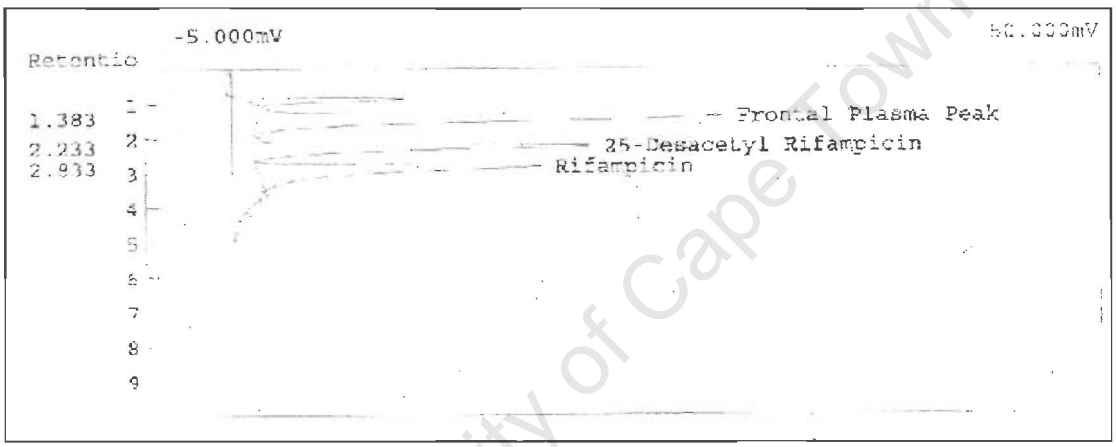


Plate 8: Chromatograph of rifampicinand its metabolites with streptomycin and co-trimoxazole run at 2:3 0.1%TFA in water:acetonitrile

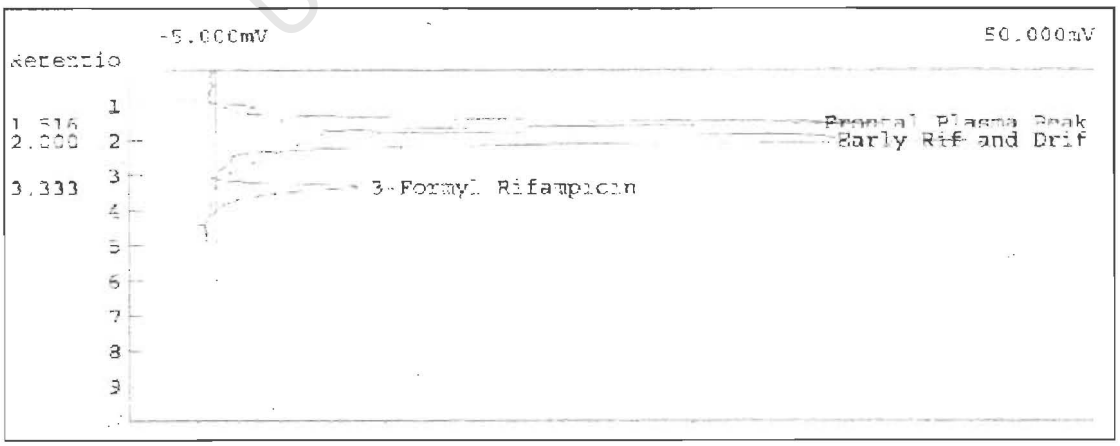


Plate 9: Chromatograph of rifampicin and its metabolites with streptomycin and co-trimoxazole run at 1:4 0.1%TFA in water:acetonitrile

4.4 Stability of rifampicin and its metabolites

The stability of the drug and its metabolites at -80°C was tested over a period of 2 months. There was no significant difference in yield of rifampicin (P=0.35), 25-desacetyl rifampicin (P=0.13) or 3-formyl rifampicin (P=0.65). The drug and its metabolite therefore did not degrade during refrigeration at -80°C and could safely be analysed up to 2 months after freezing. None of the samples were analysed later than 2 months after collection. Figures 13-15 show the observed concentration recovered without refrigeration and after 1 week, 2 weeks, 1 month and 2 months of refrigeration at -80°C. The starting concentration of rifampicin and its two metabolites was 10µg/ml. The error bars indicate SD.

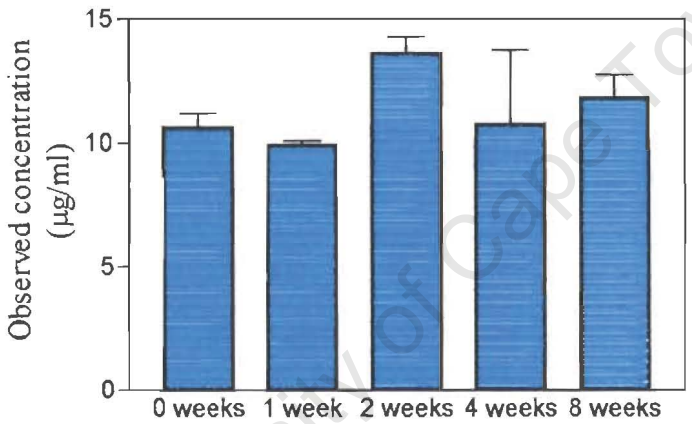


Figure 13: Observed concentration of rifampicin 0,1,2,4 and 8 weeks after refrigeration (n=2)

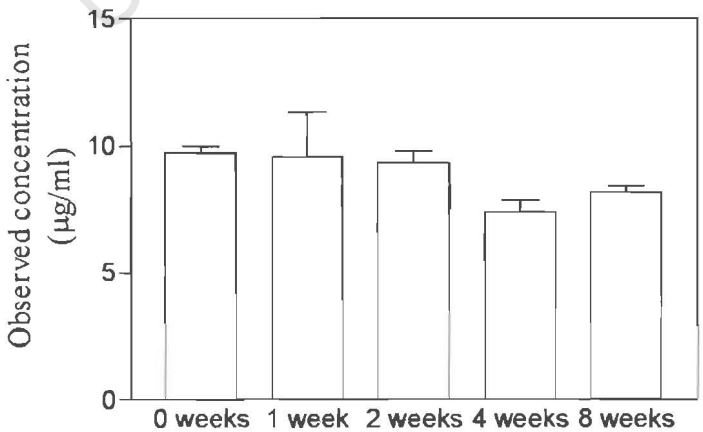


Figure 14: Observed concentration of 25-desacetyl rifampicin 0,1,2,4 and 8 weeks after refrigeration (n=2)

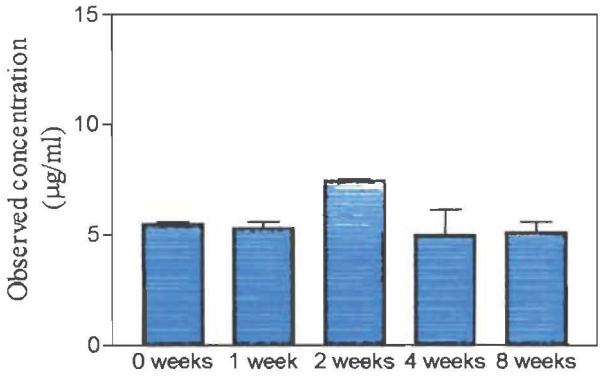


Figure 15: Observed concentration of 3-formyl rifampicin 0,1,2,4 and 8 weeks after refrigeration (n=2)

The HPLC and extraction methods that were developed and validated were used in the analysis of the data obtained from the clinical trial. The results from the clinical trial are described below.

5. Clinical trial results

5.1 Subject selection

Over a period of 2 months 68 subjects were screened, 21 of whom participated in the clinical trial. It was initially decided to screen 100 patients based on a previous trial conducted in the department of Pharmacology, UCT, in which 6 of the 100 trial subjects were reported to have raised liver enzyme levels. The aim was to obtain at least 10 test subjects with abnormal liver enzymes for the trial. Once this had been achieved, the screening was stopped. The number of patients on the trial was limited mainly due to budget constraints. The test group consisted of 12 patients, and the control group, exhibiting normal liver enzyme levels, consisted of 9 patients. The average age ± SD of the test group was 41.58 ± 2.42 years and that of the control group was 35.00 ± 2.93 years. There was a significant difference in age (P=0.046) between the groups. The average weight ± SD of the test group was 43.08 ± 1.95kg and that of the control group was 46.39 ± 2.47kg, with no significant difference between the two. The test group had an average body mass index (BMI) ± SD of 16.72 ± 0.67kg/m^2 and the average BMI of the control group was 17.55 ± 0.74kg/m^2. Both groups had BMI's below the normal range of 18-24 kg/m^2, with no significant

difference between the two. The test group had an average body mass index (BMI) ± SD of 16.72 ± 0.67kg/m^2 and the average BMI of the control group was 17.55 ± 0.74kg/m^2. Both groups had BMI's below the normal range of 18-24 kg/m^2, with no significant difference between the two groups (P=0.46). This may be indicative of malnutrition. The test group consisted of 5 male subjects and 7 female subjects and the control group consisted of 4 males and 5 females. Subjects were matched as closely as possible for age, weight, gender and race. The test group included 2 black female patients who were HIV positive and for whom, no HIV positive match in the control group could be found during the period of the study.

	Test group (n=12)	Control Group (n=9)
Age (years)	41.58 ± 2.42	35.00 ± 2.93
Weight (kg)	43.08 ± 1.95	46.39 ± 2.47
Body Mass Index (kg/m^2)	16.72 ± 0.67	17.55 ± 0.74
Gender	5M : 7F	4M : 5F
Race	5 Black : 7 Coloured	1 Black : 8 Coloured
HIV status	10 Negative: 2 Positive	9 Negative

Table 1: Patient characteristics (mean ± SD)

5.2 Medication regimen

All patients received a combination of rifampicin, isoniazid, pyrazinamide and ethambutol for the duration of the trial. In addition to this 55.56% of the control group and 58.33% of the test group received streptomycin. The two HIV positive patients were receiving a combination of sulphamethoxazole and trimethoprim called co-trimoxazole at the time of the trial.

5.3 Rifampicin dose

Of the 21 subjects who participated in the clinical trial, 4 (19%) weighed more than 50kg and were routinely given 600mg rifampicin per day. The rest of the 19 subjects (81%) weighed less than 50kg and were given 480mg rifampicin per day. The mean

dose of rifampicin ± SD administered was 11.79 ± 0.44mg/kg for the test group and 10.92 ± 0.31mg/kg for the control group. Figure 16 shows that there is no significant correlation (r^2=0.35) between the dose of drug administered and the maximum concentration of rifampicin serum levels. It was therefore decided not to subgroup subjects on the basis of the daily dose of rifampicin they received.

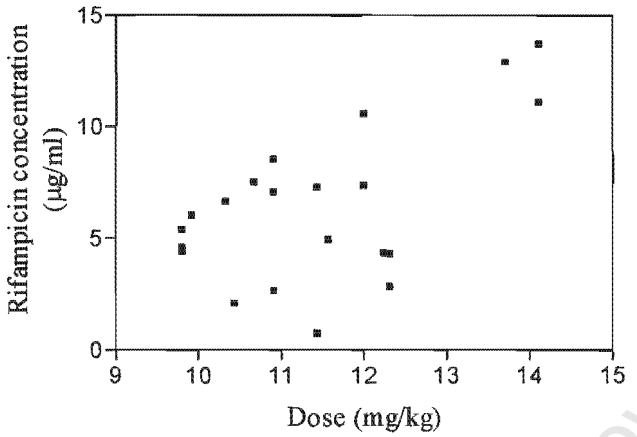


Figure 16: Correlation between rifampicin dose and rifampicin serum concentration

5.4 Alcohol consumption

Although the subjects claimed to have stopped drinking at the time of hospitalisation, all except 2 subjects had consumed large amounts of alcohol (3-6 bottles of beer or 1-6 litres of wine) on a daily basis prior to admission. One third of the trial subjects had raised mean corpuscular volumes (MCV) indicative of an inability to utilise folate or B12, which often arises as the result of alcohol exposure. Macrocytosis was evident in 57.14% of the subjects. Macrocytosis is a good indicator of alcohol consumption and responds slowly to alcohol withdrawal. None of the patients exhibited typical alcoholic AST:ALT ratios, which are greater than 2.

5.5 Virology results

Of the 68 subjects screened, there were 23 (33.82%) HIV positive subjects, 3 of whom tested positive for hepatitis B surface antigen and 2 who tested positive for hepatitis C antibody. There were also 3 HIV negative subjects who tested positive for hepatitis B antigen. None of the subjects tested positive for both hepatitis B and C. The incidence of viral hepatitis in HIV positive subjects in this study (21.74%) was

greater than the incidence of viral hepatitis in HIV negative subjects (6.67%). All subjects who tested positive for viral hepatitis were excluded from the clinical trial, as viral hepatitis is a confounding factor in the cause of liver disease.

	HIV positive	HIV negative
Subjects screened (n = 68)	23	45
Reactive Hepatitis C antibody	2	0
Reactive Hepatitis B antigen	3	3

Table 2: HIV and viral hepatitis results

5.6 Chemical pathology results

Only 3 subjects had slightly raised levels of conjugated bilirubin and none of the subjects had jaundice. One patient had urea levels 5 times greater than normal and was not included in the analysis of the data as his renal impairment would have constituted too great a confounding factor in the assessment of drug-induced liver disease. Rifampicin has been shown to cause acute renal failure and pyrazinamide has been shown to increase the uric acid concentration in serum (Girling, 1982), however, it cannot be stated with any degree of certainty that these two drugs were the cause of renal function impairment in this patient. Two patients in the test group had slightly low urea levels, perhaps indicative of malnutrition. Globulin levels were found to be high in 81% of the trial subjects. Albumin levels were below the lower limit of the reference range in 66.67% of the subjects. The average albumin level ± SD was 30.33 ± 1.88g/L in the test group and 34.78 ± 1.62g/L in the control group with no significant difference between the two. These results are indicated in the table below. The macrocytosis results have been addressed above on pg. 68.

	Test group (n=12)	Control group (n=9)	Reference Ranges	P-value
Albumin (g/l)	30.33 ± 6.50	34.78 ± 4.87	35 - 50	0.12
Creatinine (µmol/L)	60.33 ± 11.45	63 ± 12.39	50 - 100	0.64
Urea (mmol/L)	2.84 ± 0.33	3.23 ± 0.15	1.7 – 6.7	0.59
Total Protein (g/L)	76.83 ± 9.43	82.78 ± 9.64	60 - 80	0.16
Total Bilirubin (µmol/L)	5.92 ± 2.55	5.11 ± 2.35	1 – 17	0.47
Conjugated Bilirubin (µmol/L)	2.67 ± 1.63	2.22 ± 1.66	0 - 4	0.33

Table 3: Chemical pathology results (mean ± SD)

5.7 Haematology results

Both the control and test groups had subnormal haemoglobin and haematocrit and 57.41% of the subjects had anaemia of chronic disorders. This is very common in tuberculosis patients. Anaemia often goes hand in hand with alcoholism and nutritional deficiency, both of which are prevalent in the D.P Marais Hospital population. Of the control subjects, 2 had abnormal international normalising ratios (INR) indicating abnormal liver synthetic function or vitamin K deficiency. The patients were not on warfarin, which would increase the INR. One of the control subjects had an INR of 1.4 and the other of 1.7. Of the 12 test subjects, 4 had abnormal INR's of 1.4, 1.5, 1.6 and 2.0. Both test and control groups exhibited above average white cell counts, and the test group exhibited an above average level of neutrophilia. Both test and control groups had high platelet counts.

average white cell counts, and the test group exhibited an above average level of neutrophilia. Both test and control groups had high platelet counts.

	Test group (n=12)	Control group (n=9)	Reference Ranges
Haemoglobin (g/dL)	10.21 ± 3.59	10.48 ± 3.96	13.3 – 17.3
Haematocrit (ratio)	0.36 ± 0.047	0.37 ± 0.054	0.37-0.53
White Cell Count (x 10^9/L)	13.18 ± 4.56	13.26 ± 4.78	4 - 11
RDW (units)	19.67 ± 2.33	17.39 ± 2.70	7.4 – 13.6
Mean Cell Volume (fl)	87.83 ± 10.99	94.44 ± 9.89	80 - 95
Platelets (x 10^9/L)	526.33 ± 237.24	579.11 ± 335.55	150 - 450
Neutrophils (x 10^9/L)	9.55 ± 3.57	6.57 ± 2.89	1.8 – 7.7
Lymphocytes (x 10^9/L)	2.57 ± 0.67	3.63 ± 0.98	1 - 4
INR (ratio)	1.37 ± 0.25	1.34 ± 0.14	< 1.4

Table 4: Haematology results (mean ± SD)

5.8 Liver enzyme levels

Out of the total 68 subjects that were screened, 33 (52.94%) fitted this criterion, however 8 subjects were excluded from the trial because they tested positive for Hepatitis B or C and 13 subjects refused to participate in the trial. Out of 68 subjects 5 (7.35%) had abnormal ALT levels, 7 (10.29%) had abnormal AST levels, 19 (27.94%) had abnormal ALP levels and 33 (48.53%) had abnormal γGT levels. The incidence of abnormal liver enzymes of any kind was higher in subjects who were HIV positive than in those who were HIV negative. Of the 23 HIV positive subjects,

only one had raised ALT, AST, ALP and γGT levels together, 3 subjects had an isolated raised γGT level, 1 patient had an isolated raised ALP level and 7 had raised ALP and γGT levels together. Table 5 shows the distribution of raised liver enzyme levels among HIV positive and negative patients and among the control and test groups. The reference ranges of ALT, AST, ALP and γGT are 1-41 units, 1-38 units, 39-117 units and 7-49 units respectively.

		ALT (reference range: 1-41 units)	**AST (reference range: 1-38 units)**	**ALP (reference range: 39-117 units)**	**γGT (reference range: 7-49 units)**
Total screened patients (n = 68)	**mean ± SD**	21.47 ± 16.22	26.03 ± 11.49	115.43 ± 47.93	70.58 ± 64.75
	% with raised levels	7.35%	10.29%	27.94%	48.53%
HIV positive (n=23)	**mean ± SD**	21.30 ± 2.12	28.52 ± 1.45	121.78 ± 12.07	80.74 ± 11.89
	% with raised levels	0.086%	8.7%	21.74%	52.17%
HIV negative (n=45)	**mean ± SD**	21.56 ± 2.78	24.76 ± 1.96	112.18 ± 6.31	66.31 ± 10.24
	% with raised levels	0.067%	0.11%	31.1%	46.7%
Test subjects (n = 12)	**mean ± SD**	22.17 ± 3.94	22.08 ± 4.84	141.08 ± 12.82	69.08 ± 12.82
	% with raised levels	8.33%	8.33%	75%	91.67%
Control subjects (n = 9)	**mean ± SD**	16.33 ± 1.94	18.44 ± 0.88	81.89 ± 6.38	35.67 ± 3.38
	% raised levels	0%	0%	0%	0%

Table 5: Distribution of raised (1-2 times the upper limit) enzyme levels in subjects (mean ± SD)

5.9 Correlation between clinical results and liver enzyme levels

All the subjects' (n = 21) liver enzyme levels were plotted against albumin, total protein, body mass index (BMI), mean corpuscular volume (MCV), international normalising ratio (INR), platelet count and haemoglobin (Hb). Table 6 shows the squared correlation coefficient for all the above parameters. There was no linear relationship for any of the factors. The mean values of the health markers were compared in the test and control groups (figure 17) using the Mann Whitney test. There was no significant difference between the control and the test groups for any of the markers. Table 7 shows the P values for the graphs in figure 17.

	ALT (n=2)	AST (n=2)	ALP (n=9)	γGT (n=11)
Albumin	0.013	0.000	0.007	0.039
Haemoglobin	0.130	0.111	0.011	0.017
BMI	0.004	0.046	0.000	0.014
INR	0.081	0.075	0.000	0.049
MCV	0.000	0.035	0.171	0.000
Total protein	0.000	0.003	0.035	0.219
Platelets	0.050	0.005	0.008	0.002

Table 6: Squared correlation coefficient (r^2) for health markers vs. liver enzyme levels

	Albumin	BMI	Hb	INR	MCV	Platelets	Total Protein
P-value	0.12	0.46	0.88	0.80	0.08	0.80	0.15

Table 7: P-value of health markers of control vs. test groups

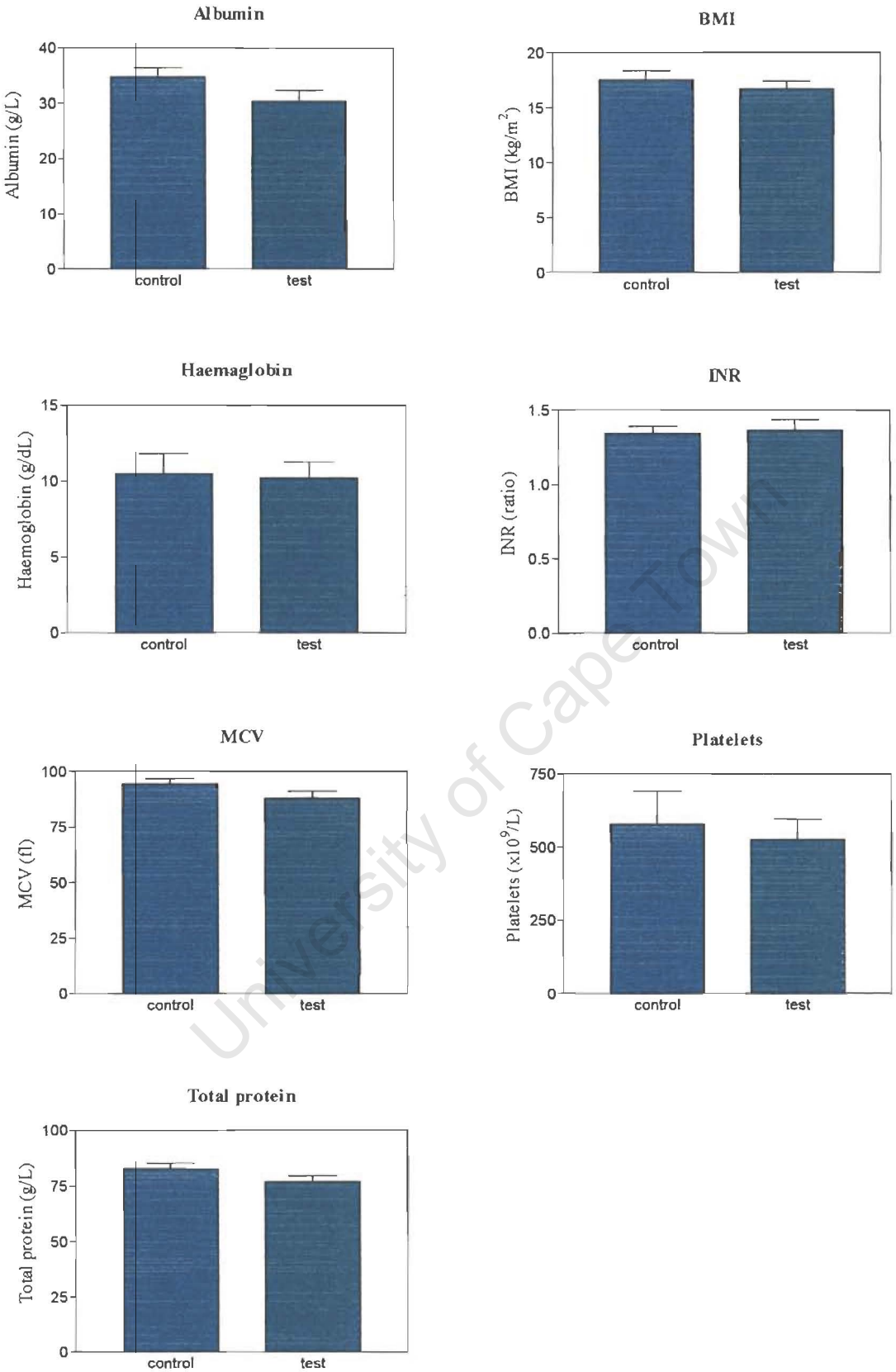


Figure 17: Mean ± SD of health markers of control and test groups

6. Pharmacokinetic parameters of rifampicin and its metabolites

6.1 3-formyl rifampicin

After analysing the complete data from four patients, it was evident that the concentration of 3-formyl rifampicin was well below the limit of detection. This metabolite was undetectable in the serum of the patients in all the samples tested. This is consistent with the literature, which states that 3-formyl rifampicin is produced in the urine and is found in minute concentrations in the serum, if at all (Ishii and Hiroyasu, 1988 and Lecaillon *et al*, 1978).

6.2 Rifampicin and 25-desacetyl rifampicin

Three parameters were examined for rifampicin and its metabolite 25-desacetyl rifampicin: the area under the curve (AUC), the maximum concentration (Cmax) and the terminal half-life ($t_{1/2}$). Table 8 shows the mean ± SD of these parameters for the control group (n = 9) and for the test group (n = 12).

		Control Group	Test Group
rifampicin	**AUC (μg/ml*hours)**	36.87 ± 14.35	58.28 ± 26.83
	Cmax (μg/ml)	5.44 ± 3.76	7.17 ± 2.94
	$t_{1/2}$ (hours)	4.59 ± 1.46	4.04 ± 6.68
25-desacetyl rifampicin	**AUC (μg/ml*hours)**	2.64 ± 1.30	4.64 ± 4.76
	Cmax (μg/ml)	0.41 ± 0.34	0.49 ± 0.42
	$t_{1/2}$ (hours)	9.12 ± 1.46	8.84 ± 6.68

Table 8: AUC, Cmax and $t_{1/2}$ of rifampicin and desacetyl rifampicin

Figures 18 and 19 compare the AUC's for the control and test groups of rifampicin and 25-desacetyl rifampicin respectively. The AUC's of the rifampicin test group were significantly higher than the AUC's of the rifampicin control group (P = 0.05). However there was no significant difference between the AUC's of the 25-desacetyl rifampicin

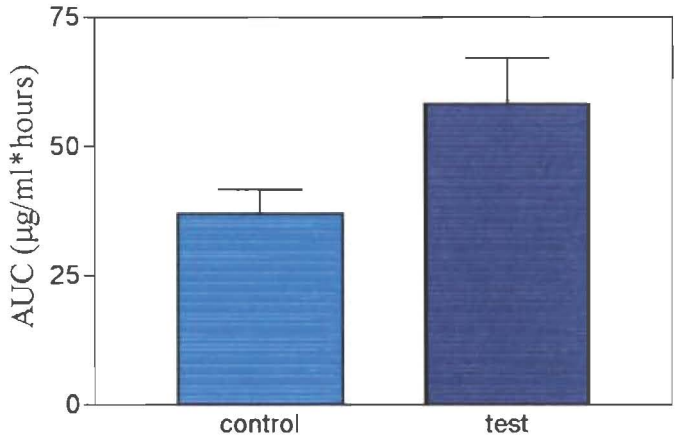


Figure 18: AUC of rifampicin in control and test groups

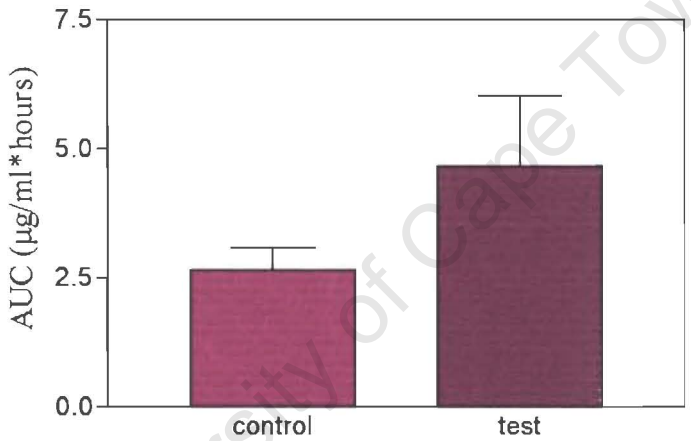


Figure 19: AUC of 25-desacetyl rifampicin in control and test groups

Since there was a correlation between the AUC of rifampicin and the presence of raised liver enzyme levels in patients, further analysis was done on the correlation between AUC of rifampicin and the specific liver enzymes that were tested (ALT, AST, ALP and γGT). Figures 20-23 show the AUC of rifampicin vs. the liver enzyme levels of all 21 patients for each of the liver enzymes specified above. There is no linearity in any of the graphs as can be seen from the squared correlation coefficient (r^2), indicating that no direct correlation can be shown between the AUC of rifampicin and a specific liver enzyme level.

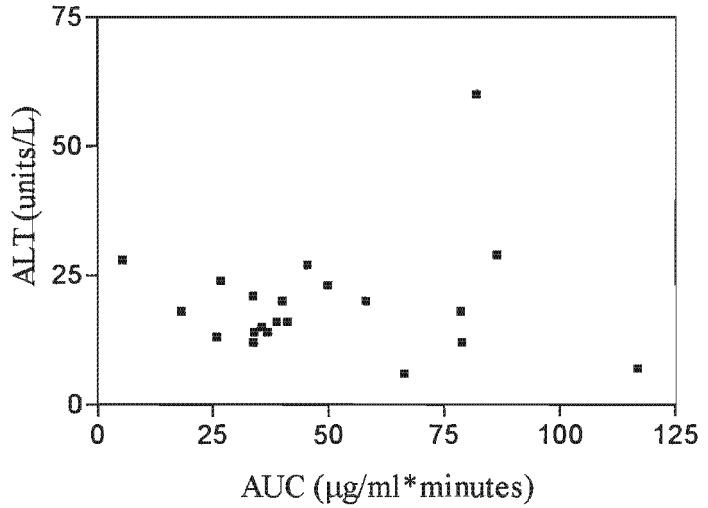


Figure 20: ALT levels vs. AUC of rifampicin ($r^2 = 0.0046$)

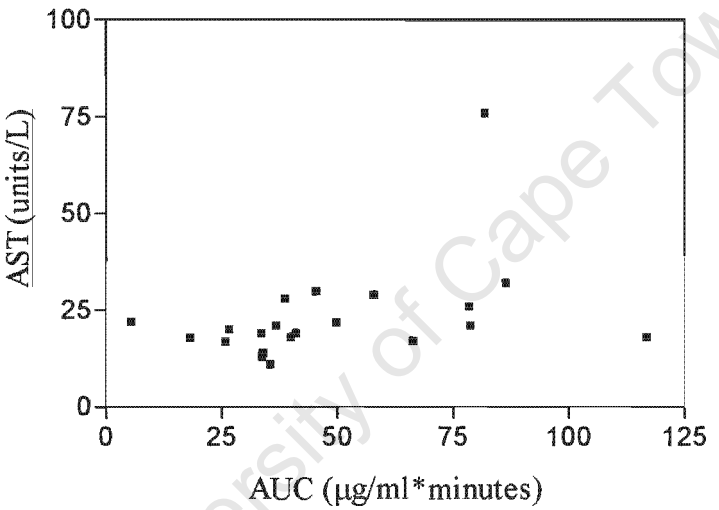


Figure 21: AST levels vs. AUC of rifampicin ($r^2 = 0.1394$)

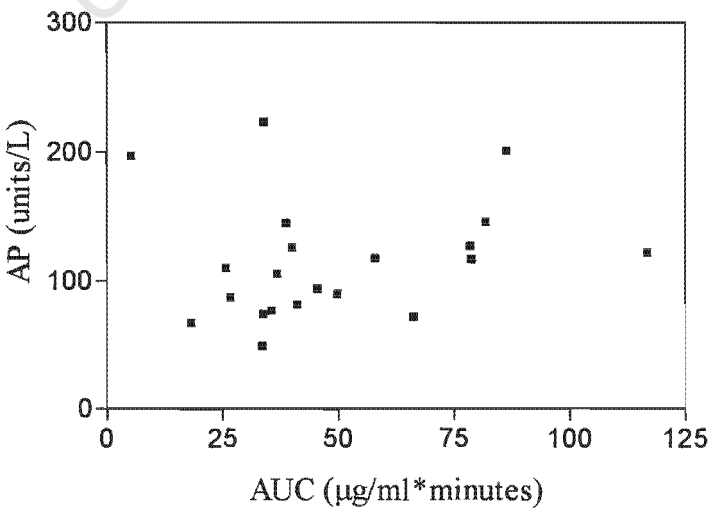


Figure 22: ALP levels vs. AUC of rifampicin ($r^2 = 0.0212$)

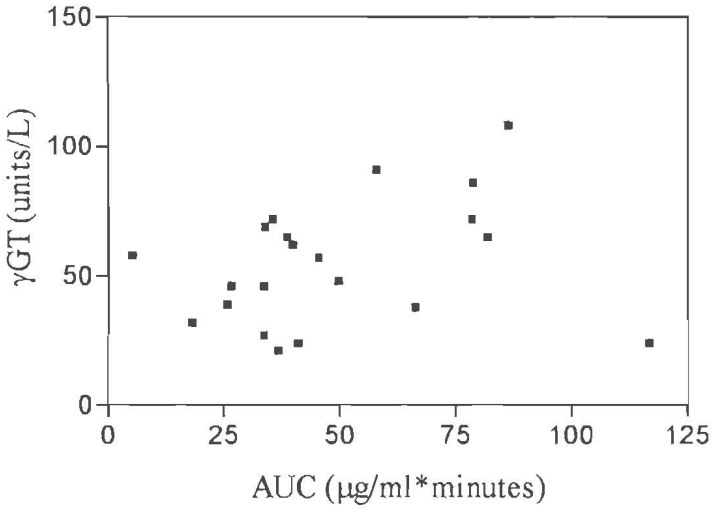


Figure 23: γGT levels vs. AUC of rifampicin (r^2 = 0.0727)

Although no significant difference could be seen between the AUC of 25-desacetyl rifampicin control and test groups, the AUC of the test group was higher than that of the control group. To determine whether the effect of rifampicin AUC on raised liver enzymes was one which was determined by the total concentration of drug + metabolites, the total AUC for both rifampicin + 25-desacetyl rifampicin was calculated by adding the rifampicin and 25-desacetyl rifampicin concentrations at each time point and calculating a new combined AUC. This combined AUC was plotted for the control and the test groups (Figure 24). There was a significant difference between the two groups (P = 0.05). Error bars indicate SD.

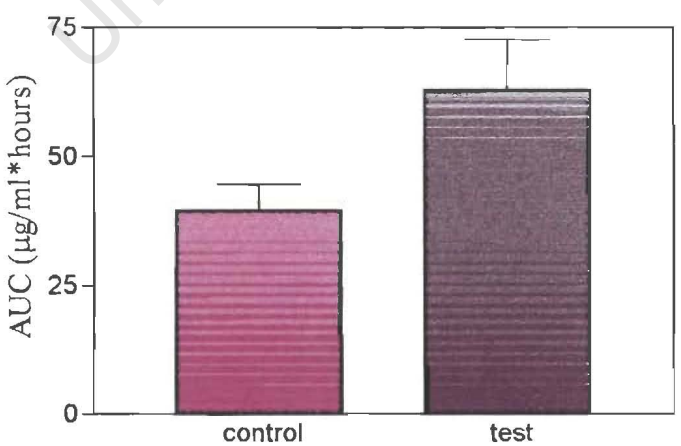


Figure 24: Sum of the AUC's of rifampicin + 25-desacetyl rifampicin for control and test groups

Figures 25 and 26 compare the Cmax for the control and test groups of rifampicin and 25-desacetyl rifampicin respectively. There was no significant difference between the Cmax of rifampicin control and test groups (P = 0.27) or between the Cmax of 25-desacetyl rifampicin control and test groups (P=0.78). There was no significant difference in the Cmax of rifampicin or 25-desacetyl rifampicin in patients with and without HIV. The rifampicin Cmax ± SD of HIV positive patients was 11.08μg/ml and 4.36μg/ml (7.72 ± 3.36μg/ml) and of negative patients was 6.29 ± 3.36μg/ml (P=0.76). The Cmax ± SD of 25-desacetyl rifampicin in the HIV positive and negative patients were 0.15 ± 0.02μg/ml and 0.48 ± 0.09μg/ml respectively (P=0.17). Error bars indicate SD.

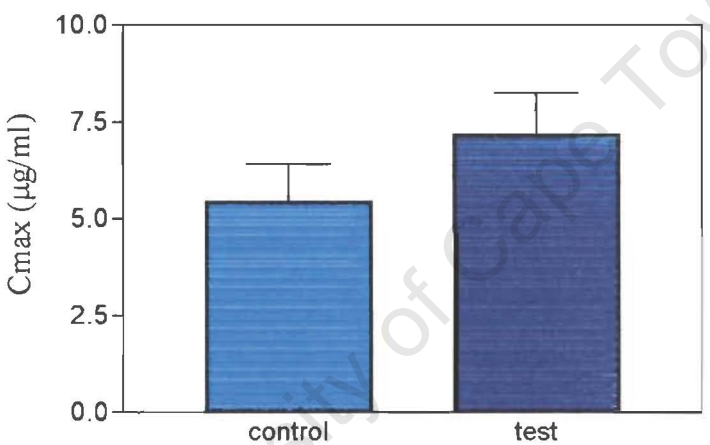


Figure 25: Cmax of rifampicin control and test groups

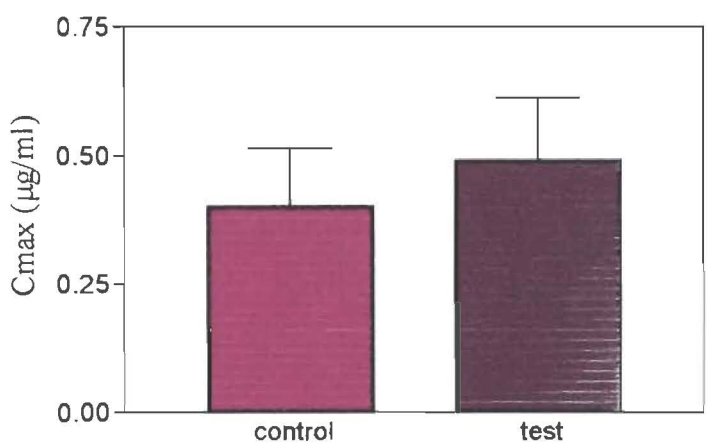


Figure 26: Cmax of 25-desacetyl rifampicin control and test groups

Figures 27 and 28 compare the half-life ($t_{1/2}$) for the control and test groups of rifampicin and 25-desacetyl rifampicin respectively. There was no significant difference between the $t_{1/2}$ of rifampicin control and test groups (P=0.49) or between the $t_{1/2}$ of 25-desacetyl rifampicin control and test groups (P=0.77). The error bars indicate SD.

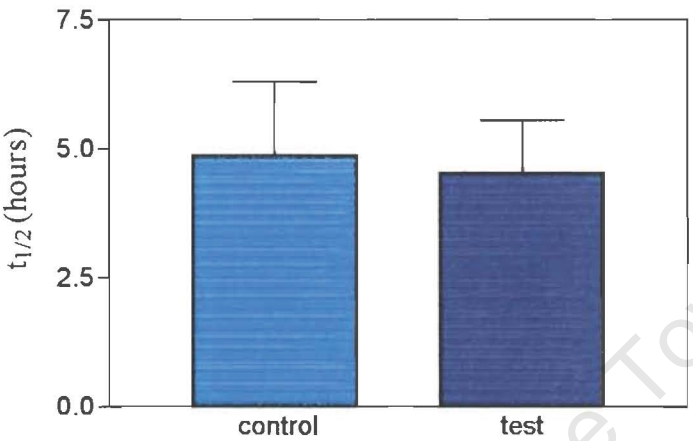


Figure 27: Half-life of rifampicin control and test groups

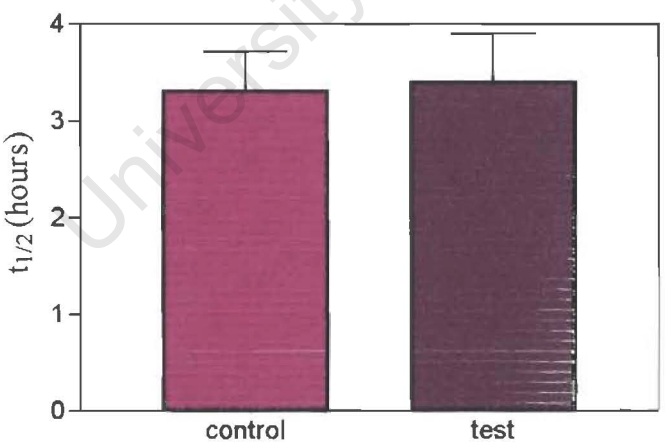


Figure 28: Half-life of 25-desacetyl rifampicin control and test groups

The ratio of metabolite to drug is a good indicator of the ability of the patient to metabolise the drug. Figures 29–31 represent the ratios of the AUC, Cmax and $t_{1/2}$ of 25-desacetyl rifampicin to rifampicin for control and test groups. There was no

significant difference between the test and the control groups for AUC (P = 0.97), Cmax (P = 0.50) or $t_{1/2}$ (P = 1). The error bars indicate SD.

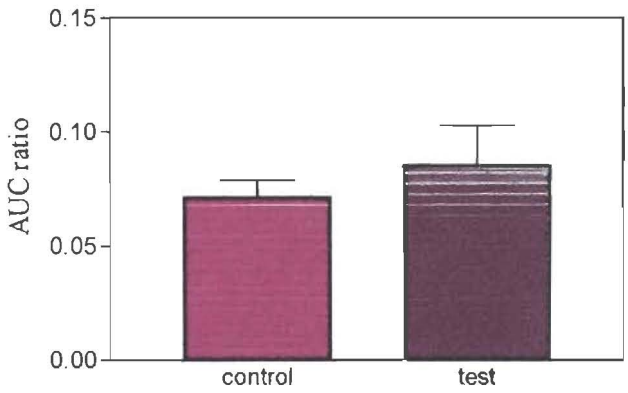


Figure 29: Ratio of 25-desacetyl rifampicin AUC and rifampicin AUC in control and test groups

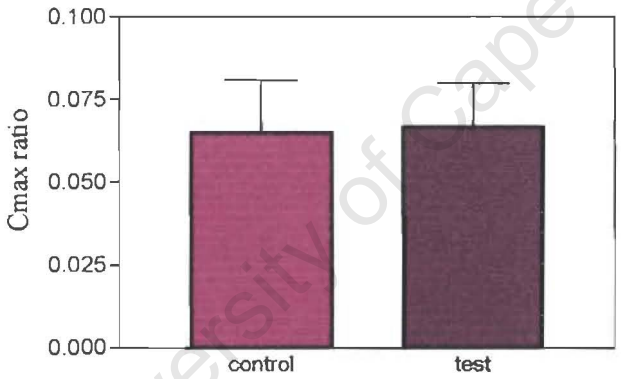


Figure 30: Ratio of 25-desacetyl rifampicin Cmax and rifampicin Cmax in control and test groups

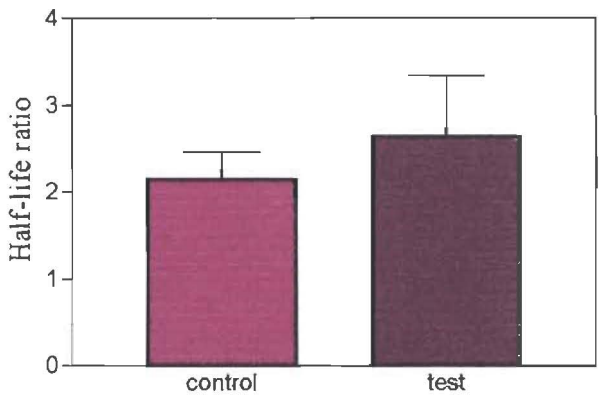


Figure 31: Ratio of 25-desacetyl rifampicin $t_{1/2}$ and rifampicin $t_{1/2}$ in control and test groups

There was no linear correlation of the pharmacokinetic parameters (AUC, Cmax or $t_{1/2}$) with the specific liver enzymes (ALT, AST, ALP or γGT). Table 9 shows the square of the correlation coefficient (r^2) of each of the pharmacokinetic parameters with each enzyme.

		ALT	AST	ALP	γGT
AUC (μg/ml*hours)	**rifampicin**	0.0046	0.1394	0.0212	0.0727
	25-D rifampicin	0.0014	0.0274	0.0029	0.0047
	25-D rifampicin / rifampicin	0.0007	0.0000	0.0431	0.0005
Cmax (μg/ml)	**rifampicin**	0.0308	0.2092	0.0417	0.0111
	25-D rifampicin	0.0115	0.0891	0.0000	0.0011
	25-D rifampicin / rifampicin	0.0002	0.0093	0.0216	0.0003
$t_{1/2}$ (hours)	**rifampicin**	0.0514	0.0293	0.0099	0.0287
	25-D rifampicin	0.0086	0.0739	0.0035	0.0810
	25-D rifampicin / rifampicin	0.0195	0.0768	0.0259	0.0158

Table 9: The squared correlation coefficient (r^2) of the pharmacokinetic parameters vs. the subjects' liver enzyme levels

6.3 Subject profiles

The figures in appendix 13 show the plasma concentrations of 25-desacetyl rifampicin and rifampicin over a 24-hour period in the test and the control subjects. Figures 32 and 33 show a summary plot of the mean pharmacokinetic curve of the Cmax of rifampicin in the test and control groups respectively. Figures 34 and 35 show the same for 25-desacetyl rifampicin. The error bars indicate SD.

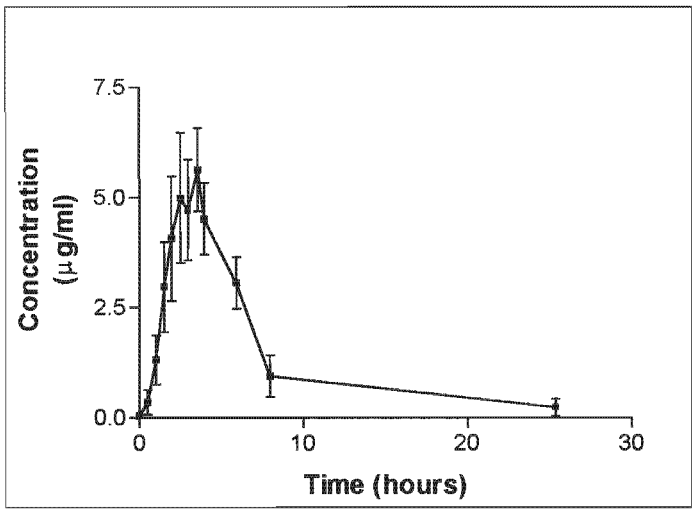


Figure 32: Summary plot of the Cmax of rifampicin in the test group

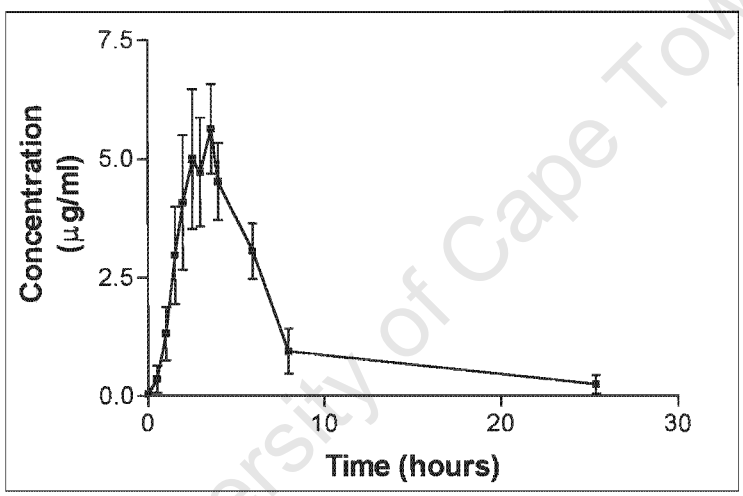


Figure 33: Summary plot of the Cmax of rifampicin in the control group

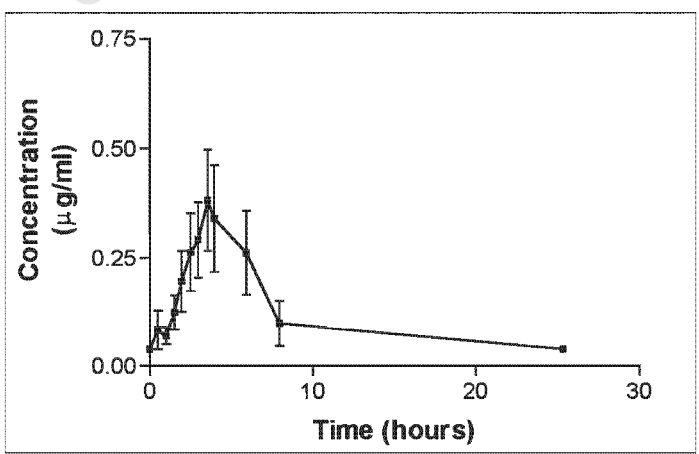


Figure 34: Summary plot of the Cmax of 25-desacetyl rifampicin in the test group

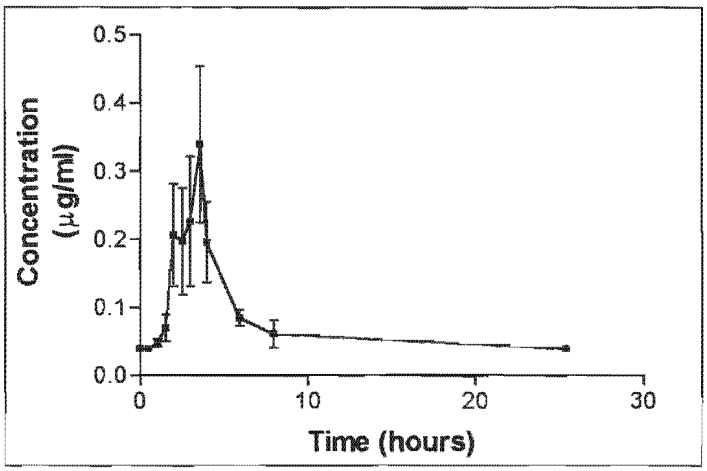


Figure 35: Summary plot of the Cmax of 25-desacetyl rifampicin in the control group

Chapter 5

Discussion

It is well established that antituberculosis treatment carries with it a risk of hepatic toxicity. The mechanism by which isoniazid causes liver disease is well understood, however rifampicin still remains an enigma in this regard. The main aim of this project was to conduct a pilot study in order to establish whether there is a correlation between raised liver enzyme levels in tuberculosis patients and the concentration of rifampicin and its metabolites in their serum.

1. Method validation

In order to determine the concentration of rifampicin and its metabolites in serum it was necessary to validate the existing high performance liquid chromatography (HPLC) method for the detection of rifampicin and 25-desacetyl rifampicin (Zent and Smith, 1995) and to develop a new method for the detection of 3-formyl rifampicin. Standard curves in acetonitrile (pg.48 figures 1-3) and plasma (pg. 51 and 52: figures 4-6) were plotted for both methods and the resulting graphs were linear, indicating that the area under the peak of the chromatograph is directly proportional to the concentration of drug or metabolite injected onto the column. The standard curves could therefore be used to determine the concentration of rifampicin and its metabolites in an unknown plasma sample. The HPLC methods were validated for specificity, reproducibility and sensitivity.

Common concomitant therapy such as ethambutol, streptomycin and co-trimoxazole were shown not to interfere with the detection of rifampicin or its metabolites (pg. 53 plates 6 and 7) while other antituberculosis drugs such as isoniazid and pyrazinamide had previously been shown not to interfere with the method (Zent and Smith, 1995). These substances did not elute in the extraction method and did not effect the recovery of rifampicin or its metabolites (pg. 56: plates 8 and 9). The methods were

shown to be reproducible by numerous repetitions of standard curve determinations. The limit of detection of rifampicin, 25-desacetyl rifampicin and 3-formyl rifampicin were 0.156μg/ml, 0.313μg/ml and 0.625μg/ml respectively. Rifampicin and its metabolites were shown to be stable at -80°C for up to two months (pgs. 57 and 58 Figures: 14-16). None of the trial samples were analysed more than two months after collection. The same C18 extraction columns were used throughout the experimental work and these columns were shown to be reproducible (pg. 54: figures 7-9). The recoveries of rifampicin, 25-desacetyl rifampicin and 3-formyl rifampicin were 75.47% ± 3.77%, 79.65 ± 2.83 and 59.34 ± 3.39 respectively.

2. Health status of the subjects

D.P Marais Hospital is a SANTA-run facility dedicated solely to the treatment of tuberculosis. Most of the patients in the hospital come from disadvantaged backgrounds. Malnutrition can play an important role in the development of liver disease because glutathione metabolism is a major drug detoxification route, and malnourished people tend to have depleted glutathione. The mean BMI of the patients in the control and test group were below the average range, another indication of malnutrition.

The albumin levels of the subjects were also low. Albumin can be an indicator of poor synthetic function, a negative acute phase reactant associated with infection or malnutrition. Low levels of albumin can also indicate chronic liver disease, however in this study it is more likely to be a negative acute phase reactant associated with infection. The patients on the study did not have overt liver disease and therefore the low albumin levels could be attributed to a combination of malnutrition and infection. Tuberculosis is a cachectic state and hence exacerbates malnutrition. Albumin has a fairly long half-life of 17-20 days with less than 5% turnover daily. It is therefore not a good indicator of acute or mild liver injury.

The haematology results presented a picture of a chronic inflammatory process in the subjects. Most patients had anaemia of chronic disorders. This is to be expected in patients who are diseased with tuberculosis due to the sequestration of iron by the

body. Many patients had raised total protein levels – another marker of ongoing inflammatory disease and a typical finding in tuberculosis patients.

3. Personal characteristics of the subjects

There were two main confounding factors that distinguished between the test and the control groups: age and gender. The mean age of the test group (41.58 ± 2.42 years) was significantly higher than that of the control group (35 ± 2.93 years). It has been shown that the risk of hepatotoxicity increases in patients over the age of 35 (Dickinson, 1981). This could be one of the reasons for increased liver enzymes in the test group. The other confounding factor was gender. Females have been shown to be more susceptible to hepatotoxicity than males and in this study there were more females in the test group than there were in the control group.

4. Alcoholism as a factor

Alcohol is known to cause hepatitis and as such, was a potentially confounding factor in this study. It was therefore chosen to exclude all patients who were abusing alcohol at the time of the study. All the patients professed to be abstaining from alcohol while in hospital, however even if this was not true, due to strict hospital policy, it is unlikely that they would have consumed large quantities of alcohol. Patients who had previously abused alcohol were not excluded because the main purpose of this study was to assess the correlation between tuberculosis drug metabolites and mildly raised liver enzymes, not chronic liver disease. Also, within the population of patients infected with tuberculosis in the Western Cape, this would have been a prohibitive criterion as alcohol abuse is rife.

The short-term effect of alcohol consumption is the saturation of the cytochrome P450 system. Cytochrome P450 is the major drug-metabolising enzyme in the liver, which also metabolises alcohol. As a result of P450 saturation, rifampicin metabolism would be expected to be diminished, resulting in the accumulation of rifampicin. Chronic alcohol consumption however, has the opposite effect. The P450 system is induced, leading to increased rifampicin metabolism and supposedly the accumulation of 25-

desacetyl rifampicin. The subjects on this trial would be expected to have induced cytochrome P450's because most subjects drank alcohol prior to hospitalization, however induction may not have been sustained, especially if some patients had abstained from alcohol for more than one month. Isoniazid is metabolised by the P450 system and the mechanism by which isoniazid causes toxicity is through its metabolites. The induction of the P450 system would thus be a further compounding factor in the development of hepatotoxicity in patients receiving antituberculosis treatment. The levels of the toxic isoniazid metabolites were not measured in the subjects and thus the extent of the hepatotoxic effect of isoniazid in relation to rifampicin could not be established.

Isolated γGT elevation is sometimes indicative of excessive alcohol consumption (Sherlock and Dooley, 1993). Of the test subjects, 25% exhibited isolated γGT elevation. However elevation of liver enzymes due to alcohol tends to subside within one week of decreased intake of alcohol. The subjects had been in hospital for at least one month prior to the trial. Therefore assuming that the patients did not consume large quantities of alcohol while in hospital, alcohol could be precluded as the cause of the raised liver enzymes.

There is a clear indication of alcohol consumption among the trial subjects, which could be a serious confounding factor in this study. But because both control and test groups had similar case histories regarding alcohol consumption and similar macrocytosis levels, it is not necessarily a differentiating factor between the two groups and would not have altered the findings of the study.

5. The effect of HIV on the measured parameters

In the Western Cape 8.7% of the population is estimated to be infected with HIV. According to a 1996 WHO report, the number of tuberculosis cases in 2001 was predicted to be 36 922, of whom 36.5% would be HIV positive (Weyer and Fourie, 1996). In this trial 33.82% of the patients that were screened, tested HIV positive, verifying the predicted value. The relatively high incidence of HIV among tuberculosis patients can be attributed to the fact that HIV increases the susceptibility of a patient in the acquisition of tuberculosis and also increases the pool of infectious

tuberculosis cases.

It has been reported that HIV positive patients absorb rifampicin abnormally with resultant concentrations of drugs that are 59% or less the Cmax of healthy volunteers (Peloquin, 1997). However work done in this department has refuted the claim (Taylor and Smith, 1998) and in this study it was also not found to be true. There was no significant difference in the Cmax of rifampicin (P=0.76) or 25-desacetyl rifampicin (P=0.17) between patients that were HIV positive and those that were HIV negative. It must, however be stated that only 2 of the 21 subjects in whom pharmacokinetic profiles were done, were HIV positive.

Hepatic side effects due to antibiotics have been shown to be more frequent in AIDS patients (Westphal *et al*, 1994). From the group of subjects that was screened (n=68), the incidence of abnormal liver enzymes was indeed higher in HIV positive patients (65.22%) than in HIV negative patients (40%). The reason for this is not known, however greater exposure of HIV positive patients to antigens increases the presence of haptens. Haptens could perhaps precipitate an autoimmune reaction or increase the possibility of a drug-induced allergic reaction. HIV positive patients in the D.P Marais hospital setting would also be expected to be glutathione depleted, increasing the susceptibility of the patients to drug-induced hepatotoxicity.

6. Liver enzyme levels as a marker of liver disease

Liver enzyme levels were only checked once. This may be problematic, as liver enzyme levels are known to fluctuate. The problem was overcome as much as possible by ensuring that the pharmacokinetic sampling was conducted no later than two weeks later than the date on which the subject was screened. There was no correlation between the liver enzyme levels of patients in the test group and their INR levels (pg. 66: table 6 and pg. 67: figure 17). INR is seen to be a reliable marker of synthetic function, however the transaminases (ALT and AST) are more specific markers of hepatocellular damage. And the combination of raised ALP and γGT levels is associated with cholestasis, which may be due to enzyme-inducing drugs. Another advantage of using the liver enzyme levels as a marker of liver disease is that they are routinely tested for, whereas INR is not.

7. Biochemical and haematological parameters as markers of liver dysfunction

The albumin, BMI, haemoglobin, INR, MCV and platelet levels of the test group were plotted against those of the control group (pg. 67 figure 17). No significant difference could be seen. When the parameters were plotted against individual enzymes (AST, ALT, ALP and γGT) again no direct relationship could be found (pg. 73 tables 5 and 6). One might expect there to be a correlation if the raised levels of AST, ALT, AP and γGT indicate hepatic injury. However there are too many other variables related to the health of the patient that may have affected the parameters. The degree of hepatic injury in this group of patients with only mild raised hepatic enzyme levels could be expected to be slight. Also the above mentioned markers would only be influenced by a sustained assault. From this study it can be concluded that none of the biochemical or haematological parameters: albumin, BMI, haemoglobin, INR, MCV or platelets, can be used as a reliable indicator of the abnormal functioning of a specific liver enzyme.

8. Hepatitis viruses as a confounding factor in the study

The virology results show an incidence of 11.68% viral hepatitis among the patients that were screened. It has been shown that liver dysfunction during antituberculosis chemotherapy is significantly higher in carriers of hepatitis B virus (34.9%) when compared with non carriers (9.4%) (Yew, 2001). Another study showed that the relative risk of developing anti-tuberculosis drug-related hepatitis is respectively 5-fold and 4-fold when the patient is HCV or HIV positive (Ungo *et al*, 1998). For these reasons it was decided to exclude all patients with HBV surface antigen and HCV antibody. It was decided not to exclude HIV positive patients from the study because HIV is extremely prevalent in the Western Cape and is an important contributing factor in the disease process of tuberculosis patients.

9. Detection of rifampicin and its metabolites in serum

As was expected, 3-formyl rifampicin could not be detected in the serum of the subjects. This confirms the results of Ishii and Ogata (Ishii and Ogata, 1988). As such 3-formyl rifampicin is not expected to play a role in the cause of liver damage unless

it is toxic at very low concentrations. However this hypothesis could neither be proved nor disproved by this study given the fact that if 3-formyl rifampicin was present at all, it was present at a concentration that was below the limit of detection of the method that was developed. Unlike 3-formyl rifampicin, rifampicin and 25-desacetyl rifampicin could clearly be detected using the HPLC method.

10. The correlation between rifampicin and its metabolite and liver enzyme levels

Three parameters were examined when determining the concentration of rifampicin and its metabolite, 25-desacetyl rifampicin in serum: Cmax, half-life and AUC. Two groups of subjects participated in the trial, a group that had at least one raised liver enzyme level (AST, ALT, ALP or γGT), referred to as the test group and a group that exhibited normal liver enzyme levels, referred to as the control group. The reason that patients with enzyme levels that were greater than two times the upper limit of normal were excluded was due to the fact that beyond this limit, patients may be taken off their medication. Using patients with extremely high levels of rifampicin could have proven detrimental to their health. The Cmax, half-life and AUC of rifampicin and 25-desacetyl rifampicin were compared in both groups. The total exposure of the patient to rifampicin has been shown to play an important role in the development of hepatotoxicity (Parthasarathy, 1986). Thus the AUC would be a better reflection on the effect that rifampicin would have on hepatotoxicity than Cmax or half-life.

10.1 Rifampicin

There was no difference in the Cmax or half-life of rifampicin between the control group and the test group, however the AUC of rifampicin in the test group was significantly higher than the AUC of the control group (pg. 69 figure: 18). This result indicates that there is indeed an association between increased rifampicin exposure and raised liver enzyme levels, however no association could be found between rifampicin exposure and specific enzyme levels.

10.2 25-desacetyl rifampicin

No significant correlation could be found between the Cmax or half-life of 25-desacetyl rifampicin and enzyme levels. This was also true for the AUC of 25-desacetyl rifampicin. Although the difference between the AUC of the control and test groups was not significant, there was a clear trend in which the mean AUC of the test group was higher than that of the control group (pg. 69 figure 19). It would be necessary to study a larger cohort of patients in order to draw significant conclusions regarding the potential hepatotoxic effects of 25-desacetyl rifampicin, however it cannot be precluded as being associated with liver toxicity.

The fact that the AUC's of both rifampicin and 25-desacetyl rifampicin were higher in the test group than in the control group raised the question of whether rifampicin-induced hepatitis is related to the overall concentration of rifampicin + its metabolite or whether it was an independent effect of the drug or the metabolite on their own. To this end, the concentrations of 25-desacetyl rifampicin and rifampicin were added together at each time point and the combined AUC's of rifampicin + 25-desacetyl rifampicin were calculated and plotted against liver enzyme levels (pg. 71 figure: 24). There was a significant difference between the AUC's of the control group and the AUC's of the test group. This shows that the effect of rifampicin on the liver is due to the overall concentration of rifampicin as well as the metabolite 25-desacetyl rifampicin. The combined AUC of rifampicin and 25-desacetyl rifampicin is a useful parameter since it eliminates the inter-patient variability of rifampicin metabolism because the concentrations of both the drug and the metabolite are being taken into account.

10.3 Ratio of 25-desacetyl rifampicin and rifampicin

The ratio of 25-desacetyl rifampicin to rifampicin is an indication of the ability of a patient to metabolise the drug that is absorbed. There was no significant difference between the drug:metabolite Cmax, half-life or AUC ratios of the test group and the control group. It can thus be interpreted, that there is no significant difference in the rate at which the test group metabolises rifampicin compared to the control group. The rifampicin concentration in the test group was significantly higher than in the control group. From these results it is possible to conclude that the observed liver

enzyme derangement was not severe enough to impair rifampicin metabolism, rather the increased exposure of the patients to rifampicin may have led to raised liver enzyme levels in the test subjects. In other words, rifampicin may have had a causal relationship with enzyme abnormalities.

The main mechanism by which rifampicin is thought to induce hepatotoxicity is through its combined effect with isoniazid. Rifampicin is a cytochrome P450 inducer. Cytochrome P450 is involved in the biotransformation of isoniazid to isonicotinic acid and hydrazine, a proven hepatotoxic agent (Sarma *et al*, 1986). In this study the fact that there was no difference in the ratio of drug to metabolite in patients with and without raised liver enzyme levels indicates that both groups metabolized rifampicin similarly. The extent of induction of the cyp2E1 enzyme involved in isoniazid metabolism could not be determined by this study. The implications of this effect on the enzyme levels of the 2 groups could therefore not be assessed. The results of this study would show that there was no difference in the activity of CYP3A4 between the two groups, however the results do not necessarily also show that there was no difference in the activity of CYP2E1 between the two groups. It can therefore not be stated with certainty that the raised liver enzyme levels did not occur as a result of isoniazid toxicity.

11. Individual liver enzyme levels as markers of rifampicin toxicity

There was no linear correlation between Cmax, AUC or half-life of either rifampicin or 25-desacetyl rifampicin with any of the individual liver enzyme functions (AST, ALT, ALP and γGT). This indicates that none of the liver enzymes can be used as a reliable marker in the determination of rifampicin or 25-desacetyl rifampicin levels in patients who exhibit mild liver enzyme abnormalities. The higher proportion of raised cholestatic markers (ALP and γGT) compared to necroinflammatory markers (AST and ALT) among the test group may be indicative of impaired cannalicular function, however the normal bilirubin levels do not corroborate this statement. Rifampicin has, however, been shown to inhibit uptake and excretion of bilirubin (Capelle *et al*, 1972 and Cohn, 1969), two of the major causes of cholestatic hepatitis.

12. Possible confounding factors in the study

The study consisted of a cohort of 21 patients, which was divided into a test group of 12 patients and a control group of 9 patients. This cohort is not sufficiently large to draw any statistically significant findings. The two groups were matched as closely as possible for age, gender and race, however as stated previously, differences in gender and age may have contributed to a higher incidence of toxicity in the test group. Other factors that could have contributed to liver enzyme derangements were isoniazid and pyrazinamide concentrations as well as isoniazid metabolite concentrations in the serum. One patient had very low concentrations of rifampicin in his/her serum. This could be due to absorption problems specific to the patient. Although rifampicin concentration in the serum cannot be correlated to the dose of rifampicin given to the patients (figure 16) it should be noted that there were three patients in the test group for whom high doses of rifampicin and high rifampicin concentrations in the serum did correlate. This is an interesting observation and perhaps in a bigger cohort of patients, more attention should be given to this point because it could mean that patients who have raised liver enzymes have difficulty in metabolizing rifampicin. However in this study, due to the small number of patients, this finding was not significant. Alcohol was an important confounding factor given the history of most of the patients, however it was not quantified for each individual patient and therefore the extent to which alcohol may have contributed cannot be established. No linear correlation could be found between the liver enzyme markers and any of the pharmacokinetic parameters of the drug. This was despite the fact that a significant difference between the AUC of rifampicin and the combination of rifampicin + metabolite in the control and test groups was evident. The discrepancy between these two findings indicates that the liver enzyme levels were not sensitive enough markers of liver disease and possibly other markers such as INR should have been used as inclusion criteria for the test group.

Chapter 6

Conclusions

A reliable high performance liquid chromatography (HPLC) technique exists for the determination of rifampicin and 25-desacetyl rifampicin in serum, and in this study an additional HPLC technique was developed for the determination of 3-formyl rifampicin in serum.

The 3-formyl rifampicin metabolite could not be detected in the serum of the patients on this study. This result confirms previous findings in which 3-formyl rifampicin has either been absent or found in very low concentrations in the serum of patients treated with antituberculosis therapy (Ishii and Ogata, 1988 and Lecaillon *et al*, 1978). Rifampicin and 25-desacetyl rifampicin were however, detected in the serum of the trial subjects.

The majority of the subjects had been exposed to large quantities of alcohol on a regular basis, which was supported by the high percentage of macrocytosis observed. This may have been a confounding factor in the study in the determination of the causal effect of rifampicin on hepatitis but because both the control and the test groups consumed similar quantities of alcohol based on the patient history, and had similar levels of macrocytosis, it would not have had any effect on the findings of the study. Furthermore macrocytosis is not only indicative of alcohol consumption but also of malnutrition due to vitamin B12 and folate deficiency. No markers of general health correlated with raised liver enzyme levels.

No significant correlation could be found between the Cmax or half-life of 25-desacetyl rifampicin and enzyme levels. This was also true for the AUC of 25-desacetyl rifampicin.

Although the difference between the AUC of the control and test groups was not significant, there was a clear trend in which the mean AUC of the test group was higher than that of the control group. There was no difference in the Cmax or half-life of rifampicin between the control group and the test group, however the AUC of rifampicin in the test group was significantly higher than the AUC of the control group. From this it can be concluded that there is a correlation between raised liver enzyme levels and the AUC of rifampicin.

One third of the screened patients were HIV positive. Although the levels of rifampicin and 25-desacetyl rifampicin were not higher in HIV positive patients (only 2 HIV positive patients were used in the pharmacokinetic study and therefore no significant conclusions can be made), the incidence of abnormal liver enzyme levels was higher in HIV positive patients than in HIV negative patients (based on the 68 patients that were screened).

The ratio of the AUC of rifampicin to 25-desacetyl rifampicin did not differ between patients who had raised liver enzyme levels and those that didn't. This indicates that the liver enzyme abnormalities in the test group did not effect the patient's ability to metabolise rifampicin. The AUC's of rifampicin and 25-desacetyl rifampicin individually and in combination, were higher in the test group than in the control group. It can thus be concluded that the observed liver enzyme abnormalities in the test group did not effect rifampicin metabolism, rather the rifampicin and 25-desacetyl rifampicin may have induced the liver enzyme abnormalities.

From the results it can be seen that rifampicin-induced liver enzyme derangements is a multifactorial process to which both the drug and the 25-desacetyl rifampicin metabolite may contribute. In order to elucidate the precise mechanism by which rifampicin toxicity is caused, further studies using larger cohorts of patients need to be conducted.

Given the fact that rifampicin may play a role in causing hepatotoxicity, it may be advisable in the future to identify patients who are at risk of hepatotoxicity by measuring

rifampicin serum levels with the view to adjusting dosage if necessary. However, in order to do this it would be necessary to determine a critical concentration of rifampicin at which the hepatotoxicity is caused. However this may be an extremely difficult task to accomplish due to the inter-patient variability in rifampicin absorption and metabolism. Testing may also not be feasible due to the high costs involved given the large number of TB patients in the Western Cape and in South Africa at large.

Chapter 7

References

Acocella G (1978) Clinical Pharmacokinetics of Rifampicin, Clinical Pharmacokinetics **3**: 108-127.

Acocella G, Mattiusi R and Segre G (1978) Multicompartmental analysis of serum, urine and bile concentrations of rifampicin and desacetylrifampicin in subjects treated for 1 week, Pharmacological Research Communications **10**: 271-288.

Alderman J, Takagi T and Lieber CS (1984) Microsomal ethanol oxidation (an account for in vivo ethanol metabolism in deermice lacking alcohol dehydrogenase), Federation Proceedings, **43**: 755.

Arias IM, Jakoby WB, Popper H, Schachter D, Shafritz DA, The liver: Biology and Pathology 2nd Ed., Raven Press, New York, 1988.

Black M, Mitchell JR, Zimmerman HJ, Ishak KG, Epler GR (1975) Isoniazid associated hepatitis in 114 patients, Gastroenterology. **69**: 289-302.

Brodie BB, Gellette JR and La Du BN (1958) Annual Review of Biochemistry, **27**: 427.

Buniva G, Pagani V and Carozzi A (1983) Bioavailability of rifampicin capsules, International Journal of Clinical Pharmacology, Therapy and Toxicology, **21**: 404-409.

Capelle P, Dhumeaux D, Mora M, Feldmann G and Berthelot P (1972) Effect of rifampicin on liver function in man, Gut, **13**: 366-371.

Chopra I and Brennan P (1998) Review: Molecular action of antimycobacterial agents, Tubercle and Lung Disease, **78**(2): 89-98.

Cohen CD, Sayed AR, Kirsch RE (1983) Hepatic complications of antituberculosis therapy revisited. South African Medical Journal, **63**: 960-963.

Citron KM, Somner AR and Angel JH (1980) Short duration chemotherapy in pulmonary tuberculosis: The occurrence of hepatitis in 6months regimens containing pyrazinamide as well as rifampicin, American Review of Respiratory Diseases, **121**, supplement, 452.

Cohn HD (1969) Clinical studies with a new rifamycin derivative, Journal of Clinical Pharmacology, **9**: 118-125.

Daffe M and Ettiene G (1999) The capsule of *M. tuberculosis* and its implications for pathogenicity. Tubercle and Lung Disease, **79**(3): 153-169.

Danan G, Pessayre D , Larrey D and Benamou JP (1981) Pyrazinamide fulminant hepatitis: an old hepatotoxin strikes again, Lancet **2**: 1056-1057.

Davies PDO, Clinical tuberuculosis 2nd ed., Chapman and Hall, London, 1998.

Department of Health, The 1997/8 Annual Report; Strides and Struggles in TB Control, DOH, RSA.

Dickinson DS, Bailey WC, Hirschowitz BI, Soong SJ, Eidus L, Hodgkin MM (1981) Risk factors for isoniazid induced liver dysfunction. Journal of Clinical Gastroenterology, **3**: 271-279.

Dienstag JL and Isselbacher KJ, Acute viral hepatitis. In: Principle's of internal medicine 14th ed., Fauci AS, Braunwald E, Isselbacher KJ, Wilson JD, Martin JB, Kasper DL, Hauser SL and Longo DL (eds.), Mcgraw-Hill, USA, 1998.

Dienstag JL and Isselbacher KJ, Chronic hepatitis. In: Principle's of internal medicine 14th ed., Fauci AS, Braunwald E, Isselbacher KJ, Wilson JD, Martin JB, Kasper DL, Hauser SL and Longo DL (eds.), Mcgraw-Hill, USA, 1998.

Douglas JG, Mcleod M (1999) Pharmacokinetic factors in the modern drug treatment of tuberculosis. Clinical Pharmacokinetics, **37** (2): 127-146.

Dusheiko GM, Brink BA, Conradie JD, Marimuthu T and Sher R (1989) Regional prevalence of hepatitis B, delta, and human immunodeficiency virus infection in southern Africa: a large population survey, American Journal of Epidemiology, **129**(1): 138-145.

Ellard GA (1969) Absorption, metabolism and excretion of pyrazinamide in man, Tubercle, **50**: 144-158.

Ellard GA and Gammon PT (1976) Pharmacokinetics of isoniazid metabolism in man, Journal of Pharmacokinetics and Biopharmaceutics, **4**: 83-113.

Ellard GA, Haslam RM (1976) Observations on the reduction of the renal elimination of urate in man caused by the administration of pyrazinamide. Tubercle, **57**: 97-103.

Ellard GA, Mitichison DA, Girling DJ, Nunn AJ, Fox W (1978) The hepatic toxicity of isoniazid among rapid and slow acetylators of the drug. American Review on Respiratory Diseases, **118**: 47-48.

Feinburg LE, Drug-induced hepatitis. In: Clinical use of drugs in patients with kidney and liver disease, Anderson RJ and Schrier RW (eds.), WB Saunders Company, Philedelphia, 1981.

Fleck A, Metabolic function of the liver: proteins and immunological response. In: Liver Function, Cramp DG and Carson ER (eds.), Chapman and Hall, London, 1990.

Furesz S (1970) Chemical and biological properties of rifampin, Antimicrobial Agents and Chemotherapy, **16**: 316.

Gangadharam PRJ (1986) Isoniazid, rifampin, and hepatotoxicity, American Review of Respiratory Disease, **133**: 963-965.

Gholson CF and Bacon BR, Acute viral hepatitis. In: Essentials of clinical hepatology, Gholson CF and Bacon BR, Mosby Year Book, USA, 1993.

Gholson CF and Bacon BR, Cirrhosis and its complications. In: Essentials of clinical hepatology, Gholson CF and Bacon BR, Mosby Year Book, USA, 1993A.

Gholson CF and Bacon BR, The use of liver tests for the diagnosis and management of liver disease and jaundice. In: Essentials of clinical hepatology, Gholson CF and Bacon BR, Mosby Year Book, USA, 1993B.

Gibbon CJ, South African medicines formulary, South African Medical Association: Health and Medical Publishing Group, Cape Town, 1997.

Girling DJ (1978) The hepatic toxicity of antituberculosis regimens containing isoniazid, rifampicin and pyrazinamide, Tubercle, **59**: 13-32.

Girling DJ (1982) Adverse effects of antituberculosis drugs. Drugs, **23**: 56-74.

Gillette JR (1966) Biochemistry of drug oxidation and reduction by enzymes in hepatic endoplasmic reticulum, Advances in Pharmacology and Chemotherapy, **4**: 219-261.

Goodman LS, Gilman A (Eds.) (1979) In: The pharmacological basis of therapeutics, 4th ed., Collier-Macmillan, London, p 1245.

Gurumurthy P, Krishnamurthy MS and Nazareth O (1984) Lack of relationship between hepatic toxicity and acetylator phenotype in three thousand South Indian patients during treatment with isoniazid with tuberculosis, American Review of Respiratory Disease, **129**: 58-61.

Hift R, Kirsch R, Bass N, Drug-induced liver disease. In: Diagnosis and management of liver disease, Kirsh R, Simon Robson and Trey C. (eds.), Chapman and Hall Medical, Britain, 1995.

Hift R and Trey C, Alcohol and the liver. In: Diagnosis and management of liver disease, Kirsh R, Simon Robson and Trey C. (eds.), Chapman and Hall Medical, Britain, 1995.

Holdiness MR (1984) Clinical pharmacokinetics of the antituberculosis drugs. Clinical Pharmacokinetics, **9**: 511-544.

Ishii M and Ogata Hiroyasu (1988) Determination of rifampicin and its main metabolites in human plasma by high performance liquid chromatography, Journal of Chromatography, **426**: 412-416.

Isselbacher KJ, Bilirubin metabolism and hyperbilirubinemia. In: Principle's of internal medicine 14th ed., Fauci AS, Braunwald E, Isselbacher KJ, Wilson JD, Martin JB, Kasper DL, Hauser SL and Longo DL (eds.), Mcgraw-Hill, USA, 1998.

Isselbacher KJ and Podolsky DK, Approach to the patient with liver disease. In: Principle's of internal medicine 14th ed., Fauci AS, Braunwald E, Isselbacher KJ, Wilson JD, Martin JB, Kasper DL, Hauser SL and Longo DL (eds.), Mcgraw-Hill, USA, 1998.

Jeena PM and Pillay T (1999) An overview of childhood TB with special emphasis on the interaction of HIV-1 infection and TB, South African Respiratory Journal, **5**(1): 6-13.

Jenner AM, Timbrel JA (1994) Influence of inducers and inhibitors of cytochrome P450 on the hepatotoxicity of hydrazine in vivo, Archives of Toxicology, **68**: 349-357.

Kaplan and Isselbacher, Jaundice. In: Principle's of internal medicine 14th ed., Fauci AS, Braunwald E, Isselbacher KJ, Wilson JD, Martin JB, Kasper DL, Hauser SL and Longo DL (eds.), Mcgraw-Hill, USA, 1998.

Kenny MT and Strates B (1981) Metabolism and Pharmacokinetics of the antibiotic rifampin, Drug Metabolism, **12**(1): 159-218.

Kirsch R, Bass N and Arias I, Biochemical evaluation of liver disease. In: Diagnosis and management of liver disease, Kirsh R, Simon Robson and Trey C. (eds.), Chapman and Hall Medical, Britain, 1995.

Konno K. *et al* (1973) Mode of action of Rifampin on Mycobacteria – Electron Microscopy study of the effect of rifampin on *Micobacterium tuberculosis*, American Review of Respiratory Disease, **107**: 1002-1005.

Kumar P and Clark M (eds.), Clinical Medicine 4th Ed., WB Saunders, Italy, 1998.

Kumar A, Misra PK, Mehotra RAJ, Govil YC and Rana GS (1991) Hepatotoxicity of rifampin and isoniazid, American Review of Respiratory Disease, **143**: 1350-1352.

Lacroix C, Hoang P, Nouveau J, Guyonnaud C, Laine G, Duwoos H and Lafont O (1989) Pharmacokinetics of pyrazinamide and its metabolites in healthy subjects, European Journal of Clinical Pharmacology, **36**: 395-400.

Lecaillon JB, Febvre N, Metayer JP and Souppart C (1978) Quantitative assay of rifampicin and three of its metabolites in human plasma, urine and saliva by high performance liquid chromatography, Journal of Chromatography – Biomedical Applications, **145**: 319-324.

Lees AW, Allan GW, Smith J (1971) Toxicity from rifampicin plus isoniazid and rifampicin plus ethambutol therapy , Tubercle, **52**: 182-190.

Leibovitz BE, Siegal BV (1980) Aspects of free radical reactions in biological systems: Aging. Journal of Gerentology, **35**: 45-57.

Lieber CS (1980) Alcohol, protein metabolism and liver injury. Gastroenterology, **79**: 373-390.

Lieber CS (1984) Metabolism and metabolic effects of alcohol, Medical Clinics of North America, **68**: 3-31.

Lieber CS (1984A) Alcohol and the liver: 1984 update. Hepatology, **4**: 1243 – 1260.

Lieber CS (1984B) Alcohol and the liver. In: The Liver Annual 14, Ed: Arias IM, Frenkel M, Wilson JHP, Elsevier, New York.

Lindros KO (1978) Acetaldehyde – its metabolism and role in the action of alcohol. In: Research Advances in Alcohol and Drug problems, Ed: Israel Y and Smart RG, Plenum Press, New York.

Lauterburg BH, Smith CV, Todd EL, Mitchell JR (1985) Oxidation of Hydrazine metabolites formed from isoniazid, Clinical Pharmacological Therapeutics, **38**: 566-571.

Lauterburg BH, Smith CV, Todd EL, Mitchell JR (1985) Pharmacokinetics of the toxic hydrazino metabolites formed from isoniazid in humans, Journal of Pharmacology and Experimental Therapeutics, **235**(3): 567-571.

Lincoln E and Sewell E, Tuberculosis in children, 1st edition, New York, 1963.

Mandell GL, Bennett JE and Dolin R (Eds.), Principles and practice of infectious diseases 4th Ed., Churchill Livingstone, USA, 1995.

Mehmedagic A, Verite P, Menager S, Tharasse C, Chabenat C, Andre D, Lafont O, (1997) Determination of pyrazinamide and its main metabolites in rat urine by high performance liquid chromatography, Journal of Chromatography B, **695**: 365-372.

Melikian DM, Treatment of infectious complications. In: Clinical use of drugs in patients with kidney and liver disease, Anderson RJ and Schrier RW (eds.), WB Saunders Company, Philadelphia, 1981.

Mestdach M, Fonteyne PA, Realini L, Rossau R, Jannes G, Mijs W, De Smet KAL, Portaels F, Van den Eeckhout E (1999) Relationship between pyrazinamide resistance, loss of pyrazinamidase activity and mutations in the *pncA* locus in multidrug resistant clinical isolates of *Mycobacterium tuberculosis*, Antimicrobial Agents and Chemotherapy, **43**: 2317-2319.

Miller FJW, Tuberculosis in children: evolution, epidemiology, treatment, prevention 1st ed., Edinburgh: Churchill Livingstone, London, 1982.

Mitchell JR, Thorgeirsson UP, Black M, Timbrell JA, Snodgrass WR, Potter WZ, Jollow DJ, Keiser HR (1975) Increased incidence of isoniazid hepatitis in rapid

acetylators: possible relation to hydrazine metabolites, Clinical Pharmacology and therapeutics, **18**:70-79.

Mitchell JR, Zimmerman HJ, Ishak KG, Thorgeirsson UP, Timbrell JA, Snodgrass WR, Nelson SD (1976) Isoniazid liver injury: clinical spectrum, pathology and probable pathogenesis, Annals of Internal Medicine, **84**: 181-192.

Nicod L, Viollon C, Regnier A, Jacqueson A and Richert L (1997) Rifampicin and isoniazid increase acitamiophen and isoniazid cytotoxicity in human HepG2 hepatoma cells, Human and Experimental Toxicology, **16**: 28-34.

Nies AS and Gal J, Clinical use of drug assays, In: Clinical use of drugs in patients with kidney and liver disease, Anderson RJ and Schrier RW (eds.), WB Saunders Company, Philedelphia, 1981.

Ohno M, Yamaguchi I, Yamamoto I, Fukuda T, Yokota S, Maekura R, Ito M, Yamamoto Y, Ogura T, Maeda K, Komuta K, Igarashi T and Azuma J (2000) Slow N-acetyltransferase 2 genotype affects the incidence of isoniazid and rifampicin-induced hepatotoxicity, International Journal of Lung Disease, **4**(3): 256-261.

Parrish, Robson, Trey and Kirsch (1990) Retrospective Survey of drug-induced liver disease at Groote Schuur Hospital, Cape Town 1983-1987, South African Medical Journal, **77**: 199-202.

Parthasarathy R, Raghupati S, Janardhanam B, Ramachandran P, Santha T, Sivasubramanian S, Somasundaram PR and Tripathy SP (1981) Hepatic toxicity in South Indian patients during treatment of tuberculosis with short-course regimens containing isoniazid, rifampicin and pyrazinamide, Tubercle, **67**: 99-108.

Peloquin CA (1996) Therapeutic drug monitoring, Clinics in Laboratory Medicine, **16**(3): 717-729.

Peloquin CA (1997), Using therapeutic drug monitoring to dose the antimycobacterial drugs, Clinics in Chest medicine, **18**(1): 79-87.

Peloquin CA, Jaresko GS, Yong C, Keung ACF, Bulpitt AE and Jelliffe RW (1997) Population pharmacokinetic modelling of isoniazid, rifampin and pyrazinamide, Antimicrobial Agents and Chemotherapy, **41**(12): 2670-2679.

Pilhau JA, De Salvo MC and Koch O (1981) Liver alterations in antituberculosis regimens containing pyrazinamide, Chest, **80**(6): 720-722.

Podolsky DK and Isselbacher KJ, Cirrhosis and alcoholic liver disease. In: Principle's of internal medicine 14th ed., Fauci AS, Braunwald E, Isselbacher KJ, Wilson JD, Martin JB, Kasper DL, Hauser SL and Longo DL (eds.), Mcgraw-Hill, USA, 1998.

Podolsky DK and Isselbacher KJ, Derangements of hepatic metabolism. In: Principle's of internal medicine 14th ed., Fauci AS, Braunwald E, Isselbacher KJ, Wilson JD, Martin JB, Kasper DL, Hauser SL and Longo DL (eds.), Mcgraw-Hill, USA, 1998A.

Podolsky DK and Isselbacher KJ, Evaluation of liver function. In: Principle's of internal medicine 14th ed., Fauci AS, Braunwald E, Isselbacher KJ, Wilson JD, Martin JB, Kasper DL, Hauser SL and Longo DL (eds.), Mcgraw-Hill, USA, 1998A.

Proust AJ (1971) The Australian rifampicin trial, The Medical Journal of Australia, **2**: 85-94.

Radziuk J, Metabolic function of the liver: carbohydrates. In: Liver Function, Cramp DG and Carson ER (eds.), Chapman and Hall, London, 1990.

Raviglione MC and O'Brien RJ, Tuberculosis. In: In: Principle's of internal medicine 14th ed., Fauci AS, Braunwald E, Isselbacher KJ, Wilson JD, Martin JB, Kasper DL, Hauser SL and Longo DL (eds.), Mcgraw-Hill, USA, 1998.

Rich AR, The pathogenesis of tuberculosis, 2nd edition, 1952.

Riess W, Schmid K, Keberle H, Dettli L and Spring P (1969) Pharmacokinetic studies in the field of rifamycins, Proceedings of the 6th International Congress of Chemotherapy, University of Tokyo Press **2**: 905-913.

Riska N (1976) Hepatitis cases in isoniazid treated groups and in control group, Bulletin of the International Union Against Tuberculosis, **51**: 203-208.

Robson S and Chisholm M, Heamatological evaluation in liver disease. In: Diagnosis and management of liver disease, Kirsh R, Simon Robson and Trey C. (eds.), Chapman and Hall Medical, Britain, 1995.

Robson S and Neuberger J, Chronic hepatitis and autoimmune liver diseases. In: Diagnosis and management of liver disease, Kirsh R, Simon Robson and Trey C. (eds.), Chapman and Hall Medical, Britain, 1995.

Robson S, Spearman W and Dusheiko G, Viral hepatitis. In: Diagnosis and management of liver disease, Kirsh R, Simon Robson and Trey C. (eds.), Chapman and Hall Medical, Britain, 1995.

Sarma GR, Immanuel C, Kailasam S, Narayana AS and Venkatesan P (1986) Rifampin-induced release of hydrazine from isoniazid. A possible cause of hepatitis during treatment of tuberculosis with regimens containing isoniazid and rifampin, American Review of Respiratory Disease, **133**: 1072-1075.

Segre G, Kinetics of drugs in the hepatobiliary system. In: Liver and drugs, Orlandi F and Jezequel AM (eds.), Academic Press, London, 1972.

Sherlock S and Dooley J, Diseases of the liver and biliary system 7th Ed., Blackwell, London, 1985.

Sherlock S and Dooley J, Diseases of the liver and biliary system 9th Ed., Blackwell, London, 1993.

Steele MA, Burk RF, DesPrez RM (1991) Toxic hepatitis with isoniazid and rifampicin. A meta-analysis, Chest, **99**: 465-471.

Taylor B and Smith PJ (1998) Does AIDS impair the absorption of antituberculosis agents?, International Journal of Tuberculosis and Lung Disease, **2**(8): 670-675.

Timbrell JA, Mitchell JR, Snodgrass WR, Nelson SD (1980) Isoniazid hepatotoxicity: The relationship between covalent binding and metabolism *in vivo*,. Journal of Pharmacology and Experimental Therapeutics, **213**: 364-369.

Ungo JR, Jones D, Ashkin D (1998) Antituberculosis drug-induced hepatotoxicity: the role of hepatitis C and the human immunodeficiency virus, American Journal of Respiratory and Critical Care in Medicine, **157**: 1871-1876.

Vesell ES, Individual variations in drug response. In: Liver and drugs, Orlandi F and Jezequel AM (eds.), Academic Press, London, 1972.

Weber WW and Hein DW (1979) Clinical pharmacokinetics of isoniazid, Clinical Pharmacokinetics, **4**:401-422.

Weber A, Opheim KE, Smith AL, Wong K (1983) High pressure liquid chromatographic quantitation of rifampicin and its two major metabolites in urine and serum, Reviews of Infectious Diseases, **5**: 433-439.

Westphal JF, Vetter D and Brogard JM (1994) Hepatic side-effects of antibiotics, Journal of Antimicrobial Chemotherapy, **33**: 387-401.

Weyer and Fourie, (1996) Tuberculosis control in South Africa: Joint programme review, WHO: 96.208.

Williams RT, Detoxification mechanisms, Chapman and Hall, London, 1959.

Wilkinson D and Moore DA (1996) HIV-related tuberculosis in South Africa-clinical features and outcome, South African Medical Journal, **86**(1): 64-67.

Woo J, Chan CHS, Wa;inp A, Chan K (1992) Hydrazine: A possible cause of isoniazid-induced hepatic necrosis, Journal of Medicine, **23**: 51-59.

World Health Organisation Report 1996 (1996) Fighting Disease, fostering development, Geneva: WHO: 27-9.

Yamamoto T, Moriwaki Y, Takahashi S, Toshikazu H, Kazuya H (1987) Study of the metabolism of pyrazinamide using a high performance liquid chromatography analysis of urine samples, Analytical Biochemistry, **160**: 346-349.

Yew WW (2001) Risk factors for hepatotoxicity during anti-tuberculosis chemotherapy in Asian populations, International Journal of Tuberculosis and Lung Disease, **5**(1): 99-101.

Zent C and Smith P (1995) Study of the effect of concomitant food on the bioavailability of rifampicin, isoniazid and pyrazinamide, Tubercle, **4**: 195-202.

Zimmerman HJ, Hepatotoxicity, Appleton-Century-Crofts, New York, 1978.

Zimmerman HJ (1975) Liver disease caused by medicinal agents, Medical Clinics of North America, **59**(4): 897-907.

APPENDIX 1: INFORMATION SHEET

The correlation between rifampicin and pyrazinamide metabolites and abnormal liver enzyme levels.

1. Researchers

The department of Pharmacology at the University of Cape Town is doing this study.
The researchers names are: Dr. Helen McIlleron and Miss Galya Reuter.
We can be telephoned at (021) 4066286 during office hours.
Our address is UCT Medical School, Pharmacology Dept., Observatory, 7925.

If you have any questions about this study at any time, please feel welcome to contact us.

2. Why are we doing the study?

When you take your TB pills, they go into your body and your liver breaks them into smaller parts called metabolites. The metabolites disappear after some time. Sometimes a person's liver can't break up the medicine properly and then these smaller parts (metabolites) stay in the person's blood. Doctors do tests to see if a person's liver is working properly. Sometimes the tests are a bit higher than they should be. We want to see if those people whose have tests that are a bit higher than normal also have more metabolites in their blood than most people. We can use this information to understand how the medicine is hurting the liver.

3. Taking part in the study

If you do not want to be on the study you do not have to be, your participation is entirely voluntary. Nobody will be angry and nothing will happen to you. You will be treated exactly the same in hospital as before. You don't even have to tell us why you don't want to be on the study, you can just say: "no".

4. Confidentiality

Any information you give us will be kept confidential. The only people who will see your forms will be Dr. Helen McIlleron, Galya Reuter and the nurse who takes your blood. Once we have collected the information from the forms, your name will not be able to be linked with the information. If we publish any results from this study, your name will not be included in the paper.

5. HIV Test

Sometimes TB medicines hurt the liver. We want to know if the medicines are worse for people who have HIV and AIDS and that is why we will be testing you for HIV.

6. Ethics Committee

The University of Cape Town has a special committee called the Ethics Committee that checks if the study is legal. The Ethics Committee has looked at this study and has found it to be legal and ethical. If you want to complain about any of the researchers or staff on the study, you can write to: Research Ethics Committee, Faculty of Medicine Anzio Rd. Observatory, 7925.Telephone: (021) 406 6492

7. Personal Benefit

The study will not help you personally in any way but it will help the community because it will help us to understand how TB medicines hurt the liver.

APPENDIX 2: CONSENT TO PARTICIPATE IN A CLINICAL STUDY

The correlation between rifampicin and pyrazinamide metabolites and abnormal liver enzyme levels.

Study Leaders: Dr. Helen McIlleron and Miss Galya Reuter

I have read the information sheet.	YES ☐	NO ☐
I have had the opportunity to ask questions and talk about this study.	YES ☐	NO ☐
I have received satisfactory answers to all my questions.	YES ☐	NO ☐
I understand the following:		
Personal information will be recorded	YES ☐	NO ☐
I will be tested for HIV status and liver disease	YES ☐	NO ☐
10 Blood samples will be taken from me over 8 hours and then one more blood sample will be taken from me the next morning.	YES ☐	NO ☐
Each blood sample will be about 1 teaspoon, so all together I will be giving about 11 teaspoons of blood.	YES ☐	NO ☐
A needle will be put into my arm in the morning and will be left there for 8 hours while blood is taken from me.	YES ☐	NO ☐
I can leave the study whenever I want to without giving any explanation and there will be no effect on my treatment.	YES ☐	NO ☐
I have answered all the questions on the forms accurately.	YES ☐	NO ☐
I agree to participate in this study	YES ☐	NO ☐

_____	_____	____/____/_____
NAME OF SUBJECT	SIGNATURE	DATE
_____	_____	____/____/_____
NAME OF INVESTIGATOR/ INVESTIGATOR'S ASSISTANT	SIGNATURE	DATE

BYLAE 3: INLIGTINGSTUK

Die korrelasie tussen rifampisien en pirazienamied metaboliete en abnormale lewer ensiemvlakke.

1. Navorsers

Die Departement Farmakologie aan die Universiteit van Kaapstad voer hierdie studie uit. Die name van die navorsers is: Dr. Helen McIlleron en Mej. Galya Reuter.
Ons kan per telefoon gekontak word by: (021) 4066286, gedurende kantoorure.
Ons adres is: UCT Medical School, Pharmacology Dept., Observatory, 7925.
Indien u enige vrae het rondom hierdie studie ter enige tyd, voel asseblief vry om ons te kontak.

2. Waarom doen ons hierdie studie ?

Wanneer u TB medisyne drink, word dit deur u liggaam opgeneem en in die lewer afgebreek na kleiner deeltjies, genoem metaboliete. Die metaboliete word mettertyd verwyder en verdwyn. In sommige gevalle kan 'n persoon se lewer nie die medisyne ordentlik afbreek nie, en dan bly die metaboliete in die persoon se bloed vir 'n baie langer tydperk.
Dokters doen gereld toetse om te sien of 'n persoon se lewer normaal funksioneer. Soms is die resultate van die toetse effens hoër as wat dit moet wees. Ons wil probeer vasstel of daardie persone met hoër toetsresultate ook meer metaboliete in hulle bloed het as meeste mense. Ons kan hierdie inligting gebruik om te verstaan hoe die medisyne moontlik die lewer beskadig.

3. Deelname aan die studie

U hoef nie deel te wees van die studie indien u nie wil nie, u deelname is totaal vrywillig. Niemand sal dit teen u hou nie en niks sal met u gebeur nie. U sal presies soos tevore in die hospitaal behandel word. U hoef ons nie eens te vertel waarom u nie deel wil neem aan die studie nie, u hoef net "nee" te sê en dit sal voldoende wees.

4. Privaatheid

Enige inligting wat u aan ons verskaf sal streng konfidensieel gehou word. Die enigste persone wat u vorms sal sien is Dr. Helen McIlleron, Galya Reuter en die verpleegster wat u bloed sal trek. Sodra ons die nodige inligting van u vorms verkry het, sal u naam nie meer aan die inligting gekoppel kan word nie. Indien ons enige van die resultate van die studie publiseer, sal u naam nie ingesluit word nie.

5. HIV toets

Partymaal beskadig TB medisyne die lewer. Ons wil probeer vasstel of die skade erger is in pasiënte met die HIV virus en VIGS, en dit is waarom u getoets sal word vir die teenwoordigheid van die HIV virus.

6. Etiese kommitee

Die Universiteit van Kaapstad het 'n spesiale kommitee naamlik die Etiese Kommitee wat seker maak dat die studie wettig is. Die kommitee het hierdie studie ondersoek en bepaal dat dit wettig en eties is. Indien u klagtes het oor enige van die navorsers of staf verbonde aan die studie, kan u skryf aan: Research Ethics Committee, Faculty of Medicine, Anzio rd., Observatory, 7925: telefoon: (021) 4066492.

7. Persoonlike voordeel

Die studie sal u geensins persoonlik bevoordeel nie, maar dit sal die gemeenskap baat aangesien dit ons sal help verstaan hoe TB medisyne die lewer kan beskadig.

BYLAE 4: TOESTEMMING OM AAN 'N KLINIESE STUDIE DEEL TE NEEM

Die korrelasie tussen rifampisien en pirazienamied metaboliete en abnormale lewer ensiemvlakke

Studieleiers: Dr. Helen McIlleron en Miss Galya Reuter

Ek het die inligtingstuk gelees.	JA ☐	NEE ☐
Ek is die geleentheid gebied om vrae te vra en om oor die studie te gesels.	JA ☐	NEE ☐
Ek het bevredigende antwoorde op al my vrae gekry.	JA ☐	NEE ☐
Ek verstaan die volgende :		
Persoonlike inligting sal genoteer word	JA ☐	NEE ☐
Ek sal getoets word vir HIV status en lewerfunksie	JA ☐	NEE ☐
10 Bloedmonsters sal van my geneem word oor 'n tydperk van 8 ure, en dan nog een bloedmonster die volgende oggend.	JA ☐	NEE ☐
Elke bloedmonster sal gelykstaande wees aan omtrent een teelepel vol, so altesame sal ek ongeveer 11 teelepels bloed skenk.	JA ☐	NEE ☐
'n Naald sal in my arm geplaas word in die oggend, en sal daar gelaat word vir 8 ure waartydens bloed getrek sal word.	JA ☐	NEE ☐
Ek kan ter enige tyd myself van die studie onttrek sonder om enige redes te verskaf en daar sal geen invloed op my normale behandeling wees nie.	JA ☐	NEE ☐
Ek het al die vrae op die vorms akkuraat beantwoord.	JA ☐	NEE ☐
Ek stem in om aan die studie deel te neem.	JA ☐	NEE ☐

_____	_____	____/____/_____
NAAM VAN PASIËNT	HANDTEKENING	DATUM
_____	_____	____/____/_____
NAAM VAN ONDERSOEKER/ ONDERSOEKER ASSISTENT	HANDTEKENING	DATUM

ISHILOMELO 5: Incwadana yolwazi ngophando

Unxibelelweno phakathi kwe Rifampicin ne Pyrazinamide metabolites ne sifo sezibindi.

1. Abaphandi
Oluphando lwenziwa liziko lophando ngamayeza (Pharmacology) kwi Yunivesi yaseKapa. Amagama abaphandi ngala: uGqirha Helen McIlleron kunye no Galya Reuter. Iinombolo zabo zomnxeba zezi: (021) 406 6286 ngamaxesha omsebenzi. Idilesi yabo yile: UCT Medical School, Dept of Pharmacology, Observatory, 7925
Wamkelekile ukubuza nayiphi na imibuzo ngoluphando, ngalo naliphi na ixesha.

2. Lolwani olophando?
Amayeza esifo sephepha athi asetyenzwe sisibindi emzimbeni ukwenza imithamo ekuthiwa zii-*metabolites*. Ezi-metabolites zithi ziphele emva kwexeshana. Ngamanye amaxesha sithi isibindi singawahlafuni kakuhle lamayeza ngoko ahlale egazini. Kungoko ke oogqirha bathi benze oluvavanyo lokuba isibindi siwahlafune kakuhle naa lamayeza. Olulwazi lusinceda sazi ukuba lamayeza awayongozi na kwisibindi sakho.

3. Ukuthatha inxaxheba koluphando
Umntu ngamnye akanyenzelekanga ukuba athathe inxaxheba koluphando. Kungokuthanda kwakho ukuthatha inxaxheba. Xa ungafuna okanye ungafuni akukho mntu oyakukuxoxisa kwaphela. Akukho nemfuneko yokuba unike inkcazelo yokuba kutheni na! Ungavele uthi: Hayi, awufuni!

4. Okuyimfihlelo
Yonke inkcaza oyinikezayo iyakugcinwa iyimfihlelo. Akekho omnye umntu oyakuyazi ngaphandle kwabaphandi kunye nonesi oyakube encedisa. Ekubeni siyifumene yonke inkcukacha, igama lakho alisayi kunxulumaniswa nanto engoluphando. Oku kudibanisa nopapasho kumaphepha ezophando.

5. Ukujongwa I-HIV
Oku kwenzelwa ukujongwa ukuba lamayeza esifo sephepha ayingozi kakhulu kubantu abanesifo sikagawulayo ne-HIV ngaphezulu kunabanye abantu. Yiyo lento sisenza olujongo lwe-HIV.

6. Ikomiti Ejongene namalungelo ezophando
I-Yunivesithi yase Kapa inekomiti ejongene namalungelo angophando. Lekomiti iyijongile indlela yoluphando yaze yaluvumela ukuba luqhubekeke. Kodwa ukuba unesikhalazo ngoluphando okanye ngabaphandi ungabhalela kulekomiti ngaledilesi:
Research Ethics Committee, Faculty of Medicine
Anzio Road, Observatory 7925
Umnxeba: (021) 406 6492

7. Okulungeniso
Oluphando aluyi kukunceda nangayiphi na indlela kodwa luyakunceda uluntu ngokubanzi. Luyakunceda ekubeni kwaziwe ukuba lamayeza okunyanga isifo sephepha ayingozi kusini na kwisibindi

ISHILOMELO 6: ISIVUMELWANO SOKUTHABATHA INXAXHEBA KUPHANDO LWEZAMAYEZA ESIFO SEPHEPHA (TB)

Unxibelelweno phakathi kwe Rifampicin ne Pyrazinamide metabolites ne sifo sezibindi.

Abaphandi abaphambili: Gqirha Helen McIlleron and Miss Galya Reuter

Ndiyifundile incwadana ingenkcukacha yoluphando	Ewe ☐	Hayi ☐
Ndilinikiwe ixesha lokubuza kwaye ndithethe ngoluphando	Ewe ☐	Hayi ☐
Ndiphendulwe ngokwanelisayo ngabaphandi malunga noluphando	Ewe ☐	Hayi ☐
Ndiyakuqonda oku kulandelayo:		
Kuyakushicilelwa inkcukacha ngam kwakunye nezonyango lwam.	Ewe ☐	Hayi ☐
Ndiyakuhlolelwa intsholongwane yesifo sikagawulayo kwakunye nesifo sesibindi.	Ewe ☐	Hayi ☐
Igazi liyakuthatyathwa amaxa alishumi kwixesha elingangeeyure ezisi bhozo. Emveni koko, esinye isixa siyakuthathwa ngentseni elandelayo.	Ewe ☐	Hayi ☐
Isixa ngasinye segazi siyakulingana necephana (tispuni). Lilonke ndiyakunikeza	Ewe ☐	Hayi ☐
Inaliti ebizwa ngokuba yi-cannula iyakufakwa kumthambo osengalweni yam. Iyakuhlala apho iiyure ezisibhozo ukuze igazi lithathwe ngayo	Ewe ☐	Hayi ☐
Ndinelungelo lokungangeneli oluphando okanye lokushiya phakathi ndinganikanga nkcaza. Oko akuyi kuluphazamisa unyango lwam kwaye andiyi kuxoxiswa	Ewe ☐	Hayi ☐
Ndiyavuma ukuba ndiyakwenza nayiphi na into abaphandi nabancedisi babo abandiyalela yona.	Ewe ☐	Hayi ☐
Ndiyavuma ukuthabatha inxaxheba koluphando	Ewe ☐	Hayi ☐

_____	_____	_____/_____/_____
IGAMA LOMGULI	UMSAYINO	UMHLA
_____	_____	_____/_____/_____
IGAMA LOMPHANDI/ LOMNCEDISI	UMSAYINO	UMHLA

APPENDIX 7: Ethics approval from the University of Cape Town

UNIVERSITY OF CAPE TOWN

Research Ethics Committee
Faculty of Medicine
Anzio Road, Observatory, 7925
Queries : Mandisa Tani
Tel : (021) 406-6492 Fax: (021) 406-6390
E-mail : Mtani@curie.uct.ac.za

06 December 2000

REC REF: 234/2000

Dr H McIlleron
Pharmacology

Dear Dr McIlleron

THE CORRELATION BETWEEN RAISED LIVER ENZYME LEVELS AND HIGH CONCENTRATIONS OF ANTITUBERTULOSIS DRUG METABOLITES

Thank you for your application submitted to the Research Ethics Committee on the 14 September 2000.

I have pleasure in informing you that the Research Ethics Committee has **formally approved** the above study on the 29 November 2000.

Included is a list of Research Ethics Committee Members who have formally approved your protocol.

Please quote the above Reference number in all correspondence.

Yours sincerely

PROFESSOR CR SWANEPOEL
CHAIRPERSON

APPENDIX 8: Ethics approval from the South African National Tuberculosis Association (SANTA)

SANTA
TB is curable

REG NO 000-934 NPO

19th December 2000

Ms Galya Reuter
Department of Pharmacology
K45 Old Main Building
Groote Schuur Hospital
Observatory
7925

Dear Ms Reuter

Re: The correction between raised liver enzyme levels and high concentrations of antiturberculosis drug metabolites.

Our reference number: No. 2

Thank you for your application submitted to the SANTA Ethics Committee, on September 2000.

This is to confirm our telephonic conversation. Please be informed that at the Ethics Committee Meeting on the 1st November, 2000 consideration was given to the above mentioned application and formally approved for the clinical trial to be implemented at the **D.P. Marais SANTA Centre in Cape Town.**

The following documentation was considered and approved:

- Amendments to M.Sc project proposal by Galya Reuter.
- The original project proposal approved with the above mentioned amendments.

Included is a list of the Ethics Committee Members and those who formally approved your protocol.

Your sincerely

Dr B Beyers
Medical Services manager

UNIT 37, HINGHAM FIELD OFFICE PARK
BOEING ROAD EAST, BEDFORDVIEW
PRIVATE BAG X10030, EDENVALE, 1610
TEL 011.454.0260
FAX 011.454.0096

APPENDIX 9: Personal characteristics of trial subjects

Patient	Gender	Race	Height (cm)	Weight (Kg)	BMI (Kg/m^2)	HIV status
Test						
4	Male	Black	161	35.0	740.600	-
10	Male	Coloured	170	49.0	589.800	-
22	Female	Coloured	147	42.0	514.500	-
24	Female	Coloured	150	34.0	661.800	-
30	Female	Coloured	150	40.0	562.500	-
33	Female	Coloured	167	46.5	599.800	-
42	Male	Coloured	160	41.5	616.900	-
43	Male	Coloured	176	49.0	628.600	-
46	Male	Coloured	162	42.0	621.000	-
50	Female	Black	160	34.0	752.900	+
58	Female	Black	164	49.0	548.900	+
67	Female	Black	160	55.0	465.500	-
Control						
8	Male	Coloured	157	44.0	563.780	-
29	Female	Coloured	162	55.0	477.160	-
36	Male	Coloured	174	60.5	500.300	-
45	Male	Coloured	164	45.0	672.400	-
49	Female	Black	157	49.0	547.760	-
52	Female	Coloured	160	39.0	656.510	-
57	Male	Coloured	160	49.0	522.450	-
66	Female	Coloured	163	46.0	577.590	-
68	Female	Coloured	161	39.0	664.640	-

APPENDIX 10: Pharmacokinetic results of trial subjects

Patient	Cmax Rif (μg/ml)	Cmax D-rif (μg/ml)	AUC Rif (μg/ml*hours)	AUC D-rif (μg/ml*hours)	$t_{1/2}$ Rif (hours)	$t_{1/2}$ D-rif (hours)
Test						
4	12.89	0.84	81.92	4.44	2.84	2.70
10	4.59	0.66	57.99	6.57	5.17	2.19
22	7.28	0.56	40.30	4.38	3.12	8.08
24	13.70	0.97	116.78	9.73	3.45	2.00
30	7.38	1.40	78.49	17.14	2.41	3.44
33	6.66	0.16	78.91	0.58	13.76	3.74
42	4.93	0.22	35.79	1.22	3.30	3.55
43	5.37	0.22	51.06	2.02		3.14
46	0.72	0.16	5.72	0.06	7.02	
50	11.08	0.16	86.38	1.66	2.58	2.95
58	4.36	0.16	45.76	1.48	2.79	2.85
67	7.09	0.57	38.82	3.35	3.46	2.84
Control						
8	2.64	0.22	33.95	2.53	3.56	2.85
29	8.54	1.18	49.81	5.41	3.96	5.41
36	6.05	0.36	41.38	2.27	3.08	2.51
45	10.57	0.43	66.29	2.98	3.06	2.59
49	7.52	0.58	37.06	2.59	3.35	2.75
52	2.82	0.16	33.82	0.85	14.82	6.37
57	4.42	0.38	26.09	1.98	3.77	3.03
66	2.07	0.16	18.49	0.06	3.51	
68	4.34	0.35	26.90	1.25	3.67	4.05

APPENDIX 11: Chemical pathology results of trial subjects

Patient	Albumin (g/L)	Creatinine (μmol/L)	Urea (mmol/L)	Total Bilirubin (μmol/L)	Conjugated Bilirubin (μmol/L)	Total Protein (g/L)
Test						
4	26	59	3.5	8	4	69
10	38	68	2.8	6	1	78
22	34	71	3.6	3	1	87
24	25	58	1.8	12	5	69
30	36	61	2.6	4	1	79
33	31	75	4.3	6	2	91
42	34	29	0.6	5	1	75
43	29	52	2.7	6	3	75
46	29	72	3.8	4	1	62
50	14	66	2.9	9	5	64
58	35	67	4.2	5	1	93
67	33	46	1.3	10	5	80
Control						
8	35	85	3.1	10	5	82
29	40	69	3.7	3	0	78
36	28	62	2.6	5	1	65
45	29	49	3.4	7	2	81
49	30	63	3.	3	1	90
52	34	56	3.8	3	1	91
57	40	77	2.6	6	2	84
66	40	55	3.6	7	2	86
68	37	51	3.3	7	2	88

Reference ranges:

Albumin (g/L)	Creatinine (μmol/L)	Urea (mmol/L)	Total Bilirubin (μmol/L)	Conjugated Bilirubin (μmol/L)	Total Protein (g/L)
35 - 50	50-100	1.7 – 6.7	1 – 17	0 - 4	60 - 80

APPENDIX 12: Haematology results of trial subjects

Patient	MCV (fl.)	Hb (g/dL)	RDW (units)	Platelets (x 10^9/L)	INR (ratio)	Neutrophils (x 10^9/L)	Haematocrit (ratio)
Test							
4	87	11.9	18.7	756	1.2	11.07	0.38
10	85	12.6	17.5	526	1.4	12.01	0.40
22	80	13.0	19.0	441	1.1	7.39	0.39
24	81	8.4	20.3	766	1.5	17.53	0.28
30	81	11.9	19.4	202	1.2	8.16	0.38
33	92	11.5	18.2	657	1.2	8.49	0.37
42	105	9.3	19.0	888	1.2	9.28	0.31
43	79	8.7	19.8	570	1.6	11.60	0.29
46	85	12.1	21.6	648	1.3	7.21	0.39
50	112	9.0	17.6	414	2.0	7.46	0.28
58	75	13.8	27.8	359	1.3	9.44	0.45
67	92	11.5	17.1	89	1.4	4.91	0.37
Control							
8	81	11.7	18.1	533	1.3	12.61	0.36
29	103	13.9	16.0	407	1.2	7.23	0.42
36	87	10.7	16.6	532	1.7	5.55	0.33
45	91	10.2	19.2	361	1.3	6.84	0.33
49	93	10.8	16.5	556	1.4	10.12	0.34
52	101	11.5	15.2	303	1.3	3.10	0.38
57	97	13.3	15.2	544	1.3	4.78	0.40
66	98	11.8	23.7	1439	1.3		0.37
68	99	13.3	16.0	537	1.3	2.31	0.42

Reference ranges

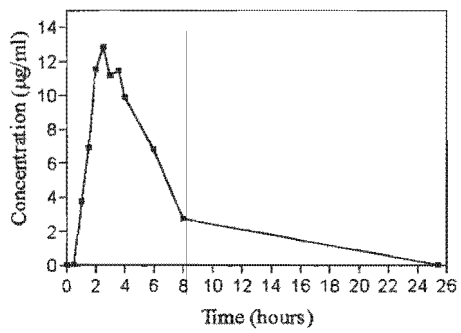
MCV (fl.)	Hb (g/dL)	RDW (units)	Platelets (x 10^9/L)	INR (ratio)	Neutrophils (x 10^9/L)	Haematocrit (ratio)
80 - 95	13.3 – 17.3	7.4 – 13.6	150 - 450	< 1.4	1.8 – 7.7	0.37-0.53

APPENDIX 13: Pharmacokinetic profiles of subjects

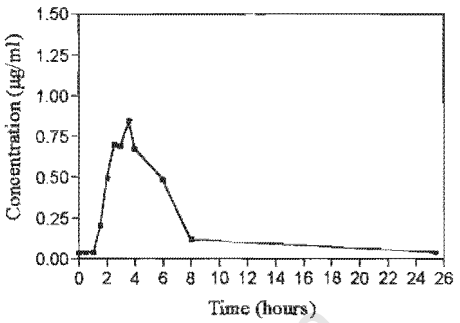
Test subject profiles

(the number in the bracket denotes the patient number)

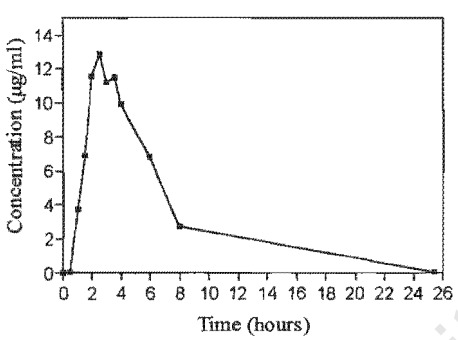
rifampicin (4)



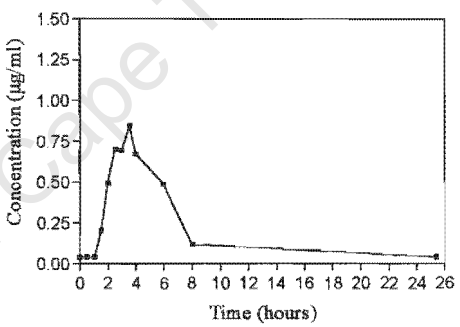
25-desacetyl rifampicin (4)



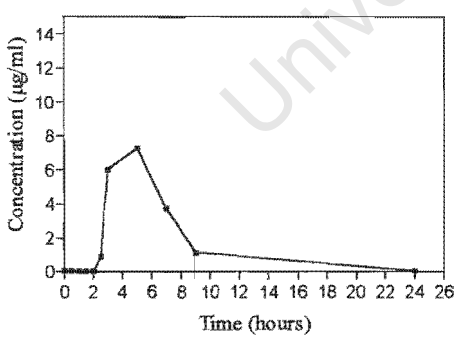
rifampicin (10)



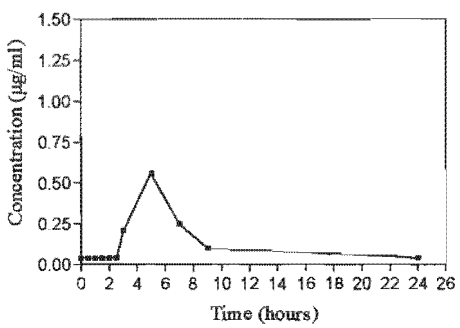
25-desacetyl rifampicin (10)



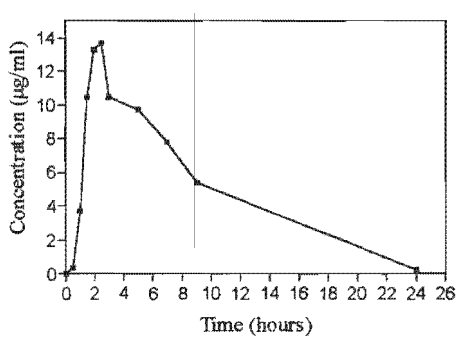
rifampicin (22)



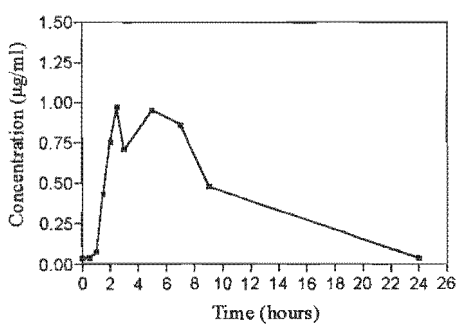
25-desacetyl rifampicin (22)



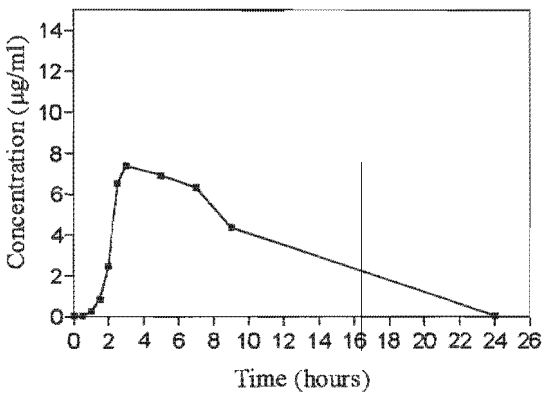
rifampicin (24)



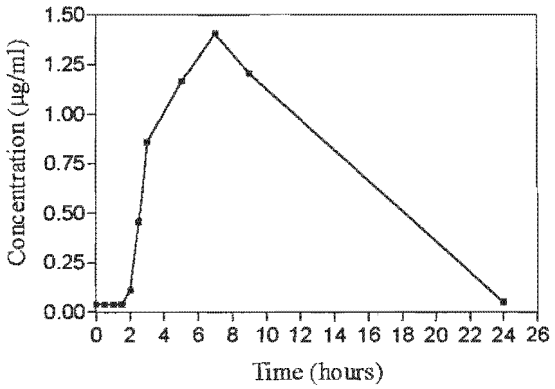
25-desacetyl rifampicin (24)



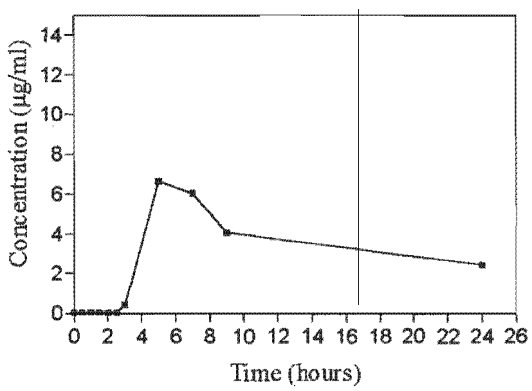
rifampicin (30)



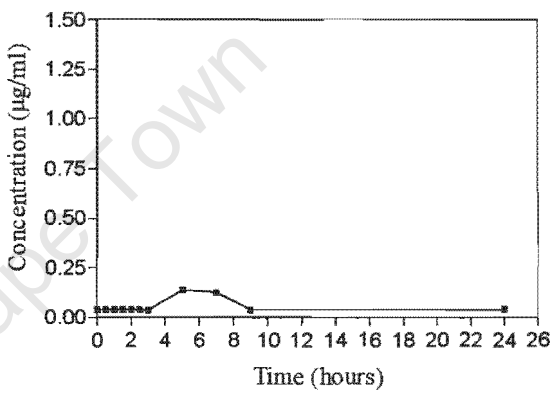
25-desacetyl rifampicin (30)



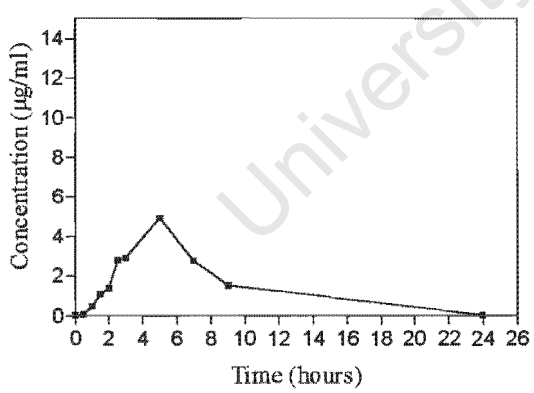
rifampicin (33)



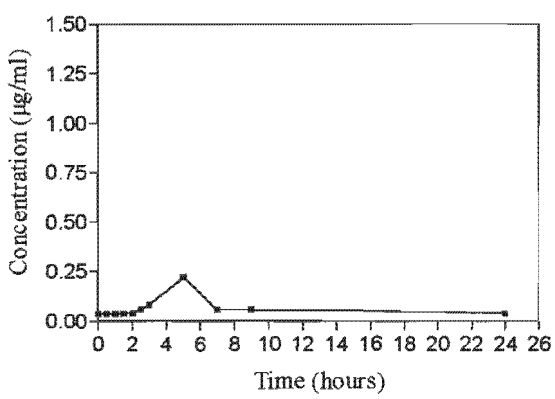
25-desacetyl rifampicin (33)



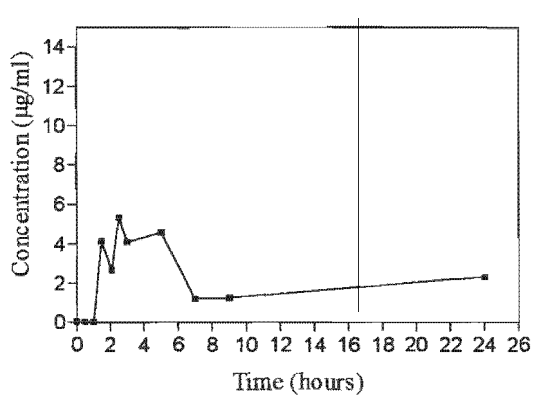
rifampicin (42)



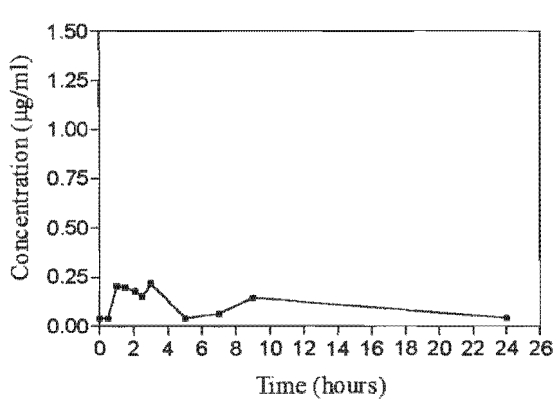
25-desacetyl rifampicin (42)



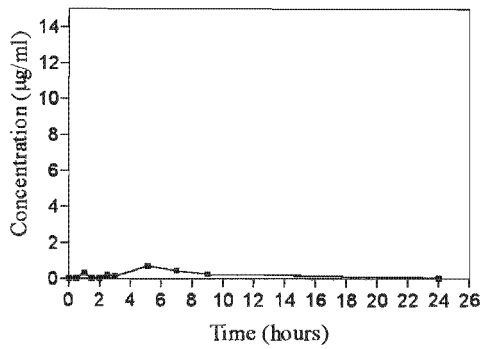
rifampicin (43)



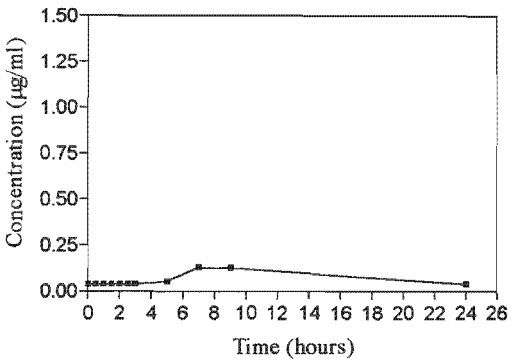
25-desacetyl rifampicin (43)



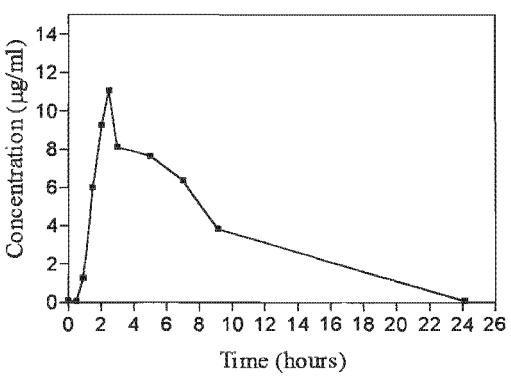
rifampicin (46)



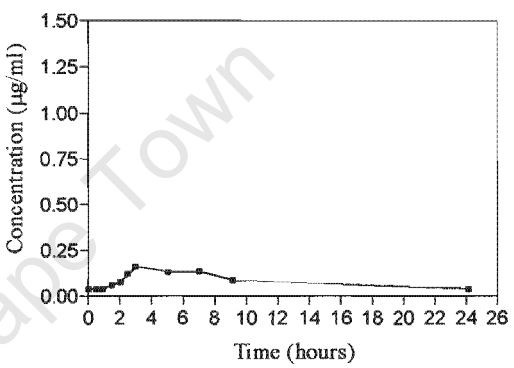
25-desacetyl rifampicin (46)



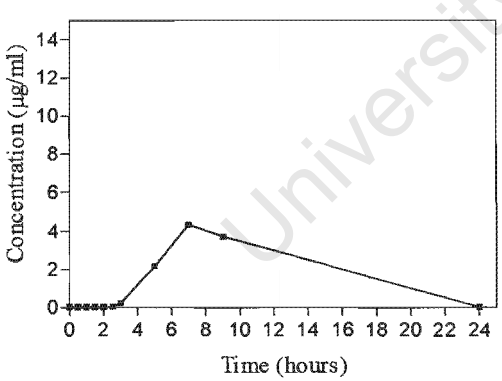
rifampicin (50)



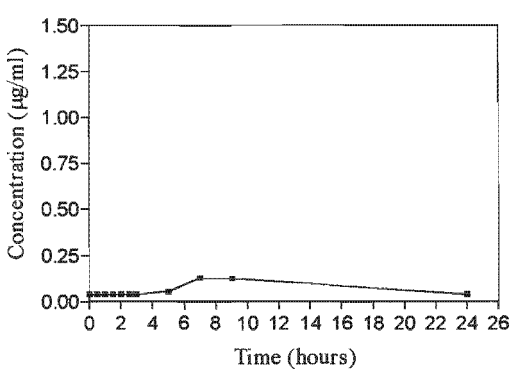
25-desacetyl rifampicin (50)



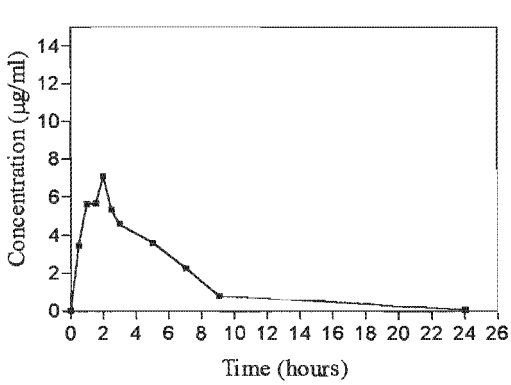
rifampicin (58)



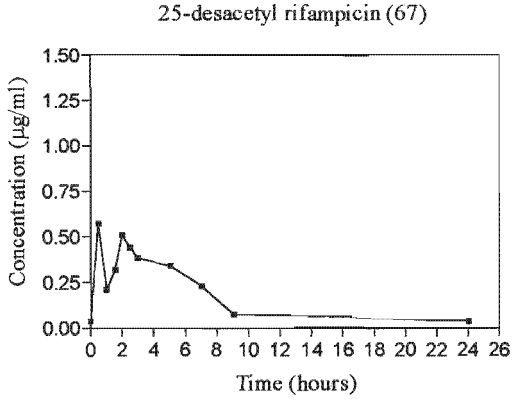
25-desacetyl rifampicin (58)



rifampicin (67)

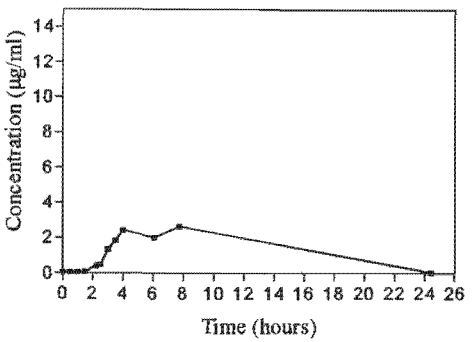


25-desacetyl rifampicin (67)

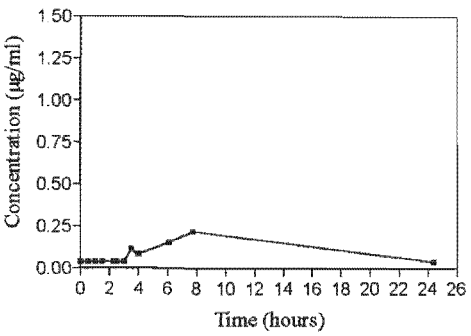


Control subject profiles

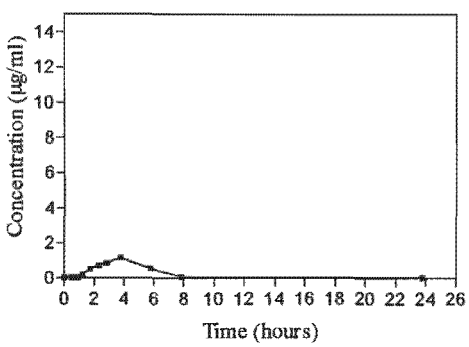
rifampicin (8)



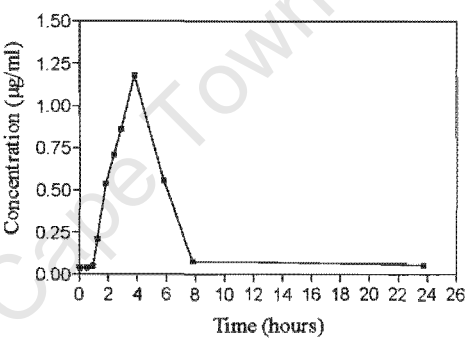
25-desacetyl rifampicin (8)



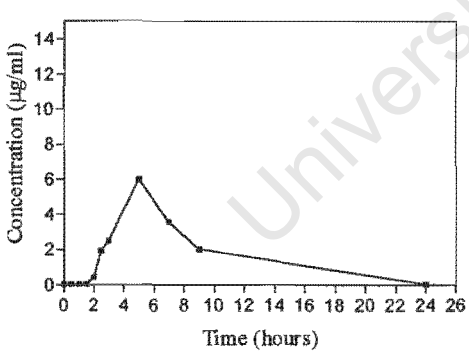
rifampicin (29)



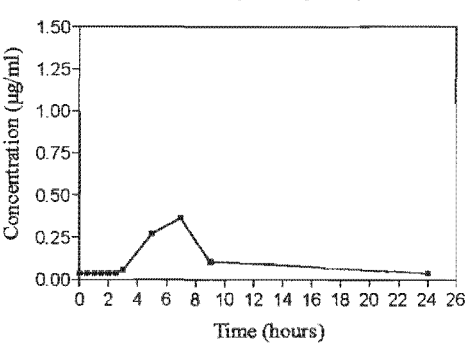
25-desacetyl rifampicin (29)



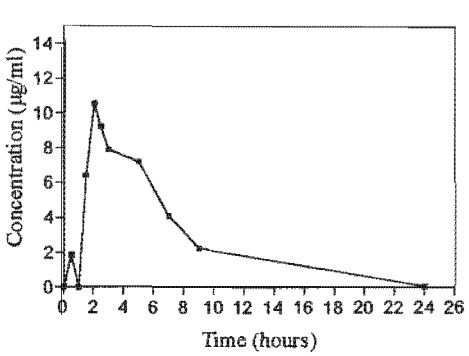
rifampicin (36)



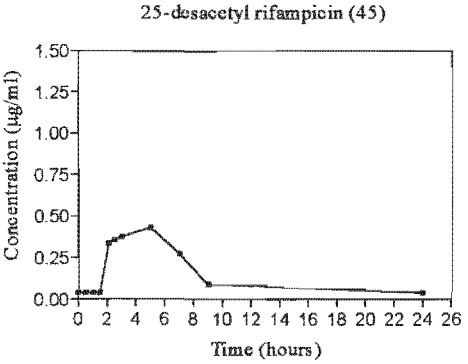
25-desacetyl rifampicin (36)



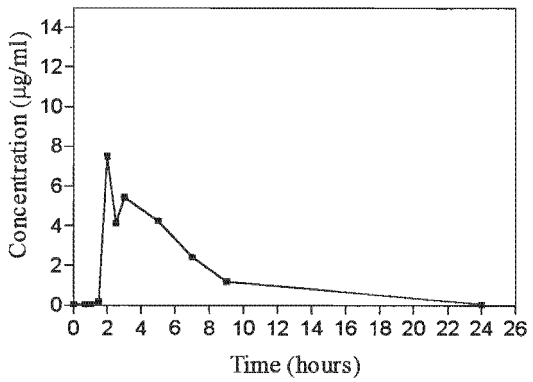
rifampicin (45)



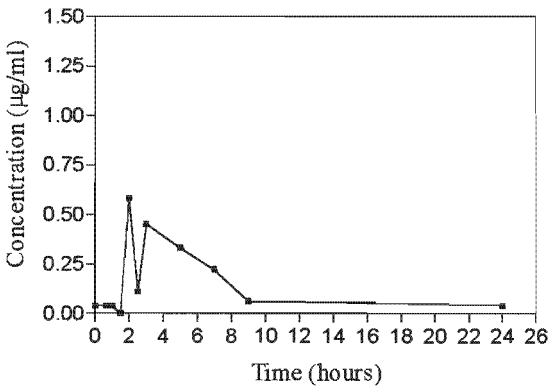
25-desacetyl rifampicin (45)



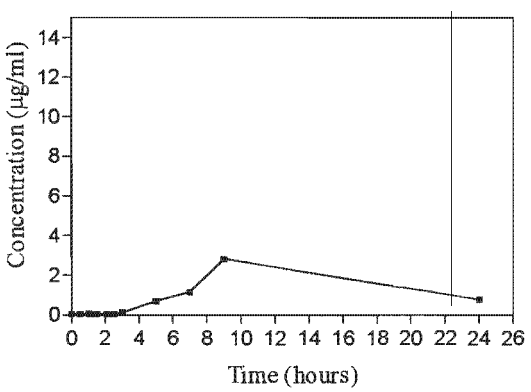
rifampicin (49)



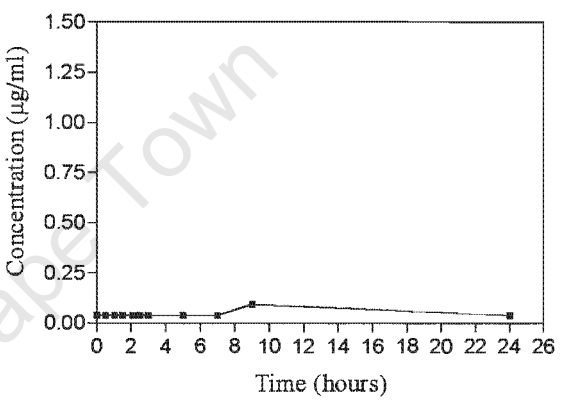
25-desacetyl rifampicin (49)



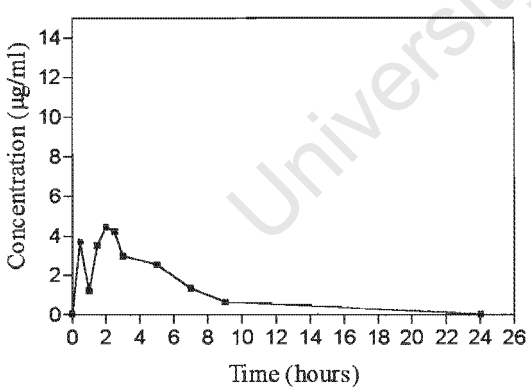
rifampicin (52)



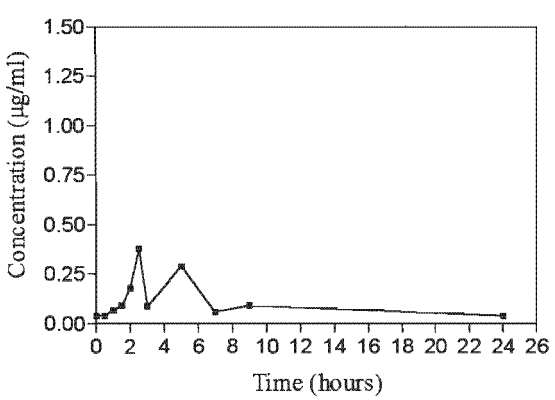
25-desacetyl rifampicin (52)



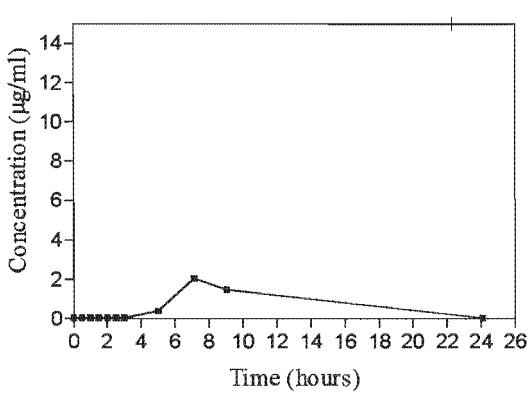
rifampicin (57)



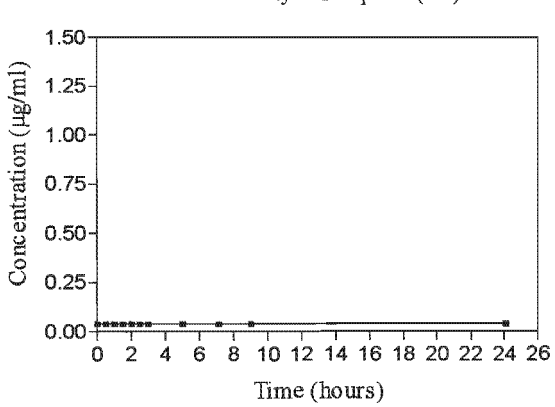
25-desacetyl rifampicin (57)



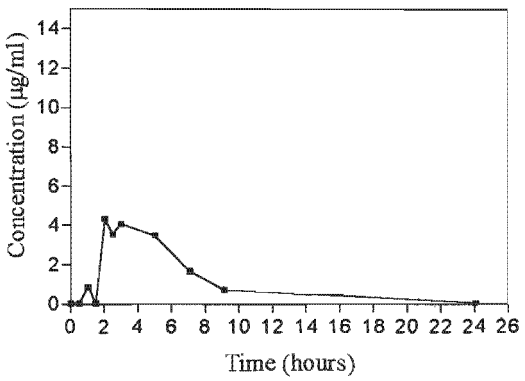
rifampicin (66)



25-desacetyl rifampicin (66)



rifampicin (68)



25-desacetyl rifampicin (68)