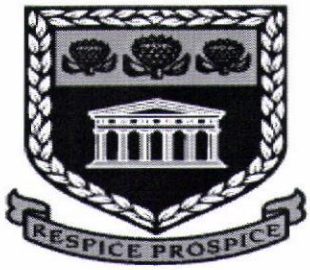

UNIVERSITY *of the*
WESTERN CAPE

DETERMINATION OF PLASMA CONCENTRATIONS USING LC/MS AND PHARMACOKINETICS OF OFLOXACIN IN PATIENTS WITH MULTI-DRUG RESISTANT TUBERCULOSIS AND IN PATIENTS WITH MULTI-DRUG RESISTANT TUBERCULOSIS CO-INFECTED WITH HIV

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A thesis submitted in fulfilment of the requirements for the degree of Magister Pharmaceuticiae in the School of Pharmacy, University of the Western Cape, Bellville, South Africa.

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Determination of plasma concentrations using LC/MS and pharmacokinetics of ofloxacin in patients with multi-drug resistant tuberculosis and in patients with multi-drug resistant tuberculosis co-infected with HIV

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Key words

Area under the curve

Clearance

Elimination rate

Half-life

HIV

Liquid chromatography

Mass spectrometry

Maximum plasma concentration

Multi-drug resistant tuberculosis

Ofloxacin

Pharmacokinetics

Plasma levels

Time to maximum concentration

Volume of distribution

ABSTRACT

Many studies have investigated the pharmacokinetics of anti-tuberculosis drugs in patients infected with tuberculosis. However, little is known about the pharmacokinetics of the drugs that are used in the treatment of multi-drug resistant tuberculosis (MDR-TB). Therefore, the objective of the present study was to investigate the steady state concentrations and the pharmacokinetics of ofloxacin, one of the drugs used in the treatment of MDR-TB in patients infected with MDR-TB and patients with MDR-TB co-infected with HIV

Plasma samples were drawn at different times over 24 hours after ofloxacin oral administration. For the determination of ofloxacin plasma concentrations, the liquid chromatography coupled with mass spectrometry analysis method was used.

The method was validated over a concentration range of 0.1-10 µg/ml. The lower limit of ofloxacin detection was 0.05µg/ml, while the lower limit of quantification was 0.1µg/ml. The response was linear over the range used with a mean recovery of 97.6%. Ofloxacin peak was well separated at a retention time of 9.6 minutes.

The pharmacokinetic parameters obtained were presented as mean ± standard deviation (SD). The peak concentration of ofloxacin (C_{max}) was 4.71± 2.27 µg/ml occurred at T_{max} 3±1.29 hours after ofloxacin oral administration. The mean (±SD) for the area under the concentration-time curve (AUC_{0-24}) and the area under the concentration-time curve (AUC_{0-8}) were 68.8±42.61 µg/ml.hr and 91.93±76.86 µg/ml.hr, respectively. Ofloxacin distributed widely with a mean (±SD) volume of distribution (V_d) 2.77±1.16 L/kg and it was eliminated with a mean (±SD) total clearance rate of 0.27±0.25 L/hr/kg. Ofloxacin mean (±SD) half-life was 9.55± 4.69 hours and mean (±SD) of the mean residence time (MRT) was 1512± 6.59 hours.

In summary, compared with the previous findings in the literature, ofloxacin pharmacokinetic was altered in MDR-TB patients with or without HIV co-infection.

The AUC and C_{max} were reduced, while the half-life and the time to reach the peak concentration were prolonged. This suggests that, both the rate and the extent of ofloxacin absorption were decreased. Furthermore, ofloxacin was highly eliminated in patients, which may be related to the altered liver function in this group of patients.

Further studies investigating the effect of HIV, liver and kidney dysfunctions on ofloxacin pharmacokinetics are recommended in large number of patients infected with MDR-TB.in addition to the therapeutic drug monitoring to maintain the desired concentration of ofloxacin in the patients.

DEDICATION

DECLARATION

I declare that the thesis "Determination of plasma concentrations using LC/MS and pharmacokinetics of ofloxacin in patients with multi-drug resistant tuberculosis and in patients with multi-drug resistant tuberculosis co-infected with HIV" is my own work, that it has not been submitted before for any degree or examination in any other University, and that all the sources I have used or quoted have been indicated and acknowledged by complete references.

E.Taha

February 2009

Signed: 

UWC, Bellville

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ABBREVIATIONS AND ACRONYMS

ACN	Acetonitrile
AIDS	Acquired immunodeficiency syndrome
ALT	Alanine transaminase
Anti-TB	Anti-tuberculosis
ARV	Antiretroviral
AST	Aspartate transaminase
AUC_{0-24}	Area under the concentration-time curve from zero to 24 hours
AUC_{0-8}	Area under the plasma concentration-time curve from zero to infinity
BCH	Brooklyn Chest Hospital
Cl_{tot}	Total clearance
C_{max}	Maximum concentration
Cp_{last}	Last measurable concentration
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DOT	Directly observed treatment
ELISA	Enzyme-linked immuno-sorbent assay
EMB	Ethambutol
ETH	Ethionamide
GFR	Glomerular filtration rate
GGT	Gamma glutamyl transpeptidase
GIT	Gastro intestinal Tract
HAART	Highly active anti-retroviral therapy
HBV	Hepatitis B virus
HIV	Human immunodeficiency virus
INH	Isoniazid
KAN	Kanamycin
K_e	Elimination rate constant

KFT	Kidney function test
LC	Liquid Chromatography
LFT	Liver function test
LLD	Low limit of detection
LLQ	Low limit of quantification
MDRD	Modification of diet in renal disease
MDR-TB	Multi-drug resistant tuberculosis
MIC_{90}	Minimum inhibitory concentration for 90%
MRT	Mean residence time
MS	Mass spectrometry
MTB	*Mycobacterium tuberculosis*
NCA	Non compartmental analysis
NNRT	Non nucleoside reverse transcriptase inhibitor
PI	Protease inhibitor
UV	Ultra violet
PK	Pharmacokinetic
PZA	Pyrazinamide
RIF	Rifampicin
RSD	Relative standard deviation
SD	Standard deviation
T½	Half-life
TB	Tuberculosis
TCA	Trichloroacetic acid
TDM	Therapeutic drug monitoring
HPLC	High performance liquid chromatography
TFA	Trifluroacetic acid
T_{max}	Time to reach maximum concentration
UCB	Un-conjugated bilirubin
V_d	Volume of distribution
WHO	World health organization
XDR-TB	Extensively drug resistant tuberculosis

CHAPTER TWO

LITERATURE REVIEW

2.1 Overview of multi-drug resistant tuberculosis

2.1.1 Background

Over the past decade, there has been an increase in the appearance of strains of TB that are resistant to anti-TB drugs. MDR-TB is caused by TB strains that are resistant to the first line drugs INH and RIF, which are considered to be the most potent drugs against drug-susceptible TB (Iseman, 2002; Sharma and Mohan, 2004). The molecular basis of drug resistance to TB is now largely understood. From a microbiological point of view, the resistance is caused by a spontaneous and random mutation in the MTB bacterial chromosome. This leads to amino acids substitutions in their target proteins (e.g. β-subunit of MTB RNA polymerase in case of rifampicin), which result in reduced susceptibility to specific drug, and thus, the drug, becomes ineffective against the mutant bacilli (Blanchard, 1996; Ramaswamy and Muser, 1998).

Drug resistance is categorized into primary, acquired and initial resistance (Kochi et. al., 1993; Loddenkemper et al., 2002). Primary resistance is resistance to anti-TB drugs in patients with no history of previous TB treatment, i.e. treatment-naïve patients. On the other hand, acquired resistance is resistance to anti-TB drugs from patients with previous treatment, which could be one or more treatment. Finally, initial resistance is referred to patients with primary resistance as well as those with undisclosed acquired resistance.

Causes of drug resistance include many factors such as inadequate treatment regimens prescribed, poor drug supply and quality, misuse of anti-TB drugs, poor treatment compliance, treatment default and HIV co-infection (Iseman, 1993; Jain, 1998; Sharma and Mohan, 2004). The HIV-infection epidemic has caused an explosive increase in TB incidence and may be contributing to the increase in MDR-TB infection. Outbreaks of MDR-TB infection and case-

fatality rates reaching 83% among HIV-infected patients have been reported (Fischl et al., 1992; Sacks et al., 1999; Franzetti et al., 1999). This suggests that the severe immune suppression, which occurs in HIV infected patients, appears to be a predisposing factor for the development of MDR-TB. During the HIV-infection, a notable reduction of CD4+ lymphocytes occurs, which leads to an increased risk of reactivation or re-infection by TB (Havlir and Barnes, 1999). Furthermore, acquired drug resistance has been associated with the poor absorption of anti-TB drugs in HIV-infected patients (Weiner et al., 2005; Gurumurthy et al., 2004).

2.1.2 Chemotherapy of multi-drug resistant tuberculosis

Tuberculosis treatment consists of a combination of drugs that are considered to be first line therapy, including INH, RIF, pyrazinamide (PZA) and ethambutol (EMB). These drugs are used in the standardized treatment regimens, which have an initial (intensive) phase for 2 months and a continuation phase for 4-6 months (WHO, 2003). During the initial phase, usually 4 first line drugs are used, which are INH, RIF, PZA and EMB. Then, in the continuation phase INH and RIF are used.

Without the two potent drugs INH and RIF the treatment of TB becomes difficult, because the drugs used in the treatment of MDR-TB are less potent and not as well tolerated as first line drugs (Perri and Bonora, 2004). The treatment of MDR-TB infection includes an aminoglycoside (amikacin or kanamycin), a fluoroquinolone (ofloxacin or ciprofloxacin), a glycopeptides (capreomycin), a thiomide (ethionamide or prothionamide), cycloserine, and para-aminosalysilic acid (Tomioka, 2000; WHO, 2003; Perri and Bonora, 2004). These drugs are the reserve drugs for MDR-TB treatment and labeled as second line drugs. Compared with first line drugs, second line drugs are less effective, more toxic, and costly (Perri and Bonora, 2004).

MDR-TB treatment in the developed countries is expensive and involves an individualized regimen based on drug susceptibility data and the use of reserve drugs. By contrast, in the resource poor countries, the WHO treatment regimens are used in addition to directly observed treatment (DOT) based regimens, which are supporting the national TB programmes

(Ormerod, 2005). In South Africa, the guidelines for MDR-TB treatment have been drawn from the WHO guidelines. There are two regimen approaches, the first one is a standard treatment regimen and the second one is an individualized treatment regimen (Weyer, 1999). The standard treatment regimen consists of 4 months intensive phase with five drugs (kanamycin, ethionamide, pyrazinamide, ofloxacin and ethambutol) followed by a continuous phase of 12-18 months with 3 drugs (ethionamide, ofloxacin and ethambutol). The drugs used in the two phases and the dosages of these drugs are given in Table 2.1 and Table 2.2 (Weyer, 1999; Department of health, South Africa, 2006).

Table 2.1: Second line anti TB-drugs used in the intensive phase (4 months) in South Africa (Weyer, 1999; Department of health, South Africa, 2006).

Drug	Daily dosage (mg)
Kanamycin	750-1000
Ethionamide	500-750
Pyrazinamide	1000-1500
Ofloxacin	600-800
Ethambutol	800-1200

Table 2.2: Second line anti TB-drugs used in the continuation phase (12-18 months) in South Africa (Weyer, 1999; Department of health, South Africa, 2006)

Drug	Daily dosage (mg)
Ethionamide	500-750
Ofloxacin	600-800
Ethambutol	800-1200

- Terizadone is used if the strain is resistant to ethambutol.
- Kanamycin may be substituted with amikacin.
- Ofloxacin may be substituted with ciprofloxacin.

MDR-TB treatment is complicated and frequently associated with treatment failure, relapse and high incidences of adverse drug reactions, which lead to prolonged illness, disability and death as a consequence (Goble et al., 1993; Cohn, 1995; Flament-Saillour et al., 1999). Nevertheless, cure with appropriate intensive MDR-TB treatment regimens has been reported. Starting treatment early with regular follow up, using aggressive treatment regimens and improving treatment adherence with DOT may result in high cure rate reaching 75% (Park et al., 1998; Mukherjee et al., 2004; Edward et al., 2004; Leimane et al., 2005).

Treatment of MDR-TB in patients co-infected with HIV is associated with poor outcomes and high mortality rates of 72-89% (Cohn, 1995). Deaths during treatment caused by MDR-TB infection or by other HIV-related diseases are more frequent, particularly, in the advanced HIV-infection stages (Cohn, 1995). However, starting treatment early may increase the survival time in HIV patient co-infected with MDR-TB (Edlin et al., 1993; Franzetti et al., 1999). By contrast, HIV-negative patients with MDR-TB have good response to appropriate MDR-TB chemotherapy regimens with 64% outcome of completing the course of therapy and no relapse (Telzak et al., 1995; Kwon et al., 2008).

The optimum duration of MDR-TB treatment is still unknown. The recommended duration of treatment is guided by sputum smear/culture conversion to negative. The minimal recommendation is that treatment should last for at least 18 months after culture conversion. However, patients resistant to all or most of the first line drugs and patients co-infected with HIV have been treated for 2 years after cultures convert to negative to prevent relapse (Iseman, 1993; Pozniak, 2001). In South Africa, prolonged treatment is required in MDR-TB patients and it is usually for at least 18 months (Department of health, South Africa, 2006).

2.2 Pharmacokinetics

2.2.1 Overview

The pharmacokinetics of each drug describes the fundamental processes of its disposition, namely its absorption, distribution, metabolism and elimination. Moreover, it describes the resulting concentration versus time profile of the administered drug. These processes of

absorption, distribution, metabolism and elimination determine how rapidly, in what concentration and for how long the drug will appear in the target organ (Grahame-Smith and Aronson, 1991).

The key principle to understand a drug's pharmacokinetics is by determining the PK parameters of such a drug in the blood and/or urine. These parameters are the result of the fundamental processes of the disposition, which influence the drug concentration inside the body.

2.2.2 Pharmacokinetic parameters

The drug concentration-time profile is studied by the means of several PK parameters .The basic PK parameters, which were used in the present study and quantitatively describe a drug PK are C_{max}, T_{max}, AUC, T½, K_e, MRT, Cl and V_d. These pharmacokinetic parameters are defined as follows:

C_{max} (also known as the peak height) is the maximum concentration of the drug in blood plasma achieved

T_{max} is the time after drug administration at which the maximum (peak) plasma concentration occurs, i.e. the time taken to reach C_{max}.

AUC is the area under the plasma concentration-time curve. It is the overall amount of drug in blood stream after a dose.

T½ is the plasma half-life. It is the time taken for the drug plasma concentration levels to fall by 50%.

K_e is the drug's elimination rate constant.

MRT is the mean residence time. It is the average time that the drug molecules spend in the body.

Cl is the drug clearance. It is the measure of the body ability to eliminate drug. The total body systemic clearance (Cl_{tot}) of a drug is the sum of the clearances of the drug by various organs and tissues of the body.

V_d is the drug apparent volume of distribution. It is the measure of the available apparent space in the body that is available to contain the drug.

C_{max}, T_{max} and AUC express the drug bioavailability, which is the proportion of the administered drug that reaches the systemic circulation. C_{max} and T_{max} are used to measure drug's absorption rate, while the AUC is used to measure the total amount of drug absorbed in the body (Grahame-Smith and Aronson, 1991). T½ is a useful pharmacokinetic parameter, as it indicates the time required to attain 50% of the steady state or to decay from the steady state condition. In addition, it shows the rate of accumulation of the drug in the body during multiple dosing, which helps with the dosing of the drug (Grahame-Smith and Aronson, 1991). V_d helps in planning dose regimens, interpretation of drug interaction and relating the T½ to the total Cl. Increasing V_d indicates that, the drug has a much higher concentration in the extra-vascular tissues than in the vascular parts, which means it is not homogenously distributed. The Cl_{tot} is, like the half-life, used to quantify drug elimination. Knowing a drug Cl helps in assessing the drug excretion mechanisms and calculating the dosage regimen.

By studying the pharmacokinetic parameters of a drug, one can define its processes of absorption, distribution, metabolism and elimination. Moreover, the proper mathematical description of the pharmacokinetic parameters of the drug provides information related to its pharmacological, therapeutic and/or toxic effects.

2.2.3 Factors affecting the pharmacokinetics of a drug

There are many factors that can affect the pharmacokinetic parameters of a drug, and thus, change its pharmacokinetic profile. Such factors include age, gender, body weight, genetic factor, food administration, diseases and various drug-drug interactions (Gibaldi, 1991; Katzung, 1998). Distribution and elimination of many drugs change as a function of the age. The half-life and apparent volume of distribution of a drug differ with different age groups (new born, infant, children, adults and elderly). Different body weights lead to different distribution volumes, which result in different drug concentrations in the plasma. The genetic factor is also a major cause of differences in the drug plasma concentrations and the rate of clearance. Drug-drug interactions have pharmacokinetic bases by affecting absorption, distribution, metabolism or elimination of a drug and its pharmacokinetic parameters as a consequence.

Diseases affect normal functioning of the gastrointestinal tract (GIT), renal and hepatic systems have influence on the PK of many drugs. Diseases affecting the GIT may affect drug absorption, C_{max}, T_{max} and as a consequence AUC. Liver diseases have a great impact on pharmacokinetics of drugs that are metabolized in the liver, which results in longer T½, and reduced clearance of these drugs and thus lead to toxicity. Diseases changing renal functions have the greatest effect on the pharmacokinetics of drugs eliminated unchanged mainly via renal clearance. The clearance of such drugs decreases and their T½ will increase and may lead to toxicity (Gibaldi, 1991). Some pathological states such as oedema, ascites and pleural effusion are associated with abnormal accumulation of fluids, which increases the V_d of drugs (Katzung, 1998).

2.2.4 Effect of HIV on drug pharmacokinetics

HIV-enteropathy and other AIDS associated opportunistic infections affect many organ systems involved in drugs pharmacokinetics e.g. the GIT, liver and kidney. The GIT is the main part of the body that is responsible for the drug absorption and any dysfunction results in drug malabsorption. Candidiasis, colitis, Kaposi's sarcoma and stomatitis are some of the diseases that occur frequently in patients infected with HIV and influence the GIT system normal functions (Unadkat and Agosti, 1990). In addition, diarrhea is one of the most common GIT symptoms reported in HIV patients and may result in drug malabsorption (Pillai, 1998). Moreover, the pH of the stomach is higher in AIDS patients, and it is known that, the pH at the absorption sites is an important factor in the drug absorption and thus, any change in the stomach pH affect drugs absorption (Unadkat and Agosti, 1990; Gibaldi, 1991).

Liver is an important organ of drug metabolism, and any dysfunction of the liver may lead to changes in the drug metabolism. Liver diseases and intra-hepatic opportunistic infections occur frequently in patients infected with HIV. These diseases include hepatitis, *Mycobacterium avium* complex, Kaposi's sarcoma, lymphoma and biliary disease (Bartlett and Gallant, 2004).

Kidneys play an important role in the drug elimination through glomerular filtration, renal tubular secretion and reabsorption, which is going via a high capacity transport system that

plays a major role in drugs and/or metabolite excretion. The transport system has many transporters families e.g. P-glycoprotein (P-gp) family and the multi-drug resistant associated proteins (MRP) (Launay-Vacher et al., 2006). These transporters play an important role in the drugs secretion and/or active reabsorption mechanisms that is responsible of drugs excretion. Diseases affecting renal system alter transporter proteins activity and contribute significantly to the change in drug's PK, tolerance and efficacy. This results in renal toxicity and systemic accumulation of drugs (Launay-Vacher et al., 2006). As an example, P-gp functions decreased in renal failure and contribute to the decrease in drug clearance (Sun et al., 2005; Launay-Vacher et al., 2006). Renal diseases occur often in AIDS patients. These diseases include nephropathy; HIV-infection associated glomerulonephritis and proteinuria in addition to fluids and electrolyte imbalances (Unadkat and Agosti, 1990, Bartlett and Gallant, 2004).

2.3 Laboratory findings in cases of hepatic and/or renal dysfunctions

Liver function tests (LFT) measure the concentration of various different proteins and enzymes in the blood that are either produced by the liver cells or released when the liver cells are damaged. The liver function tests that assess the ability of the liver to eliminate substances that undergo hepatic metabolism such as ^{14}C-aminopyrine breath test are limited by complexity and availability (DiPiro et al., 2008). Routine liver tests include measuring bilirubin, aspartate transaminase (AST), alanine transaminase (ALT) and gamma glutamyl transpeptidase (GGT) levels in the blood. AST and ALT are enzymes located in the cytoplasm of hepatocytes, while GGT found in bile ducts and their levels will be elevated with hepatocellular injury and viral hepatitis (Giannini et al., 2005). The degree of elevation of these enzymes is helpful in suggesting possible etiologies. The highest levels (> 20 fold increase above normal) are seen in acute drug induced viral infections e.g. hepatitis or ischaemic events associated with circulatory catastrophes such as a stroke or myocardial infarction. Liver function tests will typically be evaluated in chronic inflammatory liver disease such as hepatitis, biliary obstruction, alcoholic hepatitis and ischemic and toxic injuries (Giannini et al., 2005; DiPiro et al., 2008).

The renal functions include processes of filtration, secretion, and reabsorbtion as well as endocrine and metabolic functions. Renal function is generally assessed by measuring serum

creatinine and urea levels, or calculation of the glomerular filtration rate (GFR). The GFR is used to categorize the stages of kidney function, which range from normal renal function in case of GFR > 90 ml/min to renal failure in case of GFR < 15 ml/min (DiPiro et al., 2008).

2.4 Pharmacokinetics of anti-tuberculosis drugs in HIV patients

2.4.1 Absorption of anti-tuberculosis drugs in patients co-infected with HIV

Anti-TB drugs poor absorption has been reported in HIV-infected patients with association to the HIV-enteropathy. The poor absorption of anti-TB drugs results in drug sub-therapeutic serum concentration levels, treatment failure, relapse, and acquired drug resistance (Patel et al., 1995; Peloquin et al., 1996; Weiner et al., 2005).

In the literature, some studies have investigated the absorption of anti-TB drugs in HIV-infected and non HIV-infected patients. Many studies of TB patients co-infected with HIV have yielded similar results. In a study done by Peloquin et al. (1996) it was found that, anti-TB drugs serum (peak) concentrations were lower in TB patients co-infected with HIV and having CD4 cell count <200 cell/mm^3. Another study showed that, total systemic concentrations were reduced by 32% for RIF and 24% for PZA in HIV-infected patients compared to the healthy control group, which was probably related to the poor absorption of these drugs in HIV infected patients (Sahai et al., 1997). In Botswana, low serum concentration of INH, RIF and EMB (with delayed absorption of EMB and RIF) in HIV-positive patients infected with TB have also been reported (Tappero et al., 2005). By contrast, a study found no differences between HIV-positive and HIV-negative patients with tuberculosis, in terms of concentration levels or total absorption of INH, PZA or RIF (Choudhri et al., 1997). This last study by Choudhri et al., 1997 indicates that, the prevalence and clinical significance of poor absorption of anti-TB drugs in TB patients co-infected with HIV remain controversial. However, measuring TB drug levels remain strongly considered for preventing the poor absorption, which lead to treatment failure and acquired drug resistance (Yew, 2001; Peloquin, 2002).

2.4.2 Interactions between anti-tuberculosis and anti-retroviral drugs

Immunocompromised patients are at high risk of drug-drug interactions during their TB treatment. The drug-drug pharmacokinetic interactions between antiretroviral (ARV) drugs and anti-TB drugs have been reported in the literature (Lopez-Cortes et al., 2002; Hamzeh et al., 2003; Winston and Boffito, 2005). For first line anti-TB drugs, the major drug–drug interaction occurs between RIF and the highly active anti-retroviral therapy (HAART) drugs particularly, the protease inhibitors (PIs) and the non-nucleoside reverse transcriptase inhibitors (NNRTIs) (Burman et al., 1999; Aaron et al., 2004). RIF is an enzyme inducer; it induces the cytochrome P450 (CYP450) enzyme system in the liver, which leads to a decrease in the concentrations of PIs and NNRTIs to sub-therapeutic levels

These interactions cause a problem, because the standard short-course regimens for TB therapy are based on RIF and should be used wherever possible. There are many recommendations in the treatment of TB patients who are co-infected with HIV. Some guidelines recommend modification to the TB therapy or HIV-infection treatment drugs (Pozniak et al., 1999). Modification of antiretroviral drug dosage or the use of alternative TB treatment regimens, which do not include rifampicin, have also been recommended (Havlir and Barnes, 1999; Aaron et al., 2004).

In addition to the interaction with ARV drugs, some anti-TB drugs interact with drugs that are used in treatment of AIDS associated opportunistic infections. Rifampicin clinical interactions with anti-infective such as chloramphenicol and doxycycline, anticoagulants, antacids and glucocorticoids have been reported (Yew, 2002).

Most of the second line drugs that are used in MDR-TB treatment regimens have not had drug/drug interaction studies performed with HIV-infection treatment compounds, although the potential for adverse interactions is considerable. There are major overlapping toxicities especially those causing hepatic reactions. Therefore, an intensive management and monitoring is required for these patients (Pozniak, 2001).

Therapeutic drug monitoring (TDM) which is the measurement of the drug concentration in the plasma, can enable better management of anti-TB drugs malabsorption in addition to their interactions with ARV drugs in order to maintain relatively constant concentrations in the blood stream. By measuring the drug levels in the blood at intervals, TDM maintains TB drugs plasma concentrations within the therapeutic range and it prevents toxicity from high doses or treatment failure from low doses, which may lead to relapse and drug resistance.

The TDM of anti-TB drugs in TB patients is highly recommended (Yew, 2001; Peloquin, 2002). It allows better control of TB treatment, particularly in MDR-TB patients and in patients with co-morbid conditions such as HIV, liver and kidney dysfunctions. TDM helps in managing anti-TB drugs interactions and poor absorption, which are correlated with the low concentration of anti-TB drugs, especially in TB patients co-infected with HIV (Peloquin, 2002; Li et al., 2004).

2.5 Ofloxacin pharmacology and pharmacokinetics

2.5.1 Overview

Ofloxacin is a bactericidal, broad-spectrum antimicrobial agent and belongs to the fluoroquinolone antibiotic group. It is a synthetic fluorinated derivative of nalidixic acid and classified as second-generation fluoroquinolone.

2.5.2 Ofloxacin antimicrobial spectrum of activity and mechanism of action

Ofloxacin has a broad antibacterial spectrum *in vivo* and *in vitro* with activity similar to that of other fluoroquinolones. It is active against a wide range of gram-negative and gram-positive bacteria. Moreover, ofloxacin has antibacterial activity against intracellular pathogens such as *Legionella*, *Chlamydia* and mycobacteria including *Mycobacterium avium* complex and *Mycobacterium tuberculosis* (Walker, 1999).

Ofloxacin mechanism of action is based on the inhibition of the bacterial enzyme DNA gyrase, which is responsible for coiling the genetic material as prerequisite for bacterial multiplication (Walker, 1999).

2.5.2.1 Activity against *Mycobacterium tuberculosis*

Many *in vitro* studies reported that ofloxacin has relatively potent activity against MTB and other species of mycobacteria e.g. *Mycobacterium kansasii* and *Mycobacterium avium* complex (Vacher et al., 1999). Ofloxacin is active against drug resistant *Mycobacterium tuberculosis* with 2μg/ml as a minimum inhibitory concentration for 90% (MIC_{90}) of tested strains, other studies demonstrated the activity of fluoroquinolones including ofloxacin within human macrophage, where many tubercle bacilli reside (Wise and Honeybourne, 1999, Ruiz-Serrano et al., 2000). In addition, the antimycobacterial effect of low, non-bactericidal concentration of ofloxacin within the macrophage is enhanced by the presence of PZA, which is important for the use of these agents in TB preventive combination regimens (Jacobs, 1999; Berning, 2001).

2.5.3 Ofloxacin pharmacokinetics

In addition to the wide spectrum of activity of ofloxacin as an antimicrobial drug, it has a good pharmacokinetic. This makes ofloxacin suitable and preferable to be used in the treatment of many diseases including urinary tract infections, prostatitis, respiratory tract infections, gastrointestinal and abdominal infection (Katzung, 1998, Walker, 1999; Turnidge, 1999).

2.5.3.1 Absorption

Ofloxacin is well absorbed and has high oral bioavailability. The oral version of ofloxacin absorption is very high and reaches 95% with a peak serum concentrations reached within 1-2 hours (Walker, 1999). The blood levels achieved with ofloxacin oral administration are similar to those obtained after intravenous route of administration, which makes a benefit in conversion from intravenous route to oral therapy (Yuk et al., 1991; Walker, 1999).

Ofloxacin absorption may be blocked by divalent cations included in nutritional supplements and antacid. Co-ingestion with food also delays absorption and as a consequence the time to reach the peak serum concentration delays by one hour (Katzung, 1998, Walker, 1999).

2.5.3.2 Distribution

Ofloxacin penetrates most tissues well e.g. bronchial mucosa, lung, kidney, gall bladder and genital tract. It is widely distributed including intracellular fluids and has a high volume of distribution generally > 150 liters. This may be due to ofloxacin low protein binding which is less than 30% (Walker, 1999; Wise and Honeybourne, 1999). The wide distribution and good tissues penetration of ofloxacin is advantageous for treatment of disseminated TB (Gilks et al., 1990).

2.5.3.3 Metabolism and elimination

Ofloxacin undergoes metabolism. Between 65% and 80% of an administered oral dose of ofloxacin is excreted unchanged via the kidneys within 48 hours of dosing. The unchanged form is excreted mainly by the kidney (Walker, 1999; Wise and Honeybourne, 1999). In the case of patients with impaired renal function (creatinine clearance $\leq$ 50 ml/min), ofloxacin elimination decreases and it accumulates. This leads to the increase of its T½. Therefore, dose adjustment in patients with impaired renal function is required.

2.5.4 Ofloxacin drug-interactions

As other fluoroquinolones, the main drug interaction of ofloxacin is the impaired absorption by co-administration of multivalent metal cations, which are included in antacids, nutritional supplements, dairy products (calcium), some haematinics (iron) and sucralfate. This may lead to the decrease in AUC and C_{max} of ofloxacin. Thus, ofloxacin should be administered at least two hours before these cationic compounds are administered (Katzung, 1998, Walker, 1999; Yew, 2002). Probenecid may interfere with the renal clearance of ofloxacin, which may result in an increase in the half-life of ofloxacin. In addition, elevated serum levels of cyclosporine have been reported with concomitant use of cyclosporine with some other fluoroquinolones.

Regarding anti-retroviral drugs, the main interaction occurs with didanosine, which contains buffering agents such as magnesium and aluminum salts in its formulation that lead to a chelation base interaction. This interaction leads to a decrease in ofloxacin absorption and as a

consequence it's AUC. However, this interaction can be avoided by separating administration of ofloxacin and didanosine by at least 2 hours (Goodman et al., 2005)

2.5.5 Safety, adverse effects and precautions

Ofloxacin is well tolerated and safe. The most common adverse effects include gastrointestinal upset and central nervous system symptoms. GIT adverse effects include nausea, vomiting and diarrhea. Central nervous system symptoms occasionally experienced are headache, dizziness and insomnia. Allergic reactions such as skin rash, urticaria and photosensitivity occur in 1-2% of patients (Katzung, 1998, Walker, 1999). Ofloxacin as other fluoroquinolones is contraindicated during pregnancy, breast-feeding. It should be used with caution in patients with cerebral convulsive disorders, and careful monitoring is required in patients with impaired renal or hepatic functions

2.5.6 Ofloxacin clinical uses

Ofloxacin is used in many infections such as urinary tract infections, prostatitis, gastrointestinal and abdominal infections, bone, joint and soft tissue infections, gonorrhoea, skin infections, and respiratory tract infections including sinusitis (Walker, 1999, Goodman et al., 2005).

2.5.6.1 Use of ofloxacin in multi-drug resistant tuberculosis

The favorable microbiological, pharmacokinetic, toxicity and drug interaction profile of ofloxacin makes it a promising agent in the treatment of the respiratory tract infections and in the treatment of MTB infection (Wise and Honeybourne, 1999). Today, ofloxacin is used for prevention therapy, empirical treatment of patients with TB and retreatment of patients with MDR-TB (Berning, 2001). Furthermore, ofloxacin is reported to be safe and effective when it is used in MDR-TB combination treatment regimens with treatment success rate of 78% (Maranetra, 1999; Montoya et al., 1999).

In South Africa, ofloxacin is one of the second line drugs that are used in MDR-TB treatment regimens. It is used in the standard treatment regimens, in the intensive phase and the continuation phase (Weyer, 1999; Department of health, South Africa, 2006). The dose of

ofloxacin depends on the patient's weight with an average of 7.5-15 mg/kg and a maximum daily dose of 800 mg.

2.5.7 Resistance to fluoroquinolones and ofloxacin

The fluoroquinolones' wide use for many infections leads to a selective pressure on these broad-spectrum agents. This results in the emergence of fluoroquinolone resistance in a diversity of organisms including MTB. The primary mechanism of fluoroquinolone resistance in MTB thus far, is related to the mutation in the DNA gyrase (*gyr* A) gene (Ramaswany and Musser, 1998). There are no reports of cross-resistance between fluoroquinolones and other classes of anti-TB drugs. However, cross resistance within the fluoroquinolone class agents occurs. Therefore, susceptibility reduction to one fluoroquinolone likely confirms susceptibility reduction to all other fluoroquinolones (Ginsburg et al., 2003).

Resistance to ofloxacin in MDR-TB patients is reported in the literature (Grimaldo et al., 2001; Ginsburg et al., 2003; Shi et al., 2006; Umubyeyi et al., 2007). Ofloxacin resistance develops rapidly when it is used alone against MTB or when it is added singly to a failing therapy regimen. Co-infection with HIV may also be associated with development of ofloxacin resistance, especially in individuals with advanced AIDS and it was reported in patients with CD4+ cell count < 50 cell/ mm^3 (Perlman et al., 1997; Ginsburg et al., 2003). The rapid proliferation of MTB for long period of time in HIV patients with decreased cell mediated immunity can be associated with the CD4+ cell lymphocyte levels. The low CD4+cell levels accelerate the acquisition of resistance.

2.5.8 Ofloxacin pharmacokinetic parameters

2.5.8.1 Pharmacokinetic parameters in healthy volunteers

Ofloxacin pharmacokinetics is well known and described in the literature. Many studies have been done to investigate ofloxacin pharmacokinetic parameters with different doses and different ways of administration as oral, intravenous, and rectal administration (Lode et al., 1987, Warlich et al., 1990; Yuk et al., 1991; Guay et al., 1992, Eboka et al., 1997, Immanuel et al., 2002). Ofloxacin pharmacokinetic parameters in healthy volunteers after oral administration are summarized in Table 2.3.

Ofloxacin exhibits dose-independent pharmacokinetics. However, an increase in ofloxacin C_{max} and AUC with 800 mg dose compared to 600 mg dose has been reported (Immanuel et al.,2002).

Table 2.3 Ofloxacin pharmacokinetic parameters in healthy volunteers after different oral doses.

Dose (mg)	C_{max} (µg/ml)	T_{max} (hr)	T½ (hr)	AUC_{0-24} (µg/ml.hr)	$AUC_{0-\infty}$ (µg/ml.hr)	CL_{tot} (ml/min)	V_d (L)	Reference
200	2.96±0.30	1.9±0. 5	6.6±0.1	NA	NA	188±5.5	NA	Warlich et al., 1990
400	3.14±0.53	1.74±0.57	5.48±0.81	NA	28.36±4.32	NA	NA	Yuk et al., 1991
600	8.0 (7.4-8.6)	1.4 (1.0-1.8)	6.7 (6.2-7.2)	60.8 (54.2-67.4)	67.9 (60.9-74.9)	149 (135-163)	86 (78-94)	Immanuel et al., 2002
800	9.8 (8.2-11.4)	1.9 (1.6-2.2)	6.5 (6.1-6.9)	85.3 (69.4-101.2)	93.1 (79.7 -106.5)	147 (128-166)	83 (72-94)	Immanuel et al., 2002

C_{max}=maximum concentration; T_{max}= time to attain C_{max}; T½ = half-life; AUC_{0-24}=area under the concentration-time curve from zero to 24 hrs; $AUC_{0-\infty}$= area under the plasma concentration-time curve from zero to infinity, V_d = volume of distribution; Cl_{tot} = total body clearance

2.5.8.2 Pharmacokinetic parameters in patients with tuberculosis

The pharmacokinetic properties of ofloxacin in patients with MDR-TB infection and MDR-TB patients co-infected with HIV have not been studied extensively globally. However, there are some studies that evaluated ofloxacin pharmacokinetic parameters in TB patients. The results of these studies are summarized in Table 2.4.

Ofloxacin pharmacokinetics in TB patients is reported to be similar to that reported in healthy volunteers studies, although; a delay in ofloxacin absorption in TB patients compared to healthy volunteers has been reported (Zhu et al., 2002; Chulavatnatol et al., 2003)

Table 2.4: Ofloxacin pharmacokinetic parameters in patients with tuberculosis

Dose (mg)	C_{max} (µg/ml)	T_{max} (hr)	T½ (hr)	AUC_{0-24} (µg/ml.hr)	$AUC_{0-\infty}$ (µg/ml.hr)	V_d (L/kg)	Cl_{tot} (L/h/kg)	Reference
600	8.52	2	5.70	NA	NA	1.25	0.14	Zhu et al.,2002
800	10.5	1.03	7.34	NA	NA	1.28	0.12	Zhu et al.,2002
590.9mg (mean dose)	9.61±2.1	1.68±1.2	8.03±3.3	70.57±26.4	82.45±43.64	1.37±0.24	NA	Chulavatnatol et al., 2003
600-1000	4.92±1.4	1-2	7.8	NA	NA	NA	NA	Yew et al.,1999

C_{max} = maximum concentration; T_{max}= time to attain C_{max}; T½ = half-life; AUC_{0-24}=area under the concentration-time curve from zero to 24 hrs; $AUC_{0-\infty}$= area under the plasma concentration-time curve from zero to infinity, V_d= volume of distribution; Cl_{tot} = total body clearance; K_e= elimination rate constant.

2.6 Determination of anti-tuberculosis drugs concentrations

2.6.1 Methods used to determine plasma concentrations

Pharmacokinetics and therapeutic drug monitoring studies represent one of the reasons for development of clinical assay methodologies. The literature reveals several efficient methods and techniques for the analysis of anti-TB drugs in the biological fluids. These analysis methods can be either non-chromatographic such as microbiological, spectral and fluorometric assay methods or chromatographic methods like gas and liquid chromatography.

Although microbiological assays are simple, reproducible and do not require highly technical equipment, they are time consuming, and they lack sensitivity and specificity (Auten et al., 1991, Immanuel and Kumar, 2001). In the spectral analysis methods, the determination of the drug is based on its spectral absorption measurements. In fluorometric analysis the determination is based on absorption and re-emission of the radiation that the drug is exposed to. Some drugs do not have the absorption or emitting properties, therefore; some complexing agents are used to enhance spectral absorption or re-emission of these drugs. Chromatographic methods are based on the separation in which the drug and/or the material under analysis are distributed between two phases a stationary and a mobile phase, which move in a definite direction. Different chromatographic methods including gas and liquid chromatography for anti-tuberculosis drugs analyses have been reported in the literature (Holdiness, 1985).

2.6.1.1 Liquid chromatography/mass spectrometry

The high performance liquid chromatography (HPLC) technique has been the mainstay for anti-TB drugs bioanalytical measurements. These include quantitative measurement of the drug and qualitative differentiation between the parent drug, its metabolites, as well as unknown endogenous components in the analysis samples. Multiple approaches using HPLC coupled with UV, fluorometric or mass spectrometric detection have been used to quantify ofloxacin and other anti-TB drugs (Holdiness, 1985; Niessan, 1998; Gennaro et al., 2001; Khuhawar and Rind, 2002). Although there are many application areas where HPLC coupled with UV or fluorescence detection still provides satisfactory results, there is a growing need for

better analytical capabilities. Conventional UV and fluorescence spectroscopic detectors have limited specificity and thus, it may be difficult to differentiate the analyzed drug and its metabolites from endogenous matrix components. As a result, it is important to carry out more thorough sample preparation and/or extend the HPLC run time to allow sample extract matrix components to be resolved chromatographically from the analyte of the interest, which consume a lot of time (Brewer and Henion, 1998). Another reason is that, some drugs are very potent and administered at lower doses and thus are present at much lower levels in biological samples. Therefore, higher sensitivity and selectivity capabilities are needed, which are not provided by HPLC/UV or fluorescence techniques for determination of drugs and/or their metabolites in complex biological samples. Recently, liquid chromatography coupled with mass spectrometry has become the most popular approach in pharmacokinetics studies because mass spectrometry provides high specificity, selectivity, and sensitivity detection, which makes it capable of accurate quantitative analysis of drugs and their metabolites (Want et al., 2003; Kostiainen et al., 2003).

2.6.2 Determination of ofloxacin plasma concentrations

Several methods for the determination of ofloxacin concentration in the biological fluids have been reported in the literature, and both chromatographic and non-chromatographic methods have been used (Horstkotter and Blaschke, 2001; Ev and Schapoval, 2002; Garcia et al., 2002; Du et al., 2004). HPLC is the method most commonly used for the determination and assay of ofloxacin. Other methods including spectrophotometric, fluorometric electrophoresis and electro-analytic methods have also been used for the determination of ofloxacin. Despite their sensitivity, accuracy and selectivity, these methods are complicated in sample preparation and/or separation, because they require formation of ofloxacin complexes as in spectrophotometric and spectrofluorometric methods (Garcia et al., 2002; Du et al., 2004).

HPLC coupled with UV and fluorescence detection are the most commonly used methods for the determination of ofloxacin and other fluoroquinolones in biological samples. There are few studies, which used liquid chromatography coupled with

mass spectrometry for the determination and confirmation of ofloxacin in biological samples. LC/MS shows high sensitivity and selectivity (van Vyncht et al., 2002; Sinnaeve et al., 2003; Ballesteros et al., 2003).

In this study, HPLC was used for the assay of ofloxacin plasma concentrations in patients. Chapter three has more details about the methods, chemicals used and the LC/MS settings.

2.7 Research questions

Firstly, a study comparing all the above mentioned analysis methods would be useful. Due to time and budget constraint, it was decided to focus on LC/MS in this study in order to find whether LC/MS is a specific, accurate, sensitive and reproducible method in the determination of ofloxacin plasma concentrations as mentioned above.

Secondly, so far no study has been done on the PK of ofloxacin in MDR-TB patients in South Africa. Therefore, it would be interesting to find out if there are any differences or similarities between our PK data and findings from studies done before. Furthermore, it is important to find out whether HIV infection has any influence on ofloxacin PK.

2.8 Objectives of the study

The objectives of the present study are:

- To determine the plasma concentrations of ofloxacin using LC/MS as an analysis method.
- To investigate the pharmacokinetics of ofloxacin in MDR-TB patients and MDR-TB patients co-infected with HIV.
- To find out if HIV infection influences the PK of ofloxacin.

CHAPTER THREE
METHODS

3.1 Study site

The study was conducted (from January-July 2008) at Brooklyn Chest Hospital (BCH) in Cape Town, Province of Western Cape; South Africa. BCH is one of the South African hospitals specialized in the treatment of MDR-TB.

3.2 Patient selection

Male and female patients involved in this study were selected from other patients admitted at BCH using the criteria listed below.

3.2.1 Inclusion criteria

A patient was included in the study only if he/she complied with all the following five criteria:

1. MDR-TB sensitive to second line anti-TB drugs.
2. Adult patients (18-65 years).
3. On ofloxacin therapy for at least two weeks.
4. Signature of informed written consent form, after receiving explanation of the aims, procedures, advantages and disadvantages of the study in his/her first language (Appendix A and B).
5. Informed Consent for HIV-testing.

3.2.2 Exclusion criteria

A patient was excluded from the study in case one or more of the following criteria applied.

1. Patient request.
2. Pregnancy or breast-feeding.
3. Intolerance or hypersensitivity to ofloxacin.
4. Patients on drugs other than anti-retroviral drugs, known to interact with ofloxacin pharmacokinetic (medications containing cations, such as antacid or nutritional

supplements).

3.3 Clinical examination

Clinical examination of the patients was conducted as part of the routine care. For each patient, the body weight was checked every day. Vital signs were recorded every morning and evening. Patient's complaints including reactions to medications, if any, were recorded and assessed. Therapeutic effects were also determined for each patient.

3. 4 Laboratory tests

The following laboratory tests were done at PathCare laboratory (Pathcare Park, Neels Bothma Street, N1 city Good Wood, Cape Town; South Africa) in order to assess the kidney and liver function and to determine the haematological profile of the patients.

3.4.1 Liver function tests (LFT)

Liver function was evaluated by measuring the liver enzymes: alanine transaminase (ALT), aspartate transaminase (AST), gamma glutamyl transpeptidase (GGT) and bilirubin.

3.4.2 Kidney function tests (KFT)

Kidney function was evaluated by calculating the glomerular filtration rate (GFR) from the serum creatinine using the modification of diet in renal disease (MDRD) study following formula

GFR = 175 x (standardized S_{Cr})$^{-1.154}$ x (age)$^{-0.203}$ x (0.742 if female) x (1.210 if black).

S_{Cr} = serum creatinine.

Although MDRD equation is commonly used for estimating the GFR, it has not been validated in indigenous African population. Therefore, black ethnicity correction factor of 1.210 is established for African ethnic in this equation due to their difference of body composition, diet and muscle mass from American or Asian (Devaney and Tomson, 2006; Perazella and Reilly, 2003).

3.4.3 Haematological tests

The full blood count (FBC) was determined for each patient. However, our main interest was in the assessment of the CD4 cell count, which indicates the immuno-competence level.

3.4.4 Sputum microbiological test

The sensitivity test for MTB was identified in the sputum of patients for each of the second-line anti-TB drugs, before the start of the treatment. At BCH, the second line anti-TB drugs used are: kanamycin, pyrazinamide, ethionamide, ethambutol, capreomycin, para amino salicylic acid and terizidone.

3.4.5 HIV test

HIV-test (ELISA) and viral load level were performed for each patient after patients were provided with pre and post-test counselling regarding the HIV-test.

3.5 Study procedures

3.5.1 Demographic, clinical and therapeutic characteristics

After inclusion into the study, the demographic, clinical and therapeutic (drug history) characteristics were recorded for each patient. They were captured on a computer-spreadsheet designed for this study.

3.5.2 Ofloxacin administration

Patients received ofloxacin tablets orally at a daily dose of 800 mg. On the study day, after 8 hours overnight fast, ofloxacin was given to each patient with water at 7 a.m. They were allowed to have breakfast 30 minutes after ofloxacin ingestion. Lunch was served at 12 noon and dinner at 6 p.m.

3.5.3 Blood sampling for ofloxacin plasma concentrations

Blood samples were taken after two week of treatment, to allow ofloxacin steady state to be attained. For the measurement of ofloxacin plasma concentrations, 5 ml of blood was taken before ofloxacin administration (baseline), and then at 1, 2, 4, 8 and 24

hours after ofloxacin administration (post-dose). Blood samples were collected from a heparinized intravenous catheter fixed on a forearm vein of each patient before taking the dose. All blood samples were drawn and placed into vacuum tubes and immediately centrifuged at 3000 rpm for 5 minutes. The plasma was separated and stored at -80 °C until the day of analysis.

3.6 Determination of ofloxacin plasma concentrations

In this study, ofloxacin levels in the plasma were determined using liquid chromatography/mass spectrometry at Stellenbosch University, central analytical facility (LC/MS laboratory) in Stellenbosch; Cape Town; South Africa.

3.6.1 Chemicals

The following chemicals were used in the study:

Analytical grade dimethyl sulfoxide (DMSO), acetonitrile (ACN), trichloroacetic acid (TCA), phosphoric acid and HPLC grade trifluroacetic acid (TFA) were obtained from Sigma-Aldrich (Cape Town, Western Cape). Ofloxacin tablets (Zanocin 200mg, Ranbaxy Pty Ltd, SA) were supplied by BCH and used as working standard.

3.6.2 Chromatographic and mass spectrometric conditions

3.6.2.1 Column liquid chromatography

The liquid chromatography was performed with Waters 2695 HPLC system (*Waters, Microsep Pty Ltd*). The column utilized was a polar C18 liquid chromatography column, Waters Atlantis™ (5µm, 2.1mm x 100mm) at ambient temperature. The mobile phase used was 0.1% TFA at a flow rate of 0.20 ml/min and an injection volume of 5 µl.

3.6.2.2 Mass spectrometry setting

The instrument used was Waters API Quattro Micro triple quadrupole mass spectrometer with electrospray ionization (*Waters, Microsep Pty Ltd*). The ion source and desolvation temperature were held at 120°C and 400 °C, respectively. The capillary voltage was 3.7 kV, RFI 40. The desolvation gas at 550L/h and the cone gas was 50 L/h. The daughter ion mass to ratio (m/z) for ofloxacin quantification was

318 at collision energy 25 eV, and m/z for qualification was 261 at collision energy 30 eV as shown in Table 3.1. Waters Mass Lynx™ software was used for the data collection, integration and calibration.

Table 3.1: Mass to charge ratio (m/z) of ofloxacin

Parent (m/z)	Daughter (m/z),CE	Daugther2 (m/z),CE	Cone voltage	Mode
362.5	318, 25	261, 30	20	+

CE: Collision Energy

3.6.3 Ofloxacin stock solution and calibration standard preparation

3.6.3.1 Preparation of stock solutions

Ofloxacin stock solution was prepared at a concentration of 1 mg/ml. The stock solution was prepared by dissolving a 200 mg ofloxacin tablet in 50 ml DMSO, and then it was further diluted using acetonitrile to obtain the final concentration of 1 mg/ml. The stock solution of 1 mg/ml was serially diluted with acetonitrile to get working solutions with the concentrations 10, 5, 1, 0.5, 0.1 and 0.05 µg/ml.

3.6.3.2 Preparation of calibration standards

Ofloxacin plasma calibration standards were prepared by spiking blank plasma with ofloxacin working solutions (with the concentrations 10, 5, 1, 0.5, 0.1 and 0.05 µg/ml). Ofloxacin was extracted from the plasma using acidic extraction similar to the method reported by Zendelovska and Stafilov (2005), which is described as follows:

A 500 µl plasma sample was diluted using 100 µl of 0.1M phosphoric acid and 300 µl of 5M trichloroacetic acid-acetonitrile (1:1, V/V) solution. The mixture was vortex-mixed and then diluted again using 100 µl of acetonitrile and 300 µl of water. The final solution was vortexed and centrifuged at 10000 g for 10 minutes. The

supernatant collected and injected onto the liquid chromatography column for analysis.

3.6.4 Patient samples preparation

To prepare the patients plasma samples for the LC/MS assay:
100 µl of 0.1M phosphoric acid and 300 µl of 5M trichloroacetic acid-acetonitrile (1:1, V/V) solution were added to 500 µl of plasma, and the mixture vortexed well. Then 100 µl of the internal standard was added and the mixture vortexed, and centrifuged at 10000 g for 10 minutes. The supernatant was removed and injected onto the liquid chromatography column for analysis.

To validate the assay the linearity, recovery, lowest limit of detection, lowest limit of quantification, specificity, accuracy and precision were determined.

3.7 Determination of ofloxacin pharmacokinetic parameters

After determination of ofloxacin plasma concentrations, the plasma concentration-time profile for each patient was plotted using GraphPad Prism program. Ofloxacin pharmacokinetic parameters were calculated based on the non-compartmental analysis (NCA) as follows:

3.7.1 The maximum concentration and the time to reach maximum concentration

The maximum concentration (C_{max}) and the time to reach the maximum concentration (T_{max}) were obtained directly from the plasma concentration-time profile.

3.7.2 The elimination rate constant

The elimination rate constant (K_e) was calculated as the slope of terminal log-linear phase of the plasma concentration-time profile multiplied by -2.303

3.7.3 The half-life

The half-life ($T½$) was calculated using the formula:

$T½ = 0.639/K_e$

3.7.4 The area under the plasma concentration-time curve

The area under the plasma concentration-time curve from zero to 24 hours (AUC_{0-24}) was calculated by the trapezoidal method using GraphPad Prism software.

The area under the plasma concentration-time curve from zero to infinity ($AUC_{0-\infty}$) was calculated using the following formula:

$AUC_{0-24} + Cp_{last}/ K_e$

Where Cp_{last} is the last measurable plasma concentration.

3.7.5 The volume of distribution

The following formula was used to determine the volume of distribution (V_d)

$V_d = Dose/ (AUC_{0-\infty} \times K_e)$

3.7.6 The total body clearance

The total body clearance (Cl_{tot}) was calculated using the following formula:

$Cl_{tot} = Dose / AUC_{0-\infty}$

3.7.7 The mean residence time

The mean residence time (MRT) was calculated as:

$MRT= AUMC_{\infty}/AUC_{\infty}$

Where $AUMC_{\infty}$ is the area under the momentum curve from zero to infinity and it was calculated as

$AUMC_{\infty} = AUMC+ C_{last}/K_e+ (t_{last} \times C_{last})/ K_e$

AUMC is the area under the momentum curve and calculated using the following equation

$AUMC= (t_i c_i+t_{i-1} \times c_{i-1})/2 \times (t_i-t_{i-1})$

3.8 Statistical analysis of the data

Patients data collected during the study was organised and coded onto data collection forms before being captured into computer files. Microsoft Excel for windows was utilised for computer management of the data, which was reported as mean ± standard deviation (SD). Ofloxacin concentrations and pharmacokinetic parameters were analyzed with the use of descriptive statistics mean ± SD.

3.9 Ethical consideration

The study was conducted according to the declaration of Helsinki and ICH guidelines. The protocol was approved by the ethics committee of the University of the Western Cape. Permission to conduct the study was granted by the medical superintendent of BCH. HIV test informed consents were used and all the information obtained during the study was treated as confidential.

CHAPTER FOUR
RESULTS AND DISCUSSION

4.1 Ofloxacin LC/MS analysis method validation

A liquid chromatography/mass spectrometric method for the determination of ofloxacin in human plasma was developed and validated. The validation of ofloxacin LC/MS analysis was conducted by determining linearity, recovery, precision and accuracy, low limit of detection, low limit of quantification and specificity.

4.1.1 Calibration curve and linearity

The calibration curve was constructed by plotting the responses (peak areas) of ofloxacin against the corresponding concentrations. Figure 4.1 shows the concentrations of the calibration standards of ofloxacin (in µg/ml) on the x-axis. The response (peak areas) is shown on the y-axis of the same figure. The calibration curve was linear over the range (0.1-10 µg/ml) as shown in Figure 4.1. The correlation coefficient (r^2) of determination of ofloxacin during the validation was greater than 0.99.

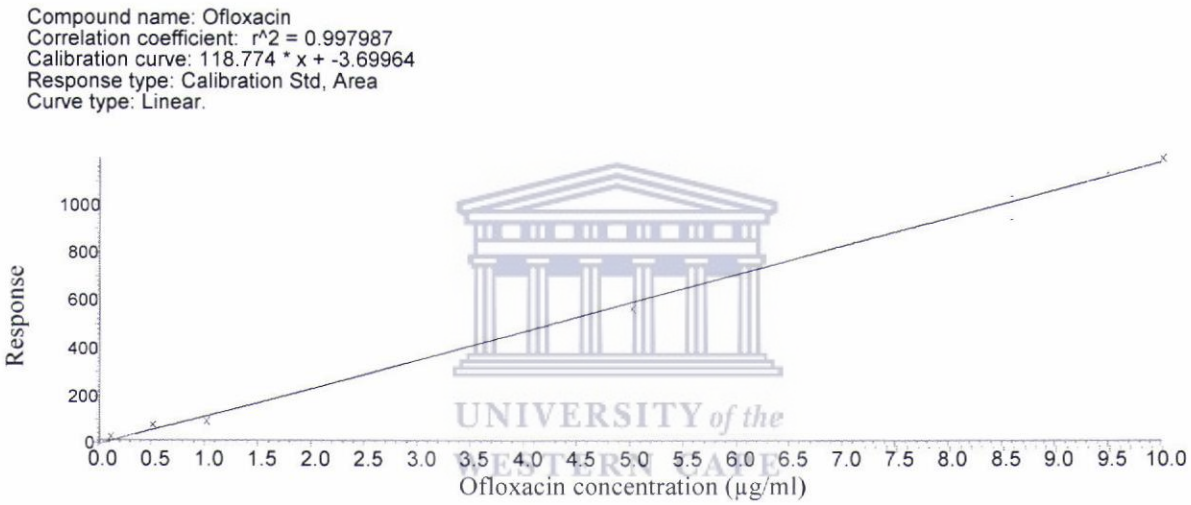


Figure 4.1: Ofloxacin calibration curve.

4.1.2 Recovery

Ofloxacin recovery was determined by comparing peak areas from the plasma samples that spiked with ofloxacin after extraction (i.e. calibration standards) with the corresponding ofloxacin standard solutions. The mean recovery of ofloxacin was 97.6 ±1.6%.

4.1.3 Precision and accuracy

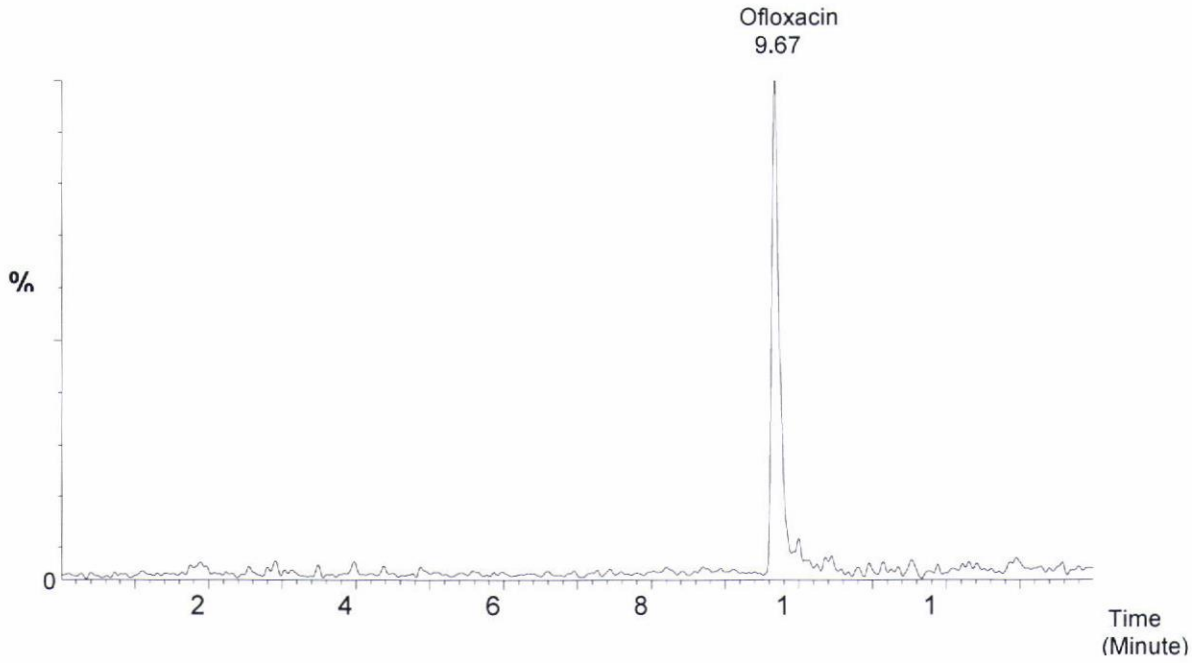
Acceptable precision and accuracy were achieved. The overall precision expressed as relative standard deviation (R.S.D %), was less than 10%. The accuracy, expressed in terms of recovery was found to range from 98.7% to 98.8% for 1 µg/ml and 92.5% to 100.5% for 10 µg/ml.

4.1.4 Lower limit of detection and quantification

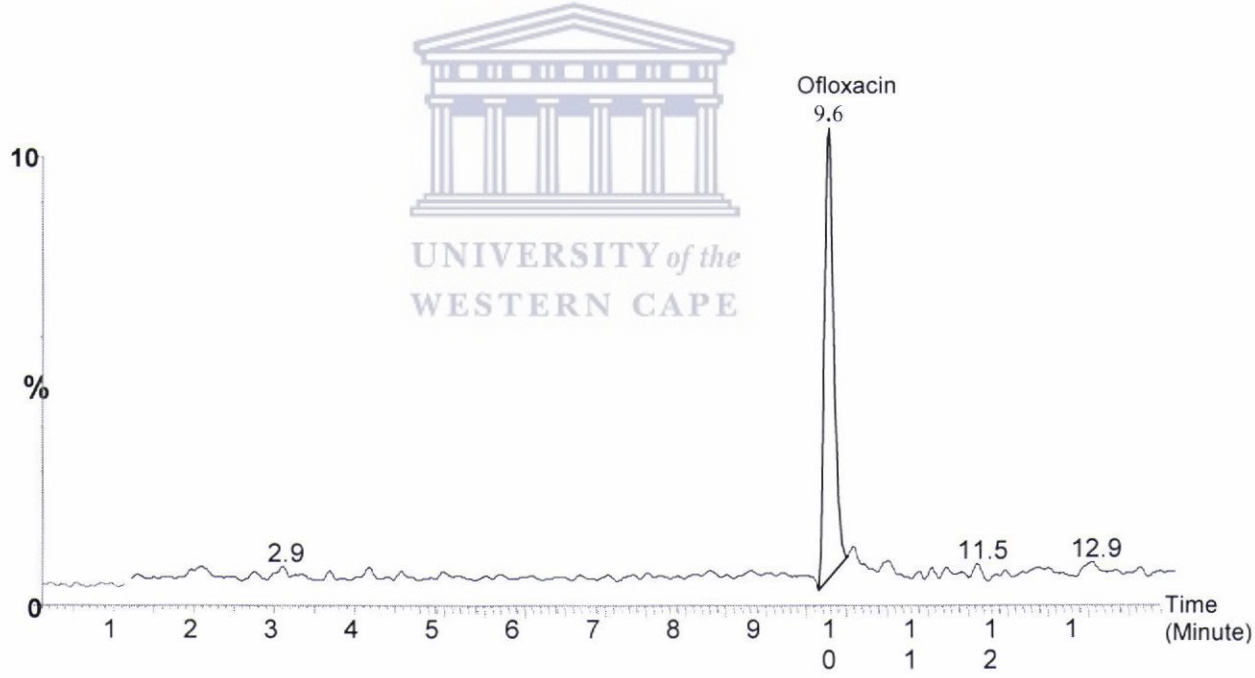
The lower limit of detection of ofloxacin concentration that could be determined (taking into consideration the concentration which produces a signal to ratio of 3 that gives a measurable response from the MS detector) was 0.05 µg /ml. Ofloxacin lower limit of quantification in the plasma was found to be 0.1 µg /ml.

4.1.5 Specificity

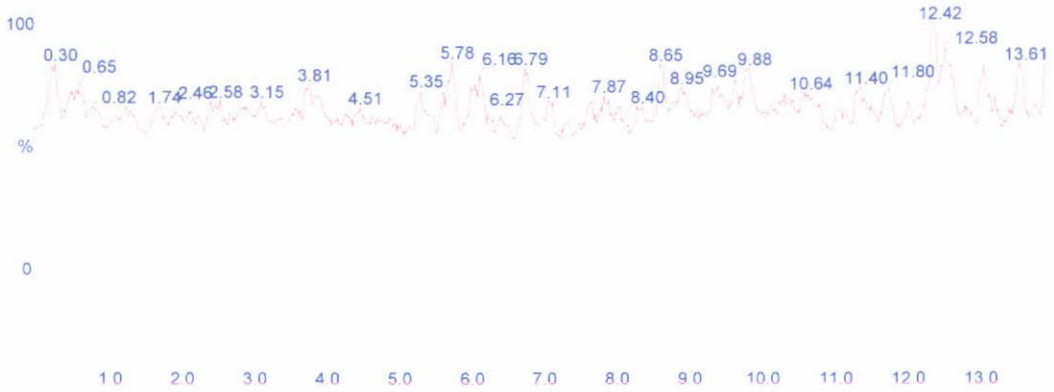
There are no interfering peaks from the plasma components with ofloxacin peak, which was detected at a retention time of 9.68 minutes. Chromatograms showing the separation of ofloxacin standard and ofloxacin plasma extract (calibration standard) in addition to the blank plasma are shown in Figure 4.2.



(A) Chromatogram of ofloxacin standard.



(B) Chromatogram of ofloxacin plasma extract (calibration standard).



(C) Chromatogram of blank plasma

Figure 4.2: Chromatograms of ofloxacin representing the separation of ofloxacin at retention time 9.6 minutes. (A) Ofloxacin standard; (B) Ofloxacin calibration standard, (C) Blank plasma.

Based on all the afore-mentioned findings, the method developed was linear, sensitive, specific, precise and accurate. Therefore, it is suitable for the quantitative analysis of ofloxacin in present study.

4.2. Patients demographic data and characteristics

Eight patients (black) with multi-drug resistant pulmonary tuberculosis participated in the study. The patient's demographic data, which are body weight in kilograms, age in years and gender, are listed in Table 4.1.

Table 4.1: Patients' demographic data

Patient	Weight (kg)	Age (years)	Gender
1	60	22	Female
2	59.3	46	Male
3	46.7	50	Male
4	55.5	31	Male
5	43.4	38	Male
6	45	18	Male
7	61.8	42	Female
8	46.6	18	Female
Mean	**52.2**	**33.1**	___
SD	**7.6**	**12.7**	___

The patients were given the drugs to which MTB had been found to be sensitive. Anti-TB drugs used with their doses and other co-existing diseases are shown in Table 4.2.

Table 4.2: Anti-TB drugs, other medications and co-existing diseases

Patient	Co-existing disease	Ofloxacin (Daily dose)	Co-administered anti-TB drugs (Daily dose)	Other medications
1	HIV	800 mg	KAN (1gm), EMB (1.2 gm) , PZA (1.5 gm), ETH (750 mg),	d4T (30mg), 3TC (150mg), EFV (600mg)
2	HIV/HBV	800 mg	KAN (1gm), PZA (1.5 gm), ETH (750 mg)	d4T (30mg), 3TC (150mg), EFV (600mg)
3	HIV	800 mg	KAN (750 mg), EMB (800mg), PZA (1gm), ETH (500mg)	–
4	HIV	800 mg	KAN (1gm), EMB (1.2 gm), PZA (1.5 gm), ETH (750 mg)	–
5	HIV	800 mg	KAN (750 mg), EMB (800mg), PZA (1gm), ETH (500mg)	d4T (30mg), 3TC (150mg), EFV (600mg)
6	—	800 mg	KAN (750 mg), EMB (800mg), PZA (1gm), ETH (500mg)	–
7	HBV/DM	800 mg	KAN (1gm), EMB (1.2 gm), PZA (1.5 gm), ETH (750 mg)	–
8	—	800 mg	KAN (750 mg), EMB (800mg), PZA (1gm), ETH (500mg)	–

DM=Diabetes mellitus; HBV= Hepatitis B virus; ETH=ethionamide;
EMB=ethambutol; KAN=kanamycin; PZA=pyrazinamide; d4T= stavudine;
3TC = lamivudine; EFV =efavirenz
All drugs were given orally except kanamycin which was given intramuscularly.

Patients' mean (±SD) age and weight were33.31±12.75 years and 52.28±7.6 kg respectively. The ofloxacin daily dose was 800 mg taken with kanamycin,

pyrazinamide, ethambutol and ethionamide. Five patients were HIV-positive; two patients have hepatitis-B and one patient was diabetic. Patients, who were co-infected with HIV (except patient 3 and 4), were on the following anti-retroviral drugs: stavudine (30 mg), lamivudine (150 mg) and efavirenz (600 mg).

Table 4.3 shows the laboratory-test results of CD4 cell count (for patients co-infected with HIV), kidney function test results as glomerular filtration rate, and liver enzymes (ALT, AST and GGT) and UCB levels.

Test	Normal range	Patient									
		1	2	3	4	5	6	7	8	Mean	SD
CD_4 Cell count (cell/mm^3)	700-1100	155	53	137	48	327	_	_	_	**144**	**113.09**
GFR (ml/min)	>90	69	116	70	128	136	142	91	122	**109**	**28.89**
GGT (u/l)	5-50	87	253	30	101	30	19	22	5	**68.37**	**81.98**
ALT (u/l)	10-40	54	64	71	32	14	21	18	1	**34.37**	**25.60**
AST (u/l)	10-40	132	137	114	54	67	34	18	44	**75**	**46.31**
UCB (µmol/L)	2-14	16	12	22	4	9	8	5	37	**14.12**	**10.97**

Table 4.3: Laboratory-tests results

GFR= glomerular filtration rate; GGT= gamma glutamyl transpeptidase; ALT= alanine aminotransferase; AST= aspartate aminotransferase; UCB=un-conjugated bilirubin

The CD4 cell counts mean (±SD) for the patient co-infected with HIV was 144±113.09 cell/mm^3, whereas the mean (±SD) of GFR rate for all patients was 109±28.89 ml/min. Regarding the liver enzymes results, the mean (±SD) of ALT and AST were 34.37±25.60 u/l and 75±46.31 u/l respectively. The mean (±SD) of GGT is 68.37±81.98 u/l and for UCB was 14.12±10 µmol/L.

4.3 Ofloxacin plasma concentrations and pharmacokinetic parameters

4.3.1 Ofloxacin plasma concentrations

Ofloxacin plasma concentrations at different time points after ofloxacin administration, over 24 hour's period are listed in Table 4.4. There are some patients with no concentration results at 1 and 2 hours because the sample tubes were broken during the centrifugation. Patients with missing 24 hour's concentrations left the hospital before their blood samples were taken.

Table 4.4: Ofloxacin plasma concentrations

Patient	Baseline (0 hr) (µg/ml)	Time (hours) after ofloxacin administration				
		1 hr (µg/ml)	2 hrs (µg/ml)	4 hrs (µg/ml)	8 hrs (µg/ml)	24 hrs (µg/ml)
1	3.53	2.73	2.84	7.87	7.51	4.13
2	BDL	**NA**	3.90	5.24	3.34	0.73
3	BDL	**NA**	2.46	3.11	3.80	**NA**
4	BDL	4.95	4.50	4.44	3.35	1.22
5	BDL	5.50	6.85	6.16	4.68	1.87
6	BDL	0.86	1.32	2.98	2.54	0.51
7	BDL	2.68	**NA**	3.90	2.65	**NA**
8	1.17	0.82	0.96	1.20	0.63	0.62
Mean	**2.35**	**2.92**	**3.26**	**4.36**	**3.56**	**1.51**
SD	**1.66**	**1.97**	**2.02**	**2.07**	**1.98**	**1.37**

NA= not available.

BDL= below detectable level (0.05 µg/ml)

Ofloxacin plasma concentration-time profiles for each patient and mean (±SD) plasma concentration-time profile for all patients are shown in Figures 4.3 and 4.4 respectively.

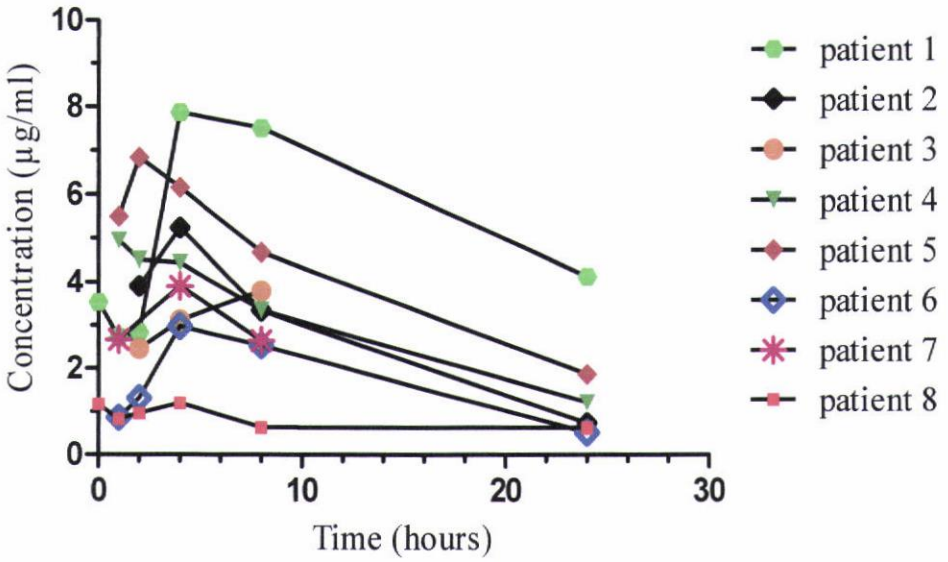


Figure 4.3: Ofloxacin plasma concentration-time profile for each patient.

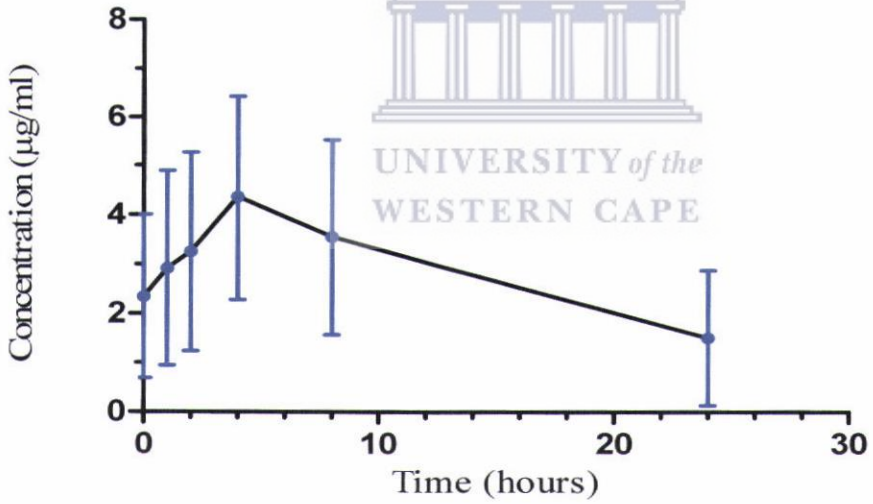


Figure 4.4: Ofloxacin mean (±SD) plasma concentration-time profile for all patients.

4.3.2 Ofloxacin pharmacokinetic parameters

Table 4.5 shows ofloxacin pharmacokinetic parameters in patients, who had been on treatment for two weeks. The parameters were calculated using the NCA method. The pharmacokinetic parameters for all the patients were expressed as mean (±SD). The range (minimum value-maximum value) for each parameter is also given. The PK parameters for patient 3 were excluded, as ofloxacin concentration at the elimination phase could not be estimated due to the 24 hour missing sample.

As reflected in Table 4.5, the mean (±SD) obtained for C_{max}, T_{max}, T½, K_e, MRT, V_d, CL_{tot}, AUC_{0-24} and $AUC_{0-\infty}$ were4.7±2.27 µg/ml, 3±1.29 hr, 9.55±4.69 hr, 0.08 ± 0.04,15.12±6.59 hr, 2.77 ± 1.1624 L/kg, 0.27 ± 0.25 L/hr/kg, 68.8 ± 42.61 µg/ml.hr and 91.9 ± 76.8 µg/ml.hr respectively. Related data from other studies in addition to the present one are included in Table 4.6 for comparison.

Table 4.5: Ofloxacin pharmacokinetic parameters

Patient	Pharmacokinetic parameters								
	T_{max} (hr)	C_{max} (µg/ml)	AUC_{0-24} (µg/ml.hr)	K_e	T½ (hr)	MRT (hr)	$AUC_{0-\infty}$ (µg/ml.hr)	Cl_{tot} (L/hr/kg)	V_d (L/kg)
1	4	7.87	137.4	0.037	18.54	29.14	249.22	0.053	1.44
2	4	5.24	58.86	0.095	7.28	11.2	66.54	0.2	2.13
4	1	4.95	65.81	0.064	10.81	10.7	84.87	0.17	2.68
5	2	6.85	93.27	0.059	11.74	17	125.51	0.14	2.49
6	4	2.98	41.16	0.100	6.90	11.1	46.26	0.28	2.88
7	2	3.90	*NA	0.097	7.17	12	50.57	0.24	2.6
8	4	1.2	16.71	0.161	4.38	14.7	20.56	0.83	5.18
Mean	**3**	**4.71**	**68.8**	**0.08**	**9.55**	**15.12**	**91.9329**	**0.27329**	**2.77143**
SD	**1.29**	**2.27**	**42.61**	**0.04**	**4.69**	**6.59632**	**76.8682**	**0.25606**	**1.1629**
Range	**1-4**	**1.2-7.87**	**16.71-140.5**	**0.037-0.16**	**4.38-18.54**	**10.7-29.14**	**20.56-249.22**	**0.053-0.83**	**1.44-5.18**

C_{max} = maximum plasma concentration, T_{max} = time to attain C_{max}; T½ = elimination half-life; AUC_{0-24} = area under the concentration-time curve from zero to 24 hrs; $AUC_{0-\infty}$= area under the plasma concentration-time curve from zero to infinity Cl_{tot} = total plasma clearance; V_d = volume of distribution; K_e = elimination rate constant; MRT = mean residence time.*For patient 7, the AUC_{0-24} is not available, as the last plasma concentration available was at 8 hrs.

Table 4.6: Ofloxacin pharmacokinetic parameters obtained in the present study and in previous studies.

Study conditions	Dose (mg)	C_{max} (µg/ml)	T_{max} (hr)	T ½ (hr)	K_e	AUC_{0-24} (µg/ml.hr)	$AUC_{0-\infty}$ (µg/ml.hr)	V_d (L/kg)	Cltot (L/hr/kg)	Reference
TB patients	800 (600-1000)	10.5 (8-14.3)	1.03 (0.5-6)	7.34 (3.53-28.3)	0.094 (0.025-0.196)	NA	NA	1.28 (0.78-2.83)	0.12 (0.02-0.32)	Zhu et al., 2002
MDR-TB patients	590.9	9.61±2.17	1.68±1.21	8.03±3.37	NA	70.57±26.4	82.45±43.64	1.37±0.24	NA	Chulavatnatol et al.,2003
Healthy volunteers	800	9.8 (8.2-11.4)	1.9 (1.6-2.2)	6.5 (6.1-6.9)	NA	85.3 (69.4-101.2)	93.1 (79.7-106.5)	NA	NA	Immanuel et al.,2002
Present study	800	4.71±2.27	3±1.29	9.57±4.69	0.08±0.04	69.55±42.93	92.54±77.69 (21.63-252.12)	2.73±1.12	0.26±0.24	–

C_{max} = maximum plasma concentration, T_{max} = time to attain C_{max}; T½ = elimination half-life; AUC_{0-24} = area under the concentration-time curve from zero to 24 hrs; $AUC_{0-\infty}$= the area under the plasma concentration-time curve from zero to infinity; Cl_{tot} = total plasma clearance; V_d = volume of distribution; K_e = elimination rate constant, NA= not available.

4.4 Discussion

4.4.1 The LC/MS analysis method

In this study, LC coupled with MS was used for the determination of ofloxacin concentrations in patients' plasma samples. The MS settings used (Table 3.1) similar to those with that previously reported in the literature, which provide high sensitivity, specificity, selectivity and rapid determination of ofloxacin in biological samples (van Vyncht et al., 2002; Ballesteros et al., 2003; Sinnaeve et al., 2003).

Good separation of ofloxacin at 9.6 minutes as a retention time with a high recovery percentage (97.6%) was obtained. Under the described chromatographic conditions, ofloxacin peak was well resolved and endogenous plasma components did not interfere with ofloxacin peak as indicated in the chromatogram (Figure 4.2, B). This revealed high specificity of the analysis method.

In summary, although other ofloxacin PK studies have used other HPLC methods coupled with UV or fluorescence detection, our validation results indicate that the LC/MS analysis method is successful and able to detect and quantify ofloxacin in plasma.

4.4.2 Demographic characteristics and concomitant infections

The numbers of patients in this study (8) (5 male and 3 female) close to the patients' numbers involved in other similar studies. The study conducted by Chulavatnatol et al. (2003) had11 patients (8 males and 3 females). Zhu et al. (2002) had 11 patients (9 males and 2 females) and Immanuel et al. (2002) study had7 patients (all males). The relatively small number of patients was due to the difficulty in obtaining consent from patients with HIV and TB. Most of the patients were also severely sick, and their inclusion in the study would have been unethical, even if they gave consent.

The mean age and weight of study group (33.12 yrs and 52.28 kg respectively), were comparable to those reported in the studies done before. For example, the mean age and weight of the patients in Chulavatnatol et al. (2003) study were 38.09 yrs and 59.34 kg respectively. For the study, which was done by Zhu et al. (2002), the mean age was 42 yrs;

and the mean weight was 64 kg. In the study conducted by Immanuel et al. (2002), the age and the weight were 34.5 yrs and 59.2 kg respectively.

Although 5 patients were co-infected with HIV, no other opportunistic infections present except MDR-TB and HBV was found in one patient only.

4.4.3 Laboratory results

The GFR is the best indicator for the kidney functions, as the kidney dysfunction stages can be evaluated using the GFR value. Stages of kidney function range from normal kidney function with a GFR more than 90 ml/min to renal failure where the GFR is less than 15 ml/min. In this study, 6 out of 8 patients (75%) had normal kidney function. Three out of the six patients were HIV-positive with CD4 cell counts 53, 48, and 327cell/mm^3. Two patients had mild decrease in their GFR values (60-89 ml/min). Therefore, they are classified as presenting mild renal failure. Both patients were HIV-positive with a CD4 cell counts 137 and 155 cell/mm^3.

With regard to the liver enzymes levels, the mean ALT value was within the normal range, however, some patients had abnormal levels. On the other hand, the AST and GGT mean values were above the upper limit of the normal range and the UCB levels within the normal range. Three patients had mild elevations in both ALT and AST (less than 5 times the upper limit of normal value, which is 200 u/l). Regarding GGT, three patients had high levels of this enzyme. One patient was co-infected with hepatitis and this might explain the patient's abnormal liver enzymes level, especially the GGT, which its elevation is an indicator of viral hepatitis. However, another patient is also co-infected with hepatitis, but the liver enzymes level within the normal range.

ALT, AST and GGT liver enzymes are markers for liver injury and alcohol abuse. Many conditions such as hepatitis, cirrhosis, drug-induced injuries and alcohol abuse can cause the elevation of these enzymes level. However, abnormal levels of these enzymes do not necessarily reflect how severely the liver is damaged (Giannini et al., 2005). The liver enzymes levels for patients in this study indicate some sort of liver up normality. Co-

infection with HBV explains this up normality in patient 2; however, for other patients it might be due to alcohol abuse or other liver disease that is not apparent.

4.4 4 Ofloxacin pharmacokinetics

The C_{max} and AUC_{0-24} results obtained in this study were lower than those reported in the literature by Zhu et al. (2002) and Immanuel et al. (2002). The mean $AUC_{0-\infty}$ was similar to that obtained by Immanuel et al. (2002) (Table 4.6). Furthermore, ofloxacin was absorbed with a longer T_{max} compared with that reported in the literature (Table 4.6). These two parameters are higher than those reported in the literature (Table 4.6). In the present study, ofloxacin T½ was longer than the one reported in previous studies (Table 4.6). Finally, K_e was similar to that reported by Zhu et al. (2002).

Ofloxacin has a high absorption rate and oral bioavailability more than 90%; therefore, its peak serum concentration was achieved within 1-2 hours (Table 2.3). The pharmacokinetic parameters of ofloxacin are dose independent; however, the absorbed amount increases linearly with increasing dose, which leads to an increase in C_{max} and AUC (Lode et al., 1987; Immanuel et al., 2002). This explains why not comparing our C_{max}, AUC_{0-24} and $AUC_{0-\infty}$ with the values obtained in Chulavatnatol et al. (2003) study.

The low C_{max} and low AUC_{0-24} in addition to the prolonged T_{max} indicate a reduction in the extent and the rate of ofloxacin absorption in our patients. Ofloxacin absorption may be blocked by divalent cations included in nutritional supplements or antacid. Co-administration of didanosine leads to a decrease of ofloxacin absorption and as a consequence, its AUC decreases (Walker, 1999; Katzung, 1998; Goodman et al., 2005). Furthermore, co-ingestion with food delays ofloxacin absorption and the time to reach T_{max} by an hour (Walker, 1999; Katzung, 1998). This suggests the influence of food or drug co-administration effect on ofloxacin absorption. None of our patients is taking a nutritional supplement or antacid, however, we cannot control the food intake in our patients. Another factor that could lead to long T_{max} is delayed gastric-emptying phenomenon, due to taking efavirenz by patients co-infected with HIV (Villani et al., 2001). This may explain the long T_{max} observed in patients 1 and 2 who were on ARVs

including efavirenz. Although, patient 5 was also taking efavirenz, he had a normal T_{max}, and this can be explained by inter-individual variations.

Impaired liver function may lead to significant alterations in the PK of many drugs, which are metabolized in the liver. Ofloxacin is a poorly metabolized drug, however, a reduction in C_{max}, AUC and prolonged T_{max} and T½ have been reported (Verbeeck and Horsmans, 1998; Wang et al., 2006). This indicates that the PK parameters of ofloxacin may be influenced by liver dysfunction. The liver enzymes levels in patients showed that some form of liver disease was likely in patients, which may explain the low C_{max}, low AUC_{0-24}, prolonged T_{max} and the long T½.

Ofloxacin has a high volume of distribution associated with its low protein binding (< 30%) (Katzung, 1998; Walker, 1999). Ofloxacin high V_d in the present study reflects that ofloxacin has higher concentration in the extra-vascular tissues than in the vascular parts, which means it is not homogenously distributed. Although the high V_d may contribute to the low C_{max} and the long T½ in patients, the high V_d cannot be related to the high Cl_{tot}, as the extent of a drug distribution is independent of its clearance (Mehvar, 2004). Patients have a high elimination rate which is consistent with the high Cl_{tot} value. The low AUC_{0-24} and low C_{max} can be correlated with the high elimination rate. During the time that plasma concentration is rising, the elimination process is also occurring, resulting in low C_{max} (Mehvar, 2004). This may also be associated with the low C_{max}, long T_{max} and the decrease in AUC_{0-24} in this study, as patients have high Cl_{tot} compared with that reported.

Ofloxacin is mainly cleared through the kidney and its elimination process decreases progressively with renal impairment. With the decrease of the GFR, ofloxacin K_e and Cl_{tot} decrease and this results in an increase of the T½ and $AUC_{0-\infty}$ (Fillastre et al., 1987; Navarro et al., 1990). Ofloxacin T½ increases in relation to the degree of renal dysfunction and can reach values five-fold higher than those obtained in normal subjects. However, the high T½ in patients cannot be related to the renal dysfunction because the GFR of most patients was within the normal range (Table 4.3). Prolonged T½ in the patients with the normal GFR may be referred to the effect of their abnormal hepatic function on ofloxacin metabolism. The low GFR for patient 1 explains the high $AUC_{0-\infty}$, long T½, long MRT and low Cl_{tot} of that particular patient compared to the other patients. It may also explain the high C_{max} in the same patient.

The mean (±SD) MRT for patients is 15.12 ± 6.59 hrs and it was longer than that reported by Chulavatnatol et al. (2003), which was 10.77±4.55 hrs. The long MRT is correlated with the long T½ in study. This can be explained by the high V_d of ofloxacin in this group of patients in addition to the abnormal liver functions.

In conclusion, ofloxacin pharmacokinetics was quite different in patients compared with previously reported studies. Many factors could have contributed to the differences in results as the genetic differences, and patients' health conditions. Furthermore, the different analytical method, which was used in the present study (LC/MS), should also be mentioned in the consideration of the different pharmacokinetic parameters obtained

4.5 Therapeutic and clinical implications

From the published data, low serum concentrations of anti-TB drugs were associated with acquisition of drug-resistant MTB, treatment failure and recurrence of TB (Berning et al., 1992; Peloquin, et al., 1993; Patel at al., 1995). The C_{max} and AUC values showed an inadequate serum concentration of ofloxacin in patients compared with previous report. The mean C_{max} of ofloxacin in study was 4.71 µg/ml (with a T_{max} of 3 hours), which was lower than the optimal plasma concentrations level for TB treatment. The efficacy of ofloxacin is correlated with the C_{max} levels in plasma, and the target C_{max} serum

concentration of ofloxacin in TB patients that provides good treatment outcome is 8-12 µg/ml with a T_{max} 1-2 hours (Root et al., 1999).

Ofloxacin low serum concentrations in the present study indicate that, patients may have poor therapeutic response, which can lead to treatment failure, relapse and further drug resistance (ofloxacin resistance/ XDR-TB). Therefore, we recommend monitoring ofloxacin plasma concentrations and adjusting the current ofloxacin dose. The optimization of ofloxacin dose may result in ofloxacin serum levels that reach the therapeutic range and lead to the desired therapeutic effect and successful treatment.

4.6 Limitations of the present study

There are many patients with MDR-TB in the hospital where the study was conducted. Within seven months, we managed to recruit 30 patients. However, 10 out of the 30 patients gave written consent and two patients of the 10 were excluded. This explains why only 8 patients were involved. Furthermore, the number of 8 patients is low for a reliable statistical analysis.

For the same reason as those mentioned above, it was difficult to get an equally high balance of patients with MDR-TB on one side and patients with MDR-TB co-infected with HIV on the other side for comparison.

It would be interesting to find out the influence of HIV and/or AIDS on the pharmacokinetics of ofloxacin. However, the small patients' number and the poor balance of distribution of our patients within the different ranges of CD4 cell count did not allow that assessment.

CHAPTER FIVE
CONCLUSIONS AND RECOMMENDATIONS

5.1 Conclusions

The objectives of the present study were to determine ofloxacin plasma concentrations and to evaluate the pharmacokinetics of ofloxacin in MDR-TB patients during their regular course of treatment. For the quantification of ofloxacin in patients' plasma, liquid chromatography coupled with mass spectrometry was used as an analysis method.

The objectives of the present study were achieved, and based on the results obtained we can conclude that:

- The LC/MS method used in the present study was simple, specific, accurate, sensitive and reproducible. Therefore, it is suitable to quantify ofloxacin plasma concentrations.

- This study found that, ofloxacin has a low rate and extent of absorption, which was reflected in the long T_{max} (3±1.29 hrs) and the low AUC_{0-24} (68.8±42.61 µg/ml.hr). In addition, the peak plasma concentration of ofloxacin was low (4.71± 2.27µg/ml). Ofloxacin distributed widely (2.77±1.16 L/kg) and was eliminated with high Cl_{tot} (0.27±0.25 L/hr/kg). The patients displayed a long T½ (9.55±4.69 hrs) and long MRT (15.12±6.59 hrs).

Due to the small number of patients and the patients' different co-morbidities, we cannot conclude whether the co-infection with HIV and impaired liver and kidney functions are associated with the altered pharmacokinetic profile of ofloxacin in MDR-TB patients.

5.2 Recommendations

This study provides information about the pharmacokinetics of ofloxacin in patients infected with MDR-TB in South Africa; however, the number of patients used in this study was small. Therefore, we recommend further studies with larger numbers of patients. We also recommend investigating the effect of HIV/AIDS, liver dysfunction and kidney dysfunction on ofloxacin pharmacokinetics. We also recommend using TDM for ofloxacin in this group of patients to maintain constant ofloxacin concentration in their plasma.

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APPENDICES

Appendix A

Patient consent form (written consent only)

Patient study number: .....

Patient statement

This study and related procedures have been explained to me to my satisfaction, I have received a copy of the patient information sheet and have been given the opportunity to ask questions which have been answered to my satisfaction. I hereby agree to voluntarily participate in this program.

I understand that if I choose to withdraw from this study, I will need to inform my Doctor in order to enable him to evaluate my status, to review the consequences of my decision, as well as to allow him to perform procedures for an orderly termination of my participation.

....

Signature of patient Date (to be completed by patient)

Witness

.....

Statement of randomizing Doctor

I declare that I have explained to the above patient (name……, age …gender…..) the nature, purpose, procedures, the possible risks and potential benefits of this research project and have provided the patient with the patient information sheet whose reference is ( )*

(* Insert reference of Patient Information Sheet given to the patient)

…….. ……

Signature of randomizing Doctor Date

Witness

……

Top Copy-patient's hospital notes Second Copy-patient Third Copy-Site Working File

* Afrikaans and Xhosa copies of this form are available.

Appendix B.1

Patient information sheet (for patients with TB)

Nature and purpose of the study

You have been found with multi-drug resistant pulmonary tuberculosis and infection due to HIV. Tuberculosis is due to a bacterium which has affected your lungs and makes you cough, get tired, lose weight, and present with the other symptoms you are complaining of. Tuberculosis is a very common disease in the Western Cape. Under normal conditions, it responds well to treatment. However, due to many factors including non-compliance with the treatment, the number of patients infected by bacteria resistant to drugs usually used to treat tuberculosis has dramatically increased.

Because of resistance to conventional anti-tuberculosis drugs used n short-course (6 months) chemotherapy, including streptomycin, isoniazid, rifampicin, pyrazinamide and ethambutol, it has become necessary to use traditional second-line anti-tuberculosis (anti-TB) drugs and newer anti-bacterial agents. The former include aminoglycosides (e.g. kanamycin), glycopeptides such as capreomycin, thiomides (e.g. ethionamide and prothionamide), cycloserine and para-amainosalicylic acid (PAS). The latter involve the newer amino glycosides such as amikacin and aminosidine, as well as some fluoroquinolones, and perhaps rifabutin and clofazimine.

The pharmacology of some of the second line anti-TB drugs is not well known.

The purpose of this study is to assess: the effect of your body on the drugs given to you (pharmacokinetics) and the influence of HIV on the pharmacokinetics of the drugs you are going to take. The information obtained will help in the development of therapeutic drug monitoring in order to optimize drug therapy and improve patient outcome.

In this study, you will receive a treatment regimen, which is recommended in South Africa for the treatment of multi-drug resistant tuberculosis. It includes kanamycin/ amikacin, ofloxacin, ethionamide, ethambutol, pyrazinamide, terizidone/cycloserine, capreomycin and para-amino salicylic acid (PAS).

If you agree to participate in the study, 2 weeks after commencement of the treatment, you will be requested to fast the first night from midnight. On the following morning, an intravenous line will be put on your arm for blood collection, just before administration of

the above-mentioned drugs. Blood samples will be collected in appropriate tubes in the following order:

1. Five (5) millilitres for liver and renal function.

2. Five (5) millilitres for haematological and virological tests.

3. Five (5) millilitres for assessment of plasma drug concentration before administration of drugs, then at 1,2,4,8 and 24 hours after administration of drugs.

You are instructed to take only the treatment prescribed by doctors in the ward where you have been admitted.

Risks and difficulties

No risks and difficulties are expected to occur. However, you may experience the following adverse effects: systemic or cutaneous allergic reactions, hepatitis, skin rashes and reddish coloration of the urine.

Benefits

During the days of hospitalization you will benefit from regular visits from the doctor, regular advice on the treatment, a close supervision of drug intake and an optimum diet.

Alternatives

If you decide not to take apart, you will be treated like any other patient in the word. Your normal treatment will in no way be affected by your refusal to participate in the study.

Consent, withdrawal and confidentiality

You do not have to take part or can end your involvement at any time. If your doctor feels that it is not in your best interest to stay in the study he will stop participation.

You will be provided with pre- and post-test counseling regarding the test for antibodies to HIV, by trained counselors. All information obtained during the study will be treated as confidential. If you want to know anything else, your doctor will answer any question you may have.

Dissemination of research results

The research results will be disseminated through the following channels:

a.Final report to the sponsor of the study

b.Presentation at departmental levels at institutions involved: University of the Western Cape, University of Stellenbosch and Cape Peninsula University of Technology.

c.Class teaching: Medical, Pharmacy, Nursing and Dental studens.

d.Conference presentations at national and international levels

e.Local radio and local news papers

f. Publication in scientific journals

Your names will always be kept confidential.

Name of investigator.....Tel.....

* Afrikaans and Xhosa copies of this form are available.

Appendix B.2

Patient information sheet (for patients with HIV infection and TB)

Nature and purpose of the study

You have been found with multi-drug resistant pulmonary tuberculosis and infection due to HIV. Tuberculosis is due to a bacterium which has affected your lungs and makes you cough, get tired, lose weight, and present with the other symptoms you are complaining of. HIV destroys your body's immune system and this causes many opportunistic infections. One of the most important infections is tuberculosis.

Tuberculosis is a very common disease in the Western Cape. Under normal conditions, it responds well to treatment. However, since the advent of HIV and some other factors, its prevalence has seriously increased and the level of recurrence and relapses of the treated patients has increased. The number of patients infected by bacteria resistant to drugs usually used to treat tuberculosis has dramatically increased.

Because of resistance to conventional anti-tuberculosis drugs used n short-course (6 months) chemotherapy, including streptomycin, isoniazid, rifampicin, pyrazinamide and ethambutol, it has become necessary to use traditional second-line anti-tuberculosis (anti-TB) drugs and newer anti-bacterial agents. The former include aminoglycosides (e.g. kanamycin), glycopeptides such as capreomycin, thiomides (e.g. ethionamide and prothionamide), cycloserine and para-amainosalicylic acid (PAS). The latter involve the newer amino glycosides such as amikacin and aminosidine, as well as some fluoroquinolones, and perhaps rifabutin and clofazimine.

The pharmacology of some of the second line anti-TB drugs is not well known.

The purpose of this study is to assess: the effect of your body on the drugs given to you (pharmacokinetics) and the influence of HIV on the pharmacokinetics of the drugs you are going to take. The information obtained will help in the development of therapeutic drug monitoring in order to optimize drug therapy and improve patient outcome.

In this study, you will receive a treatment regimen, which is recommended in South Africa for the treatment of multi-drug resistant tuberculosis. It includes kanamycin/ amikacin, ofloxacin, ethionamide, ethambutol, pyrazinamide, terizidone/cycloserine, capreomycin and para-amino salicylic acid (PAS).

If you agree to participate in the study, 2 weeks after commencement of the treatment, you will be requested to fast the first night from midnight. On the following morning, an intravenous line will be put on your arm for blood collection, just before administration of the above-mentioned drugs. Blood samples will be collected in appropriate tubes in the following order:

1. Five (5) millilitres for liver and renal function.

2. Five (5) millilitres for haematological and virological tests.

3. Five (5) millilitres for assessment of plasma drug concentration before administration of drugs, then at 1,2,4,8 and 24 hours after administration of drugs.

You are instructed to take only the treatment prescribed by doctors in the ward where you have been admitted.

Risks and difficulties

No risks and difficulties are expected to occur. However, you may experience the following adverse effects: systemic or cutaneous allergic reactions, hepatitis, skin rashes and reddish coloration of the urine.

Benefits

During the days of hospitalization you will benefit from regular visits from the doctor, regular advice on the treatment, a close supervision of drug intake and an optimum diet.

Alternatives

If you decide not to take apart, you will be treated like any other patient in the word. Your normal treatment will in no way be affected by your refusal to participate in the study.

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You do not have to take part or can end your involvement at any time. If your doctor feels that it is not in your best interest to stay in the study he will stop participation.

You will be provided with pre- and post-test counseling regarding the test for antibodies to HIV, by trained counselors. All information obtained during the study will be treated as confidential. If you want to know anything else, your doctor will answer any question you may have.

Dissemination of research results

The research results will be disseminated through the following channels:

a. Final report to the sponsor of the study

b. Presentation at departmental levels at institutions involved: University of the Western Cape, University of Stellenbosch and Cape Peninsula University of Technology.

c. Class teaching: Medical, Pharmacy, Nursing and Dental student.

d. Conference presentations at the national and international level

e. Local radio and local news papers

f. Publication in scientific journals

Your names will always be kept confidential.

Name of investigator.....Tel.....

* Afrikaans and Xhosa copies of this form are available.