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Population pharmacokinetics of terizidone and cycloserine in patients with drug-resistant tuberculosis

Mwila Mulubwa

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Supervisor: Prof Pierre Mugabo

October 2019

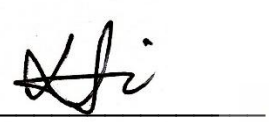
"Is there no balm in Gilead; is there no physician there? Why then is not the health *of the daughter of my people recovered?"* **Jeremiah 8:22 (KJV)**

DECLARATION

I declare that:

"*Population pharmacokinetics of terizidone and cycloserine in patients with drug-resistant tuberculosis"* is my own work, that it has not been submitted for any degree or examination at any other university, and that all the sources I have used or quoted have been indicated and acknowledged by complete references.

Mwila Mulubwa

Signed 

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ABSTRACT

Population Pharmacokinetics of Terizidone and Cycloserine in Patients with Drug-resistant Tuberculosis

Mwila Mulubwa – 2019

Introduction: Drug-resistant tuberculosis remains a major world health problem and one of the leading cause of death worldwide. Despite adequate adherence to anti-tuberculosis drugs by patients, the emergence of drug-resistance tuberculosis still occurs. This fact implies other factors leading to the emergence of resistant strains of *Mycobacterium tuberculosis*. A multidrug treatment regimen, which may consist of five to seven different drugs including terizidone, is used in the treatment of drug-resistance tuberculosis. Terizidone is part of the multidrug regimen whose pharmacokinetics is scarce in literature and plasma concentration profile unknown. Two molecules of cycloserine joined by terephtalaldehyde moiety makes up a molecule of terizidone, which is thought to undergo complete metabolism into cycloserine *in vivo*. Additionally, the current literature report that terizidone and cycloserine can be used interchangeably as they are thought to be equivalent. The aim of this thesis was first to develop and validate bioanalytical methods for determination of terizidone and cycloserine in patients' plasma samples. Secondly, to model population pharmacokinetics of terizidone and cycloserine. Thirdly, to determine the amount of cycloserine resulting from metabolism of terizidone.

Methods: This was a non-randomised prospective clinical study involving 39 patients with drug-resistant tuberculosis admitted to Brewerskloof Hospital for intensive treatment phase. All patients took 500 – 750 mg daily dose of terizidone in addition to other anti-tuberculosis drugs. Blood was sampled at pre-dose, 0.5, 1, 2, 3, 3.5, 4, 8, 16 and 24 hours post terizidone administration. Chromatographic bioanalytical methods for determination of both terizidone and cycloserine in plasma samples were developed and validated. Terizidone population pharmacokinetics was modelled solely and jointly with cyloserine using nonlinear mixed-effects modelling implemented in Monolix 2018R1 software. The R statistical software version 3.5.2 was used to

perform correlation and regression analyses between secondary pharmacokinetic parameters of cycloserine and markers of hepatic function.

Results: The HPLC-UV and UPLC-MS/MS methods were developed and validated for analysis of terizidone and cycloserine in patients' plasma samples. A one-compartment model with first-order elimination process described best the pharmacokinetics of terizidone. The absorption process was well characterised by a modified transit compartment model with mean transit time (*MTT*) and absorption rate constant (*ka*) of 1.7 h and 2.97 h^{-1}, respectively. Albumin had significant effect on the apparent distribution volume (*Vp/F*) of terizidone (13.4 L). The total terizidone apparent clearance (Cl_{tot}/F) was 0.51 L/h. In the joint model, the percentage of the total amount of terizidone in the body that was metabolised to cycloserine was 29%. The clearance of terizidone via biotransformation and other routes was 0.47 and 0.1 L/h, respectively. The apparent clearance of cycloserine was 2.94 L/h and a one-compartment model with first-order elimination characterised well its pharmacokinetics. The median and range of cycloserine amount resulting from terizidone metabolism was 51.6 (0.64 – 374) mg. Cycloserine C_{min} and C_{max} was significantly associated with increased conjugated bilirubin concentration ($p < 0.05$). C_{max} was significantly associated with increased binding affinity of unconjugated bilirubin to albumin ($p = 0.048$).

Conclusions: In this thesis, the population pharmacokinetics of terizidone and cycloserine, has for the first time, been described in patients with drug-resistant tuberculosis. The *Vp/F* in patients with drug-resistant tuberculosis is influenced by albumin concentration, which may affect drug concentration in patients with hepatic impairment. Terizidone and cycloserine is not interchangeable as the amount of cycloserine emanating from metabolism of terizidone is far lower than expected. Cycloserine plasma exposure may be a predisposing factor to the development of hyperbilirubinemia because of its relationship with conjugated bilirubin.

Keywords: *Terizidone, Cycloserine, Population pharmacokinetics, Drug-resistant tuberculosis, Metabolism*.

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RESEARCH OUTPUTS

Publications

1. Pierre Mugabo, Mwila Mulubwa. Population pharmacokinetic modelling of pyrazinamide and pyrazinoic acid in patients with multi-drug resistant Tuberculosis. *European Journal of Drug Metabolism and Pharmacokinetics*, 2019; 44 (4): 519–530. https://doi.org/10.1007/s13318-018-00540-w. (Not submitted for this thesis).

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1. Mwila Mulubwa, Pierre Mugabo. Steady-state pharmacokinetics of terizidone in patients with drug-resistant tuberculosis. *First Conference of Biomedical and Natural Sciences and Therapeutics*, 7 – 10 October 2018, Stellenbosch, South Africa. (Podium presentation).

2. Mwila Mulubwa, Pierre Mugabo. Cycloserine amount emanating from terizidone metabolism in patients with multidrug-resistant tuberculosis. *18th World Congress of Basic and Clinical Pharmacology*, 1 – 6 July 2018, Kyoto, Japan. (Poster presentation).

3. Mwila Mulubwa, Pierre Mugabo. Influence of HIV status and age on cycloserine clearance in multidrug-resistant tuberculosis patients. *3rd University of the Western Cape, School of Pharmacy Research Symposium,* 2 October 2017, Bellville, South Africa. (Podium presentation).

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LIST OF ABBREVIATIONS AND ACRONYMS

ALT	Alanine aminotransferase
AST	Aspartate transaminase
AUC	Area under the curve
BMI	Body mass index
BSV	Between-subject variability
Cl/F	Apparent clearance
Clp	Clearance of terizidone via other routes
Clpm	Clearance of terizidone via biotransformation
Cl_{tot}/F	Apparent total clearance of terizidone
C_{max}	Maximum concentration
C_{min}	Minimum concentration
CRP	C-reactive protein
DR-TB	Drug-resistant tuberculosis
EBE	Empirical Bayes estimates
FFM	Free-fat mass
GGT	Gamma-glutamyltransferase
HIV	Human immunodeficiency virus
HPLC-UV	High-performance liquid chromatography with ultraviolet detection
HQC	High quality control
IPRED	Individual predicted concentration
IWRES	Individual weighted residuals
ka	Absorption rate constant
K_{af}	Binding affinity constant of unconjugated bilirubin to albumin

LIST OF ABBREVIATIONS AND ACRONYMS

ke	Elimination rate constant
Ktr	Transit rate constant
LLOQ	Lower limit of quantification
LOD	Limit of detection
LQC	Low quality control
MBC	Minimum bactericidal concentration
MCP-1	Monocyte chemoattractant protein 1
MDR-TB	Multidrug-resistant tuberculosis
MIC	Minimum inhibitory concentration
MQC	Medium quality control
MRM	Multiple reaction mode
MTT	Mean transit time
NLME	Non-linear mixed effects
OFV	Objective function value
PK/PD	Pharmacokinetics/pharmacodynamics
PPK	Population pharmacokinetics
RSD	Relative standard deviation
RSE	Relative standard error
SAA	Serum amyloid A
SAEM	Stochastic Approximation Expectation Maximization
sIL-2Rα	Soluble interleukin-2 receptor alpha
Tlag	Lag time
T_{max}	Time at which concentration is at its maximum value

LIST OF ABBREVIATIONS AND ACRONYMS

T>MIC	Time at which concentration is above the MIC
UPLC-MS/MS	Ultra-performance liquid chromatography tandem mass spectrometry
V/F	Apparent volume of distribution
VEGF-A	Vascular endothelial growth factor A
Vm/F	Apparent distribution of cycloserine
Vp/F	Apparent distribution of terizidone
VPC	Visual Predictive Checks

Chapter One

1.0 BACKGROUND

Tuberculosis, an infectious disease caused by the bacillus *Mycobacterium tuberculosis* remains a major world health problem. Respiratory tuberculosis is the active infection of the lungs, mediastinal lymph nodes, larynx or pleural cavity. It is typically spread by droplets from infected person particularly by coughing, sneezing, talking and breathing. The extra pulmonary tuberculosis affects any other organ. Pulmonary tuberculosis infection is of particular importance, as it is highly contagious and life threatening to affected patients. Extra pulmonary tuberculosis is less common and not very contagious (Gordon, Mwandumba, 2008).

Each year, millions of people continue to fall sick with tuberculosis, which is one of the top ten causes of death in the world. Worldwide, an estimated 10 million people in 2017 developed tuberculosis disease of which 90% were adults aged above 15 years (World Health Organization, 2018). In 2017, tuberculosis caused an estimated 1.6 million deaths among HIV infected and uninfected patients (World Health Organization, 2018). Meanwhile, in 2015 tuberculosis morbidity was 10.4 million new cases while mortality was at 1.4 million deaths in addition to 0.4 million deaths patients co-infected with HIV (World Health Organization, 2016a). HIV infection is the most potent risk factor for active tuberculosis as it is directly related to the patient's degree of immunosuppression. Hence, tuberculosis is common among HIV-infected persons worldwide. In some African countries, the rate of HIV infection among tuberculosis patients reaches 70–80% (World Health Organization, 2014, Longo et al., 2013). Not only does HIV infection increase the number of tuberculosis cases, but also changes the clinical course of tuberculosis (NDoH, 2014).

The World Health Organization recommends a standardised first-line daily treatment regimen for new patients presumed or known to have drug-susceptible tuberculosis. The regimen consists of four drugs; isoniazid, rifampicin, pyrazinamide and ethambutol which are taken in the initial phase or first two months of treatment

followed by isoniazid and rifampicin in the continuation phase or the last four months (World Health Organization, 2010).

Improper use of anti-tuberculosis drugs, among other factors have led to the emergence of drug resistant strains of *Mycobacterium tuberculosis*. It is estimated globally that 3.5% of new tuberculosis cases and 18% of previously treated cases have drug-resistant tuberculosis (World Health Organization, 2018).

In South Africa, the design of standardised second-line regimen is based on first-line drug susceptibility testing at diagnosis. The drug-resistant tuberculosis treatment regimen for the intensive phase consists of kanamycin or amikacin (injection), moxifloxacin, ethionamide, terizidone, ethambutol and pyrazinamide. Isoniazid is added to the treatment for rifampicin mono-resistant tuberculosis patients. The continuation phase treatment comprises four drugs; terizidone, moxifloxacin, ethionamide and pyrazinamide which are administered daily (NDoH, 2013). The South African department of health has simplified the administration of standardised second-line regimen across four weight bands; <33 kg, 33–50 kg, 51–70 kg and >70 kg in order to accommodate the fixed dose formulations that are available in the country (NDoH, 2013).

The knowledge and subsequent application of pharmacokinetic information of anti-tuberculosis drugs in the clinical management of tuberculosis is crucial in order to achieve the desired treatment outcomes (Savic et al., 2017). Characterisation of pharmacokinetic parameters of each anti-tuberculosis drug in the regimen is indispensable in order to determine appropriate optimal dose (Chirehwa et al., 2017). In additional to maximising the anti-mycobacterial efficacy, pharmacokinetic characterisation of anti-tuberculosis drugs ensures prevention of drug resistance with minimal toxicity (Srivastava et al., 2017, Savic et al., 2017). Terizidone and cycloserine (metabolite) are among the drugs used in the treatment of drug-resistant tuberculosis whose pharmacokinetics have not been extensively studied.

1.1 PROBLEM STATEMENT

Drug-resistant tuberculosis constitutes a major threat to the management of tuberculosis worldwide and remains a public health crisis. Its treatment success

globally is low, at 55% (World Health Organization, 2016a, World Health Organization, 2018).

In South Africa, drug-resistant tuberculosis has reached alarming proportions. It is estimated that 9.6% of all tuberculosis cases in South Africa are drug-resistant, thereby ranking it as one of the 30 high drug-resistant tuberculosis burden countries in the world (Streicher et al., 2012). A high proportion of drug-resistant tuberculosis cases in South Africa had unfavourable treatment outcome, with rates in the range 41-58%. Additionally, treatment is empiric owing to a delay in Drug Susceptibility Tests results for second-line anti- tuberculosis drugs (NDoH, 2013, Brust et al., 2010). This phenomenon may partly explain the high mortality and low culture conversion rates reported in South Africa (Streicher et al., 2012). Drug-resistant tuberculosis is often associated with higher mortality rates in HIV-infected than uninfected patients (Brust et al., 2010, Streicher et al., 2012).

Treatment of drug-resistant tuberculosis in HIV-infected patients remains a challenge (Isaakidis et al., 2012).The most common risk factors of anti-tuberculosis drug-resistance are low plasma drug exposure due to drug-drug interactions, altered pharmacokinetics, inter-individual pharmacokinetic variability and inadequate dose or dosing frequency in drug-resistant tuberculosis patients co-infected with HIV (Alsultan, Peloquin, 2014, Satti, McLaughlin & Seung, 2013, Sotgiu et al., 2015).

The pharmacokinetics of terizidone, which is one of the second-line drugs used in drug-resistant tuberculosis, is poorly described in literature (World Health Organization, 2015). The recommended daily dose of terizidone is 750 mg within the weight band of 33-70 kg for both intensive and continuous phases of treatment (NDoH, 2013) but the average C_{max} and T_{max} is unknown. Furthermore, the primary parameters that describe terizidone pharmacokinetics at steady-state in drug-resistant tuberculosis patients and the sources of pharmacokinetic variability are unknown. Additionally, whether the 750 mg daily dose of terizidone is sufficient to guarantee adequate cycloserine exposure within the recommended target plasma range of 20–30 µg/ml (Lange et al., 2014) is not established.

The World Health Organization acknowledges a number of gaps in the current knowledge about the treatment of drug-resistant tuberculosis. Hence, population

pharmacokinetic studies aimed at determination of optimal drug dosing and safety is one of the research priorities in order to close these gaps (World Health Organization, 2016b). Thus, attending to the urgent need for improved care and treatment of patients with drug-resistant tuberculosis (World Health Organization, 2018). Therefore, the study of the population pharmacokinetics of terizidone and cycloserine is highly called for.

1.2 RESEARCH QUESTIONS

1. What is the best population pharmacokinetic model that describes the fate of terizidone and its metabolite cycloserine in drug-resistant tuberculosis patients?
2. What is the effect of HIV status on terizidone and cycloserine pharmacokinetic parameters?
3. What are the sources and correlates of variability in terizidone and cycloserine plasma concentrations at recommended doses of terizidone?
4. What are the clinically significant covariates influencing pharmacokinetic parameters of terizidone and cycloserine?
5. How much cycloserine is formed from terizidone metabolism in patients with drug-resistant tuberculosis?

1.3 GENERAL OBJECTIVE

The general objective of this study was to describe the steady-state population pharmacokinetics of terizidone and cycloserine in drug-resistant tuberculosis patients with and without HIV infection.

1.3.1 Specific objectives

1. To develop and validate a high-performance liquid chromatography method coupled with ultraviolet detection (HPLC-UV) for determination of terizidone in plasma.
2. To develop and validate a sensitive ultra-performance liquid chromatography tandem mass spectrometry (UPLC-MS/MS) method for determination of cycloserine in plasma.

3. To determine the plasma concentrations of terizidone and cycloserine in plasma of patients with drug-resistant tuberculosis.
4. To model population pharmacokinetics of terizidone and its metabolite cycloserine in patients with drug-resistant tuberculosis.
5. To determine the factors influencing the pharmacokinetics of terizidone and cycloserine in patients with drug-resistant tuberculosis.
6. To determine the cycloserine amount emanating from terizidone metabolism in patients with drug-resistant tuberculosis.

1.4 THESIS OUTLINE

The thesis comprises nine chapters. Each chapter has its own reference list provided at the end. The chapters are arranged as follows:

Chapter 1: Introduction

Chapter 2: Literature review

Chapter 3: Methodology

Chapter 4: Analysis of terizidone in plasma using HPLC-UV method and its application in a pharmacokinetic study of patients with drug-resistant tuberculosis.

Chapter 5: Sensitive ultra-performance liquid chromatography tandem mass spectrometry method for determination of cycloserine in plasma for a pharmacokinetics study.

Chapter 6: Steady-state population pharmacokinetics of terizidone and its metabolite cycloserine in patients with drug-resistant tuberculosis.

Chapter 7: Amount of cycloserine emanating from terizidone metabolism and relationship with hepatic function in patients with drug-resistant tuberculosis.

Chapter 8: General discussion

Chapter 9: Overall conclusions

1.5 REFERENCE

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Chapter Two

2.0 INTRODUCTION

The purpose of this chapter is to review the current literature on the clinical pharmacology (pharmacokinetics/pharmacodynamics) of terizidone and cycloserine in human subjects. Each sub-section ends with a brief summary. The bioanalytical methods for determination of terizidone and cycloserine in plasma are reviewed. Furthermore, factors that influence the pharmacokinetics of these drugs are reviewed in subsequent sections. On the other hand, the link between pharmacokinetics and the development of drug-resistant tuberculosis is discussed in light of other drugs used in tuberculosis treatment. In general, the relationship between plasma exposure (AUC, C_{max}) of several anti-tuberculosis drugs and outcomes (efficacy) is illustrated. Therefore, the role that pharmacokinetics play in the management of tuberculosis is justified. In addition, the concept of dose optimisation of anti-tuberculosis drugs performed in some studies is substantiated. In order to emphasise the role of pharmacokinetics in tuberculosis management, methods for optimal dose determination such as pharmacokinetic modelling and simulation are presented with applicable studies reviewed. The need for terizidone dose optimisation based on pharmacokinetic target is dealt with. Finally, the chapter is summarised by highlighting the current gaps in the pharmacokinetics knowledge of terizidone and its metabolite cycloserine.

2.1 QUANTIFICATION OF TERIZIDONE AND CYCLOSERINE IN PLASMA

Quantification of terizidone in plasma has only been achieved using colorimetric method (Zitkova, Toušek, 1974). In this method, an analyte of interest undergoes a chemical reaction with another reactant to form a coloured target chemical product. The intensity of the target coloured chemical product or its optical absorbance, which is measured using light with suitable wavelength, is proportional to the concentration of the analyte (Dubois et al., 1956). However, the current standard bioanalytical method employed in clinical pharmacokinetic studies is a chromatographic method

(USFDA, 2013, ICH, 2005). Chromatography is among the most versatile analytical techniques used to separate and quantify complex mixtures based on some compound's properties such as polarity, molecular weight, particle size, solubility and ionic mobility (Robards et al., 1994). To date, no chromatographic bioanalytical method has been employed to determine plasma concentrations of terizidone.

Unlike terizidone, chromatographic methods for determination of cycloserine in plasma have been employed before (Polagani et al., 2013, Patel et al., 2011, Mao et al., 2017, Yaroshenko, Grigoriev & Sidorova, 2014, Stepanova et al., 2016, Supriya, Ashish & Meena, 2012). However, the steps involved in the sample preparations are many making the routine analysis of plasma samples to be prone to imprecision and inaccuracy.

2.2 CLINICAL PHARMACOLOGY OF TERIZIDONE

Terizidone is categorised as group IV anti-tuberculosis drug by the World Health Organization and classified as a second-line drug. It is effective against *Mycobacterium tuberculosis*, used in the treatment of both pulmonary and extra pulmonary tuberculosis, and reserved for treatment of drug-resistant tuberculosis (World Health Organization, 2014). It is a dimer which is obtained by combining two molecules of cycloserine and one molecule of terephtalaldehyde (Figure 1). Terizidone and cycloserine have advantage over other drugs in that they do not show cross-resistance with other active anti-tuberculosis drug classes (NDoH, 2013). The World Health Organization Prequalification Team-Medicines states that terizidone is not detected in plasma using a bioanalytical method with a Lower Limit of Quantification of 0.2 µg/mL and appears to be pre-systemically hydrolysed completely into its active metabolite cycloserine (World Health Organization, 2015).

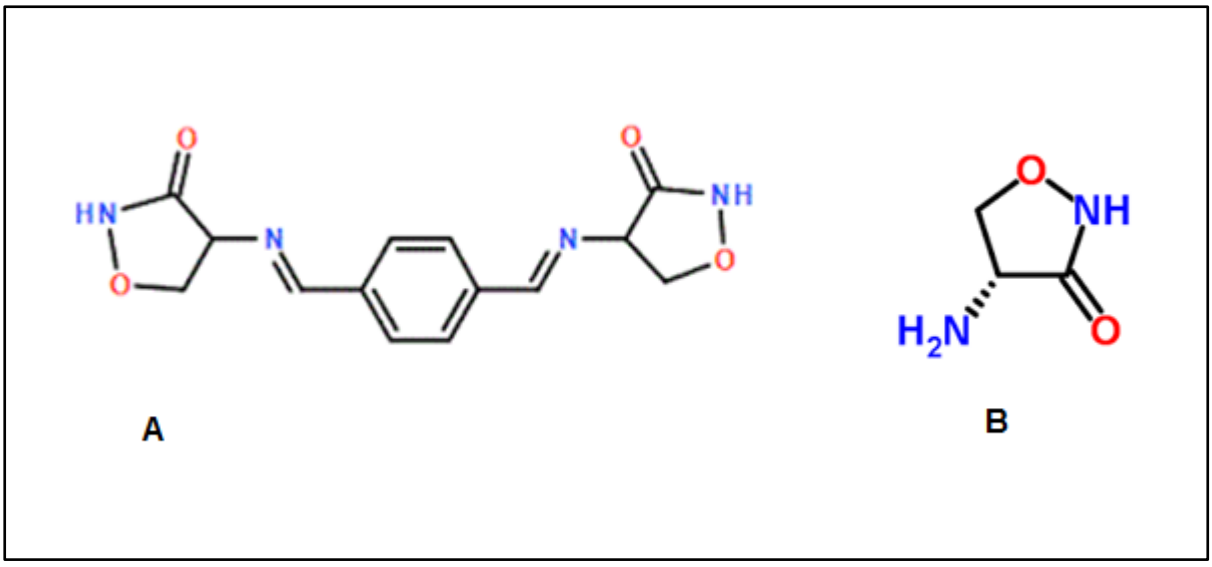

Figure 1. Terizidone molecule (A) and cycloserine molecule (B).

2.2.1 Terizidone pharmacokinetics

The pharmacokinetic properties of terizidone are scarce and poorly described in the literature. Currently, no pharmacokinetic studies of the drug have been performed in patients with drug-resistant tuberculosis. The drug was developed in the 1970s when the bio-analytical methods were limited (World Health Organization, 2015). Plasma concentration of terizidone in these pharmacokinetic studies (Zitkova, Toušek, 1974, Zitkova et al., 1983) was quantified using colorimetric method, which is unspecific and currently not in use.

Terizidone is rapidly absorbed after single oral administration of 250-750 mg dose on empty stomach (Table1) and reaches maximum concentration (C_{max}) in 2-4 hours. Absorption shows some dependence on age. Doses above 500mg do not cause a proportional increase in the concentration (Vora, 2010, Zitkova, Toušek, 1974).

It is widely distributed and the volume of distribution (V/F) increases with increasing dose. Terizidone has a low elimination rate constant, clearance and long half-life. Clearance ranges from 2.49-6.4 L/h over the dose range of 250-750mg (Zitkova, Toušek, 1974, Zitkova et al., 1983).

Thirty nine per cent of the administered dose is excreted in urine after 30 hours. The high concentrations of terizidone in urine indicate the possibility of using it in

genitourinary tuberculosis (Kottász, Babics, 1972). The urine excretion of terizidone in adults is slower than in young ones.

It is, however, worthy to note the significant shortcoming in Zitkova and Toušek (Zitkova, Toušek, 1974) study design. Blood sampling was not done at steady-state but it was performed on the first day of the trial after administration of a single dose. In order to reach steady-state, it takes about four to five elimination half-lives. The authors used an open one-compartment model to calculate the pharmacokinetic parameters of terizidone. Based on visual inspection of the concentration-time curves, rough estimates for and the observed average C_{max} were 8, 17 and 19 µg/mL following administration of 250, 500 and 750 mg single doses of terizidone, respectively (Zitkova, Toušek, 1974). However, simulations of one-compartment model using MlxR package of R software (R Core Team, 2018) with average pharmacokinetic parameters shown in Table1 yielded lower C_{max} but same T_{max}. The simulated C_{max} for 250, 500 and 750 mg single doses were 2.1, 2.6 and 2.8 µg/mL, respectively. Either this huge difference between the observed and simulated C_{max} of terizidone imply that pharmacokinetic parameters were incorrectly estimated or the model used to describe the pharmacokinetics was inappropriate. Therefore, the pharmacokinetic parameters of terizidone published in literature (Zitkova, Toušek, 1974) may be incorrect.

Table1: Pharmacokinetic parameters of terizidone in tuberculosis patients

N	Dose (mg)	ka (h^{-1})	V/F (L)	Cl/F (L/h)	ke (h^{-1})	$T_{1/2}$ (h)	T_{max} (h)	Source
10	250	1.36	112.6	2.49	0.022	33	3	(Zitkova, Toušek, 1974)
15	500	1.39	175.1	6.30	0.035	21	3	(Zitkova, Toušek, 1974)
10	750	1.17	245.6	6.40	0.028	24.8	3	(Zitkova, Toušek, 1974)

2.2.2 Terizidone pharmacodynamics

The mode of action of terizidone is similar to that of cyloserine. Its bacteriostatic effect is achieved through inhibition of cell wall synthesis by competitively inhibiting L-alanine racemase and D-alanine ligase, thereby impairing peptidoglycan formation needed for *Mycobacterium tuberculosis* cell wall synthesis. The minimum inhibitory concentration (MIC) ranges from 4-130 mg/mL in susceptible strains (Vora, 2010). Currently, there

is no information on the MIC of terizidone in drug-resistant strains. Therefore, the pharmacodynamics indices such as C_{max}/MIC ratio, AUC/MIC ratio and time > MIC (Craig, 2007) which are used to link anti-infective drug exposure and response are not available for terizidone.

Prior to the 1970s, some clinical studies were performed in tuberculosis patients mainly on terizidone safety and tolerability. However, literature that described these studies was in Portuguese, Italian, Croatian and Russian that were reviewed by Hwang et al (Hwang et al., 2013). The adverse effects profile of terizidone is similar to that of cycloserine but terizidone is better tolerated and hence leads to better compliance. The few reported side effects are slurred speech, dizziness, headache and convulsions. Others adverse effects reported include depression, tremors, insomnia, confusion and suicidal tendency (Vora, 2010). The electroencephalographic studies using light and sonic stimulation show that terizidone leads to less activating effect of the ascending part of the reticular formation of the brain stem than cycloserine. Patients with concomitant psycho-neurological impairments tolerate well terizidone dose of 250 mg taken eight hourly (Shmelev, Shabalova & Kolosovskaia, 1975).

2.2.3 Factors influencing pharmacokinetics of terizidone

There are scanty studies that have established the factors that affect the pharmacokinetics of terizidone, if any. The only study trial that was performed in the 1970s found urine excretion of terizidone lower in older tuberculosis patients than in young ones. Thus, old age was found to be affecting the renal excretion of the drug (Zitkova, Toušek, 1974). This could have been due to the decrease in both renal function and high transaminase levels which were significantly worse in elderly patients than the young ones (Zitkova, Toušek, 1974). These authors did not develop mixed effects pharmacokinetic model that could have accounted for the effects of other covariates on pharmacokinetics of terizidone. It is however, worthy to note that Zitkova and Toušek carried out their study in an era before the concept of population pharmacokinetics (non-linear mixed effects modelling) was introduced in the pharmacokinetics discipline (Sheiner, Rosenberg & Marathe, 1977).

2.3 CLINICAL PHARMACOLOGY OF CYCLOSERINE

Cycloserine is an analogue of D-alanine amino acid and a broad-spectrum antibiotic used in combination with other drugs (Brennan, Young & Robertson, 2008). The World Health Organization classifies it as a group 4 oral bacteriostatic drug used as the second-line treatment for drug-resistant tuberculosis (World Health Organization, 2014). Depending on the concentration, cycloserine may also be bactericidal on particular strains (Brennan, Young & Robertson, 2008).

2.3.1 Cycloserine pharmacokinetics

The pharmacokinetics of cycloserine in drug-sensitive and –resistant tuberculosis patients is very limited while there are currently no pharmacokinetic studies performed in HIV-infected patients receiving anti-retroviral therapy.

In patients with drug-sensitive tuberculosis, oral doses of 250 – 750 mg of cycloserine reach maximum concentration in blood within 2-3 hours (Table 2).The rate of absorption in drug-resistant tuberculosis patients is slower than in drug-sensitive tuberculosis, with absorption rate constant of 0.135 versus 1.67 ± 0.25 (Chang et al., 2017, Zitkova, Toušek, 1974).

The rate of absorption in healthy subjects is faster than in tuberculosis patients. Meanwhile, in healthy subjects, a single dose of cycloserine is rapidly absorbed and reaches peak plasma concentration in approximately 0.84 hours after oral administration of 250 – 100 mg. A fatty meal or orange juice increases the T_{max} in healthy subjects but the extent of cycloserine absorption is not affected. In fasting state of healthy subjects, cycloserine has lag time of 0.4 hours (Zitkova, Toušek, 1974, Zhou et al., 2015). The C_{max} after a dose of 500 mg and 250 mg ranges between 12.4 – 42.9 µg/mL 7.21 – 30 µg/mL, respectively (Brennan, Young & Robertson, 2008, Zhou et al., 2015, Zhu et al., 2001, Zheng-sheng et al., 2014). However, C_{max} in drug-resistant tuberculosis patients has not yet been reported in literature. The concentration of cycloserine in cerebrospinal fluid of tuberculosis patients after a dose of 250 mg reaches maximum concentration of 12.8 µg/mL in 2 hours (Holdiness, 1985).

Cycloserine is widely distributed to most body fluids and tissues, including cerebral spinal fluid, lymph tissue, breast milk, lungs, bile, sputum, pleural, synovial fluids and

crosses the placental barrier. The concentration reached in cerebrospinal fluid is 79% of the serum concentration (Brennan, Young & Robertson, 2008, Holdiness, 1985).

The V/F in drug-sensitive tuberculosis patients is higher than in healthy subjects, 115-255 L and 15.2-16.8 L, respectively (Zitkova, Toušek, 1974, Zhou et al., 2015). The clearance is higher in tuberculosis patients than in healthy subjects, 3.2 - 8.6 L/h and 0.91-1.1 L/h, respectively. In drug-resistant tuberculosis patients, the V/F is surprisingly comparable to that in healthy subjects (Table 2). The pharmacokinetic properties of cycloserine are linear at doses ranging from 250-1000 mg in healthy subjects (Zitkova, Toušek, 1974, Zhou et al., 2015).

Excretion is primarily renal, with 38-70% excreted unchanged within 12 to 30 hours (Brennan, Young & Robertson, 2008, Zitkova, Toušek, 1974).

Critical review of the study by Zitkova and Toušek (Zitkova, Toušek, 1974) show some flaws in the estimated pharmacokinetic parameters. Simulations of C_{max} using the pharmacokinetic model, which the authors used to estimate the pharmacokinetic parameters of cycloserine, yielded very low values compared to the observed C_{max}. The simulated C_{max} at single doses of 250, 500 and 750 mg yielded 2.01, 2.51 and 2.73 µg/mL compared with 8, 14 and 17 µg/mL observed average C_{max}, respectively. The simulated T_{max} was equally different from the observed one except for the 750 mg dose which was same as the observed one. Therefore, the pharmacokinetic parameters of cycloserine, which Zitkova and Toušek estimated, may be incorrect and this could be the reason why they seem to be different from the rest of other studies shown in Table 2.

Furthermore, terizidone and cycloserine were co-administered in this study and hence there was also cycloserine emanating from hydrolysis (metabolism) of terizidone which authors did not consider in their pharmacokinetic calculations. It is for this reason that the authors noted that increase in blood concentration of terizidone was not proportional to the two molecules of cycloserine that make up a molecule of terizidone even after administration of same dose of both drugs (Zitkova, Toušek, 1974). The sampling of blood for pharmacokinetic analyses in this study was not done at steady-state. These factors highlighted above contributed to the inaccuracy of cycloserine pharmacokinetic parameters

Table 2. Pharmacokinetic parameters of cycloserine in tuberculosis patients and healthy volunteers

Subjects	N	Dose (mg)	Tlag (h)	ka (h^{-1})	V/F (L)	Cl/F (L/h)	$T_{1/2}$ (h)	ke (h^{-1})	C_{max} (µg/mL)	T_{max} (h)	AUC (µg•h/mL)	Source
TB patients	10	250	-	1.60	115	3.23	25	0.028	-	2	-	(Zitkova, Toušek, 1974)
	15	500	-	1.47	176.5	8.31	15.8	0.047	-	3	-	(Zitkova, Toušek, 1974)
	10	750	-	1.94	255.8	8.65	21.8	0.033	-	2	-	(Zitkova, Toušek, 1974)
	22	250	-	-	-	-	-	-	12.8[c]	2[c]	-	(Holdiness, 1985)
Drug-resistant TB	14	250-500 bd	-	0.135	10.5	1.38	-	-	-	-	-	(Chang et al., 2017)
Healthy volunteers												
Fasting	12	500	0.4	3.97	0.41[†]	0.033[‡]	8.26	0.083	14.8[a]	0.75	214*	(Zhu et al., 2001)
Fatty meal	12	500	-	-	-	-	-	-	12.4[a]	3.5	217*	(Zhu et al., 2001)
Orange juice	12	500	-	-	-	-	-	-	13.8[a]	1.5	225*	(Zhu et al., 2001)
Anti-acid	12	500	-	-	-	-	-	-	19.2[a]	0.88	216*	(Zhou et al., 2015)
	12	250	-	-	16.82	1.11	12.33	-	19.42[b]	0.86	283*	(Zhou et al., 2015)
	12	500	-	-	15.26	0.91	13.27	-	42.9[b]	0.83	643*	(Zhou et al., 2015)
	12	1000	-	-	16.61	0.99	13.13	-	84.76[b]	0.84	1224*	(Zhou et al., 2015)
	16	250 bd	-	-	10.7	1.2	20.3	-	24.9	4	242.3	(Park et al., 2015)
Fatty meal	10	250	-	-	-	-	11.4	-	9.56	1.8	135*	(Zheng-sheng et al., 2014)
Fasting	10	250	-	-	-	-	12.5	-	7.21	2.8	125*	(Zheng-sheng et al., 2014)
	-	250 bd	-	-	-	-	10	-	25-30	-	-	(Brennan, Young & Robertson, 2008)

[a]Median value, [b]mean value, *$AUC_{0-\infty}$, [†]L/kg, [‡]L/h/kg, [c]measured in cerebrospinal fluid.

2.3.2 Cycloserine pharmacodynamics

Cycloserine achieves bacteriostatic effect by inhibiting alanine racemase and d-alanine ligase which are both essential enzymes for the synthesis of peptidoglycan and subsequently cell-wall biosynthesis and maintenance. It has *in vitro* potency against *Mycobacterium tuberculosis* with MIC ranging from 6.2-25 µg/mL and depends on the media used, pH and the presence of d-alanine which inhibits its activity. Cycloserine can be bacteriostatic or bactericidal depending on local concentration effects along with efficacy against the particular bacteria strain involved. About <1log (80%) of mycobacterium tuberculosis is killed in macrophages with cycloserine plasma concentration of 50 µg/mL (Brennan, Young & Robertson, 2008, Peloquin, 2008).

The C_{max}/MIC ratio of 1.7 is achieved after a daily dose of 500 mg cycloserine at steady-state. A dose of 500 mg every 12 hours at steady-state results in C_{max}/MIC ratio of about 2.4 and 24-hour-concentration/MIC ratios of about 1. The time in which the cycloserine serum concentrations exceed the MIC is about 8 hours. However, the precise cycloserine pharmacodynamic index that requires optimisation is not known (Berning, Peloquin, 1999). The target C_{max} range after 250-500 mg doses is 20-35 µg/mL. For therapeutic drug monitoring, a dose increase is recommended if the C_{max} is less than 15 µg/mL also a dose decrease if the C_{max} is above 40 µg/mL (Alsultan, Peloquin, 2014, Peloquin, 2002).

The main adverse effect of cycloserine is central nervous system (CNS) toxicity which includes depression, dizziness, hyper-excitability, memory loss, lethargy, anxiety and confusion. The CNS effects may be worse with plasma concentrations greater than 35 µg/mL. The lethargy is often seen at normal plasma concentrations (Peloquin, 2008).

2.3.3 Factors influencing pharmacokinetics of cycloserine

In health individuals, high fat meal lowers the C_{max}, delay the T_{max}, decrease the ka and increase the absorption half-life but does not affect $AUC_{0-\infty}$. The orange juice or anti-acids (aluminium hydroxide or magnesium hydroxide) does not significantly affect cycloserine disposition (Zheng-sheng et al., 2014, Zhu et al., 2001).

Gender in health subjects affects Cl/F and elimination half-life. The Cl/F is greater in women than in men while half-life in men is longer than in women (Zhou et al., 2015).

Meanwhile, in drug-resistant tuberculosis patients, only one study investigated the effects of covariates on cycloserine ka, Cl/F and V/F. The covariates such as age, sex, body weight, body mass index, serum albumin and serum creatinine did not have any effect on ka, Cl/F and V/F (Chang et al., 2017). There was however, interindividual variability in Cl/F and V/F of 22.3% and 35.1%, which could not be accounted for by any covariate investigated (Chang et al., 2017). In this study, the influence of HIV infection and antiretroviral therapy on pharmacokinetic parameters of cycloserine was not investigated.

Generally, pharmacokinetic variability is affected by:

- Pathophysiologic and physiologic factors such as renal and hepatic impairment, co-morbidities (other disease states) and pregnancy.
- Environmental factors that include exposure to pollutants, diet and smoking.
- Drug–drug interactions,
- Genetic phenotypes that affect the clearance of drugs through hepatic metabolism by polymorphic cytochrome P450 isoforms such as CYP2D6, 2C19, 2C9 and 2A6.
- Other factors such as circadian rhythm, adherence, food effect and the timing of meals, activity and posture (Ette, Williams, 2004).

2.4 ANTI-MYCOBACTERIAL PHARMACODYNAMICS AND REGIMEN EFFICACY

Pharmacodynamics relates the time course of pharmacokinetics or plasma drug levels to the therapeutic action or toxicological effects. For antimicrobials, it describes the relationship that exist between the plasma drug concentration to which the bacteria is exposed at various sites of infection and bacterial killing (David, Willian, 2005). The major parameters that have been used to measure *in vitro* activity of antimicrobials against various bacteria are MIC and minimum bactericidal concentration (MBC). Even though MIC and MBC are excellent predictors of the potency of an antimicrobial agent, they do not provide information on the time course of antimicrobial activity (Craig, 2007). Using the MIC as a measure of the potency of drug-organism interactions, the pharmacokinetic parameters that determine efficacy such as C_{max} and AUC can be converted to pharmacokinetic/pharmacodynamics (PK/PD) indices. The C_{max}/MIC and

AUC/MIC ratios have been the PK/PD parameters that correlate well with *in vivo* efficacy for concentration dependent killing anti-tuberculosis drugs. Time above MIC (T>MIC) is the PK/PD parameter for anti-tuberculosis drug that exhibit time dependent killing (Craig, 2007).

Some proteins have been proposed as surrogate biomarkers of anti-tuberculosis treatment response. The serum inducible protein of 10 kDa (IP-10) decreases significantly after anti-tuberculosis treatment. Therefore, it can be a useful marker for monitoring therapy and efficacy in patients with active tuberculosis (Hong et al., 2014). In drug-resistant tuberculosis patients, chemokines and acute inflammation markers [monocyte chemoattractant protein 1 (MCP-1), IP-10, soluble interleukin-2 receptor alpha (sIL-2Rα), serum amyloid A (SAA), C-reactive protein (CRP) and vascular endothelial growth factor A (VEGF-A)] predict the microbiological outcome and hence predictive of treatment response (Ferrian et al., 2017). No studies that have shown how pharmacokinetics of anti-tuberculosis drugs in drug-resistant tuberculosis patients are related to these biomarkers. However, it is undoubted that plasma exposure to anti-tuberculosis drugs (pharmacokinetics) plays a vital role since drug-resistant strain induces the production of these chemokines (Basile et al., 2017) which correlate well with treatment outcome (Ferrian et al., 2017).

In patients with pulmonary tuberculosis, conversion of sputum mycobacterial cultures from growth to negative growth of mycobacteria tuberculosis is considered the most important indicator of treatment response or efficacy of anti-tuberculosis pharmacologic treatment for drug-resistant tuberculosis (World Health Organization, 2010b). From the literature, the sputum conversion at 2 or 3 months is taken as a good treatment response in pulmonary tuberculosis (Brust et al., 2011, Holtz et al., 2006, Kurbatova et al., 2012, Kwon et al., 2008, Yew et al., 2000). The efficacy of the second-line regimen in drug-resistant tuberculosis patients whether HIV-infected or not, ranges between 24 – 85% in terms of the rate of sputum culture conversion. The median time to culture conversion ranges between 60 - 90 days (Brust et al., 2011, Holtz et al., 2006, Loveday et al., 2012, Kurbatova et al., 2012, Kwon et al., 2008, Yew et al., 2000). It is worthy to note that the efficacy in these studies was not related to the plasma concentration of each individual drug in the regimen. Therefore, variations in the efficacy could be a result of inadequate individual drug exposure.

There are, however other factors that influence the efficacy of the second-line anti-tuberculosis regimen. Factors such as baseline positive (Kurbatova et al., 2012) smear, smoking, previous use of second-line drugs, older age, male gender and bilateral radiological involvement (Mota et al., 2012, Qazi et al., 2011).

2.5 MECHANISMS OF DRUG-RESISTANT TUBERCULOSIS

Multidrug-resistant tuberculosis (MDR-TB) is defined as the infection with *Mycobacteria tuberculosis* strains which are resistant to rifampicin and isoniazid, the key first-line anti-tuberculosis drugs (Zumla et al., 2012). Drug resistance in tuberculosis is epidemiologically classified as primary, acquired and initial. Primary drug resistance occur in treatment naïve patients who get infected with resistant strain of bacteria. Acquired drug resistance applies to patients previously treated with drug susceptible tuberculosis and later become resistant to medications mainly due to non-adherence or inappropriate/irregular treatment (Long, 2000). The sub-optimal drug therapy causes spontaneous mutations in the mycobacteria chromosomal genes that leads to production of resistant strains (Almeida Da Silva, Pedro Eduardo, Palomino, 2011). Initial or mixed drug resistance is a combination of primary and acquired drug resistance as patients in this category have unconfirmed drug history although claim to be treatment naïve (Long, 2000).

There are six genotypes of *Mycobacterium tuberculosis* strains that have been identified in MDR-TB patients in the Western Cape Province of South Africa. These are Beijing, Latin American and Mediterranean (LAM), Haarlem, X, Family 11(F11), Family 28 (F28) and IS6110 low copy number clades (Johnson et al., 2010, Marais et al., 2006, Streicher et al., 2004). Beijing is the most prevalent genotype family followed by LAM. Infection with either Beijing or Haarlem strain increases the likelihood to have drug resistant tuberculosis than other strain families (Marais et al., 2006). The Beijing cluster 220 genotypes with -15 inhAC-T promoter and rpoB gene mutations confer isoniazid and rifampicin resistance, respectively (Johnson et al., 2006).

The action of rifampicin on *Mycobacterium tuberculosis* is bactericidal with MIC range of 0.05-10 µg/mL in different media. It targets the β-subunit of DNA dependent RNA polymerase and interferes with the synthesis of RNA (Zhang, Yew, 2009). The β-subunit of the RNA polymerase is encoded in the 81 base pair region of *rpo*B gene.

Mutations in the *rpo*B gene (amino acid 507-533) are accountable for most rifampicin resistance and cross-resistance to other rifamycins (Laurenzo, Mousa, 2011, Zhang, Yew, 2009). Furthermore, mutations in codons 531 (S531L) and 526 (H526P/D/Y) give rise to high-level rifampicin resistance with MIC above 64 µg/mL (Laurenzo, Mousa, 2011).

Isoniazid is a prodrug that is activated by catalase peroxidase leading to production of multiple reactive species such as oxygen radicals and isonicotinic acyl radicals, which lead to the killing of *Mycobacterium tuberculosis* infected cells (Zhang, Yew, 2009). The enzyme, enoyl-acyl carrier protein reductase (InhA), involved in mycolic acid synthesis is thought to be the primary target of inhibition by isoniazid. Mutations in two genes *Kat*G and *Inh*A account for majority of isoniazid resistance as they result in loss of catalase peroxidase activity (Laurenzo, Mousa, 2011).

2.5.1 Factors contributing to the development of drug-resistant tuberculosis

Poor treatment outcome such as acquired drug-resistant tuberculosis are mainly caused by patient factors like non-adherence, inappropriate treatment regimen, inadequate supply of anti-tuberculosis drugs or supply of poor quality drugs (World Health Organization, 2014). Comorbidities and undernutrition may also cause malabsorption of anti-tuberculosis drugs leading to low bioavailability and sub-therapeutic concentrations (Murray, Cohen, 2009, World Health Organization, 2014). Low exposure to anti-tuberculosis drugs or to a single drug because of inappropriate regimen or poor treatment adherence provides survival advantage for drug-resistant bacteria (Pablos-Mendez, Lessnau, 2000). Similarly, if new cases of tuberculosis are not successfully treated due to clinical mismanagement, result in the increase of drug-resistant tuberculosis prevalence by ten-fold (Pablos-Mendez, Lessnau, 2000).

Comorbidity like HIV infection was found to be associated with drug-resistance and resistance to any other drug even after adjusting for confounding effect of previous anti-tuberculosis therapy (Murray, Cohen, 2009). This is thought to occur because of non-adherence due to polypharmacy in HIV infected patients, overlapping toxicities and drug interactions between anti-tuberculosis and anti-retroviral drugs (Murray, Cohen, 2009). Drug-resistant tuberculosis has been associated with previous history of pulmonary tuberculosis and episodes and previous treatment with category 2 drugs

(streptomycin, kanamycin, amikacin and capreomycin) (Workicho, Kassahun & Alemseged, 2017, Dessalegn et al., 2016). Being younger than 30 years is associated with higher chance of having drug-resistant tuberculosis than being older. This could be due to non-adherence or reluctance in adhering to the anti-tuberculosis drugs in younger patients (Workicho, Kassahun & Alemseged, 2017). A case-control study found type II diabetes and past smoking status to be associated with drug-resistant tuberculosis (Rifat et al., 2014). In another study, diabetes mellitus was independently associated with drug resistance even after adjusting for other factors (Baghaei et al., 2016). It is suggested that in type II diabetes, low immunity may enhance propensity to infection with resistant strains (Rifat et al., 2014). Consumption of alcohol and chronic anti-acid use were found to be significant predictors of drug-resistant tuberculosis (Mulisa et al., 2015). Furthermore, indulgence in both alcohol and tobacco influence the outcome of treatment with category 4 drugs in drug-resistant tuberculosis patients (Yadav et al., 2016).

The factors that lead to development of drug-resistant tuberculosis are largely those that eventually result in low plasma exposure to anti-tuberculosis. These are non-adherence, comorbidities, malabsorption and drug-drug interactions through the use of medication for comorbidities and social drugs such as tobacco and alcohol. Erratic supply of anti-tuberculosis and use of wrong treatment regimens ultimately lead to drug resistance resulting from inadequate drug exposure.

2.6 THE ROLE OF PHARMACOKINETICS IN PREVENTION OF DRUG-RESISTANT TUBERCULOSIS

Despite the high rate of adherence to anti-tuberculosis medication by patients, emergence of drug-resistant tuberculosis still occur (Calver et al., 2010). Pharmacokinetic variability in anti-tuberculosis drugs is the more likely cause of the emergence of drug-resistant tuberculosis (Srivastava et al., 2011) since not only is associated with drug-resistant tuberculosis but also treatment failure (Pasipanodya, Srivastava & Gumbo, 2012).

2.6.1 Relationship between drug exposure and tuberculosis treatment outcome

Rifabutin AUC_{0-24} and median C_{max} were found significantly lower in patients who had acquired rifampicin resistance (ARR) than those who did not. Subsequent adjusted

analyses showed that rifabutin AUC_{0-24} of less than 4.5 mg*h/mL was associated with relapse or failure (Weiner et al., 2005). Isoniazid median AUC_{0-12} was also significantly lower in patients with relapse or ARR failure (Weiner et al., 2005). A controlled trial revealed a strong association between isoniazid AUC_{0-12} and treatment failure or relapse while C_{max} did not show any association (Weiner et al., 2003). The AUC_{0-12} was significantly lower among patients with relapse or failure. The trial on the contrary did not find any significant associations between rifampin AUC (or C_{max}) and treatment outcome (Weiner et al., 2003). The time over a certain concentration threshold as described by AUC is the better determinant pharmacokinetic parameter for treatment outcome of isoniazid than C_{max}.

In drug-sensitive tuberculosis, the 24-hour AUCs for isoniazid, rifampicin and pyrazinamide were found to be most predictive of long-term outcomes (relapse, treatment failure and death). Furthermore, target plasma concentrations of these drugs were not associated with long-term outcomes (Pasipanodya et al., 2013). Only pyrazinamide peak concentration was found the highest predictor of 2-month sputum conversion and sterilizing activity (Pasipanodya et al., 2013). Another study showed that standard dosing of rifampin resulted in sub-therapeutic plasma levels and poor clinical response. Dose increase resulted in improved clinical outcome with no change in side effects profile (Mehta et al., 2001).

Lower than expected C_{max} values of ethambutol, rifampin and isoniazid occurred frequently in a cohort of tuberculosis patients (Chideya et al., 2009). Meanwhile, poor tuberculosis treatment outcome was associated with low pyrazinamide C_{max} and more frequently among HIV-infected patients with a CD4 cell count of less than 200 cells/mL (Chideya et al., 2009). Surprisingly, in HIV negative patients, sub-therapeutic plasma levels of ethambutol, isoniazid, rifampicin and ofloxacin sampled at 1-2 hours were reported (Kimerling et al., 1998). Additionally, improvement in the clinical outcomes was noted after drug doses were adjusted upwards. Drug interactions and malabsorption could not be ruled out as probable reason since patients were reported to be smokers, alcohol and laxative abusers (Kimerling et al., 1998).

On the contrary, one study revealed that despite the presence of low concentrations of rifampicin, isoniazid and pyrazinamide in the patients, treatment response was good (Burhan et al., 2013). There was no any association found between plasma

concentrations and sputum culture results at four and eight weeks (Burhan et al., 2013). However, this study assessed only two time points and this could have led to the underestimation of the C_{max} especially in the presence of delayed drug absorption. Similarly, despite a substantial number of drug-resistant tuberculosis patients not achieving the minimum therapeutic concentrations of cycloserine, moxifloxacin and prothionamide, the sputum conversion after two months of therapy was not affected (Lee et al., 2015). These concentrations, nevertheless, may have not represented the typical C_{max} as each patient sample was drawn between 2 to 6 hours (Lee et al., 2015). Another reason for this observation is that the authors could not use AUC as the predictor of sputum conversion as their study design could not allow. In another study, lower than recommended plasma concentrations of cycloserine were frequent among the drug-resistant tuberculosis patients. However, the majority of the patients achieved cure defined as five consecutive negative sputum cultures taken every 30 days during the last 12 months of treatment (Hung et al., 2014). This study had some limitations to consider; low doses of cycloserine were administered and not the recommended (Hung et al., 2014). The important investigations with respect to the impact of low cycloserine plasma concentrations were not performed and blood was sampled at two time points (Hung et al., 2014).

In anti-tuberculosis drugs, pharmacokinetics plays a major role in either prevention or contributing factor to development of drug-resistant tuberculosis. Achieving therapeutic plasma drug levels is cardinal for clinical efficacy of anti-tuberculosis treatment. Strong evidence from a meta-analysis of controlled clinical trials confirms that variability in the pharmacokinetics of a single drug in the anti-tuberculosis drug regimen is significantly associated with acquired drug resistance and treatment failure (Pasipanodya, Srivastava & Gumbo, 2012). This evidence supports more use of individualised dosing than standardised dosing recommended in directly observed therapy programmes (Pasipanodya, Srivastava & Gumbo, 2012).

2.7 POPULATION PHARMACOKINETICS AND MODELLING

Population pharmacokinetics is defined as the study of sources and correlates of variability in drug concentrations between individuals (patients) when standard dosage regimens are administered. The essence of population pharmacokinetics is to recognise any demographical features (age, weight, and race), pathophysiological

(renal and hepatic impairment), concomitant therapies or disease state that can significantly alter the pharmacokinetic parameters (Aarons, 1991). It seeks to identify the pathophysiological factors that cause changes in the dose-concentration relationship that can potentially result in altered therapeutic response. The population pharmacokinetics make provision for appropriate dose adjustment or modification (Sun et al., 1999).

The concept of population pharmacokinetics was first proposed by Sheiner and co-workers in 1977 and has now become a standard tool in the clinical setting and pharmaceutical industry (Sheiner, Rosenberg & Marathe, 1977). In population pharmacokinetic modelling, a non-linear mixed-effects modelling approach is employed. This approach considers the population rather than the individual as the unit of analysis for estimating the pharmacokinetic parameters distribution and covariate relationship within the population of interest. All pharmacokinetic parameters and precision of these parameters are estimated simultaneously (Kiang et al., 2012). Population pharmacokinetic modelling is robust and has several advantages, which include:

- Ability to accommodate flexible study designs which occur during treatment,
- Allow analysis of sparse or dense data from each patient,
- Ability to screen and quantification of covariates for explaining variability in the pharmacokinetic parameters,
- Ability to distinguish between inter-individual and intra-individual variability in the pharmacokinetic parameters,
- Allow estimation of the magnitude of the unexplained part of the variability in the patient population (Charles, 2014, Ette, Williams, 2004).
- Utilisation of information generated for individualised dosing.
- Overcome ethical barriers that does not allow performing of traditional Phase 1 studies in children (Bonate, 2011).

2.7.1 Role of pharmacokinetic modelling and simulation in anti-tuberculosis drugs

Pharmacokinetic modelling characterises and summarises concentration-time data while simulation is applied modelling. Simulation uses models to predict outcomes for

a given set of inputs. Monte Carlo simulations unlike deterministic simulations take into consideration random variation component of the model to make predictions (Bonate, 2011).

The Monte Carlo simulations were performed on final population pharmacokinetics models for second-line anti-tuberculosis drugs in patients with drug-resistant tuberculosis with an aim to evaluate World Health Organization and American Thoracic Society recommended dosages. The observed data in comparison with simulated data revealed none or small proportion of patients achieving target concentration range. Hence, authors recommended the following doses: 500 to 750 mg of cycloserine, 200 mg six hourly of moxifloxacin for patients weighing less than 50 kg, 750 to 1000 mg IM of kanamycin and 3.3 to 6.6 g twelve hourly or 3.3 g eight hourly of para-aminosalicylic acid (Chang et al., 2017). Meanwhile, simulations of the 600 mg standard dose of rifampicin in another study showed inability to prevent emergence of resistance, as probability of target attainment especially in pulmonary epithelial lining fluid was less than 36% (Goutelle et al., 2009). However, using 1200 mg dose produced better results in terms of target attainment (Goutelle et al., 2009). The mean bactericidal effect after simulations of 300 mg daily dose of isoniazid was 11% lower for fast than slow acetylator subjects with MIC of 1 mg/L (Lalande et al., 2015). Increment of daily dose to 450 mg resulted in a 22% increase in bactericidal effect for fast acetylator subjects. These results implied that fast acetylator patients infected with low-level resistance mycobacterium tuberculosis might benefit from higher doses of isoniazid (Lalande et al., 2015).

The final pharmacokinetic models in one study (Zvada et al., 2014) were used to perform steady-state Monte Carlo simulations of the measures of exposure (AUC and C_{max}) for tuberculosis-infected patients using World Health Organization recommended guidelines (World Health Organization, 2010a). The simulated results predicted decreased AUCs by 50% and 56% for isoniazid and rifampicin, respectively. Children in the lowest weight band (5.0-7.9 kg) had lower exposures than those in the reference adult population (Zvada et al., 2014). These simulation results showed that children within the lowest weight band were under-dosed. Furthermore, a dose of 50mg/kg pyrazinamide was found optimal for children weighing between 5.0 to 7.9 kg, as it would achieve the same AUC as in adults. Authors observed wide variability in

drug exposure when dosed according to World Health Organization programmatic weight bands (Zvada et al., 2014).

From the studies discussed above, it can be concluded that pharmacokinetic modelling and simulation play a role in the determination of effective dose or dosing interval for the target patient population. It can further be utilised as a tool for optimal individualised anti-tuberculosis drug dose determination and subsequent adjustments.

2.7.2 The need for terizidone dose optimisation

There is scanty information available in English language on the use of terizidone in tuberculosis besides no studies currently in the scientific literature have attempted to optimise terizidone dose in drug-resistant tuberculosis patients. Terizidone in a dose of 600-900 mg per day demonstrated a good level of tolerance in five dialysed tuberculosis patients (Galietti et al., 1991). Similarly, in the two studies of pulmonary tuberculosis patients with concomitant psycho-neurological impairments, tolerated well a dose of 250 mg three times daily (Shmelev, Shabalova & Kolosovskaia, 1975, (Kottász, Babics, 1972). While terizidone in the above studies was reported to be well tolerated at these doses, no plasma concentrations were measured to establish the corresponding tolerated plasma concentration range.

According to South African policy guidelines on the management of drug-resistance tuberculosis, patients weighing 33-70 kg take terizidone daily dose of 750 mg in both intensive and continuous phase (NDoH, 2013). In addition, the guideline does not state the target plasma concentration. Considering the patient weight-band of 33-70 kg and the daily dose of 750 mg, it is reasonably justified to speculate that patients within this weight-band do not achieve similar plasma concentrations of terizidone. Heavier patients are more likely to be under-dosed compared to the lighter ones.

The plasma concentration profile of terizidone at steady-state in drug-resistant tuberculosis patients has not been established. Hence, not only is there a need to optimise the dose but also establish effective terizidone exposure (C_{max} or AUC) in order to prevent resistance and improve treatment outcomes.

2.8 SUMMARY

There is only one study published in English language on pharmacokinetics of terizidone in drug sensitive tuberculosis. The pharmacokinetic parameters were not accurately estimated due to the use of a non-specific bioanalytical assay (outdated) and inappropriate pharmacokinetic model. Cycloserine is the secondary metabolite of terizidone. Nevertheless, no pharmacokinetic studies have established the pharmacokinetic relationship between terizidone and cycloserine as a secondary metabolite. The influence of HIV infection, anti-retroviral therapy and other anti-tuberculosis drug in the regimen on pharmacokinetics of terizidone in drug-resistant tuberculosis patients is not known. The drug-resistant tuberculosis develops as a result of low plasma drug exposure caused by several factors that account for pharmacokinetic variability of some drugs in the regimen. Terizidone daily dose of 750 mg for patients weighing 33-70 kg may not provide sufficient plasma concentrations especially in patients with higher body weight. Good treatment outcomes indicated by 2 or 3 months sputum culture conversion and other biomarkers are primarily driven by adequate plasma concentration of each drug in the regimen. Population pharmacokinetic modelling is the best method that can be used to establish pharmacokinetic parameter profile of terizidone and cycloserine as its metabolite at steady-state.

2.9 REFERENCE

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Chapter Three

3.0 INTRODUCTION

The overarching aim of this chapter is to describe the methods and various tools employed in order to perform the study objectives outlined in Chapter one. Briefly, the chapter describes the study design, site and states the ethical considerations of the study. Additionally, the criteria that were applied to either include or exclude the patients in the study are outlined. The procedure on how the blood samples from patients were obtained and processed has been highlighted. The analytical methods to determine the plasma concentrations of terizidone and cycloserine are briefly outlined as they have been described in Chapters four and five in detail, respectively. The information about the patients' demographic and clinical characteristics collected is provided. The various steps in process of pharmacokinetic modelling of terizidone and cycloserine concentration-time data, including the selection of the base, error and covariate models have been described. Finally, the chapter concludes by explaining the procedure on how the final pharmacokinetic model was validated and used in the simulation of current terizidone dose across three weight-bands.

3.1 STUDY DESIGN AND SITE

This prospective, non-randomised (non-intervention) clinical study was conducted at Brewelskloof Hospital in Worcester, Western Cape province of South Africa. Brewelskloof Hospital is one of the designated drug-resistant tuberculosis specialised centre in the province. It is a referral hospital to both public and private health institutions within the catchment area. The areas catered for by the hospital include Caledon, Grabouw, Touws River, De Doorns, Robertson, Montagu, Stellenbosch, Swellendam, Bredasdorp, Riviersonderend, Ceres, Kleinmond, Hermanus, Heidberg, Paarl, Matjiefontein and Klein Bay.

3.1.1 Ethics statement

The study protocol was approved by the University of the Western Cape ethics committee (Reference number: 07/6/12) and University of Cape Town ethics committee (Reference number: 777/2014). The study was conducted according to the principles stated in the declaration of Helsinki (General Assembly of the World Medical Association, 2014) and the guidelines of the National Department of Health of South Africa (Department of Health, 2006). The patients were identified by codes. Data collected was handled with strict confidentiality. Each patient signed informed consent form. Participation in the study was voluntary. Patients had the right to withdraw their participation at any stage of the study without consequences.

3.1.2 Study sample size

Thirty-nine (39) adult patients with drug-resistant tuberculosis participated in this study. Twenty-seven (27) of them were co-infected with human immunodeficiency virus (HIV). It was not possible to calculate the minimum number of patients and blood samples per patient for pharmacokinetic modelling, as there is no information in literature about the population pharmacokinetic model of terizidone.

3.2 PHARMACOKINETIC SAMPLING DESIGN: COLLECTION OF BLOOD SAMPLES

The full population pharmacokinetics sampling design was applied in order to get several

drug concentrations per patient at different times and to allow for precise estimation of pharmacokinetics parameters from the concentration-time data (USFDA, 1999). The blood sampling in patients co-infected with HIV was performed as per protocol of the main approved study. Blood samples from each patient were collected in heparinised tubes at 0h (baseline), 1(±0.5)h, 2(±0.5)h, 3(±0.5)h, 4(±0.5)h, 5h, 8h, 16h, and 24h after witnessing intake of terizidone and other second-line anti-tuberculosis drugs, including antiretroviral drugs for HIV-infected patients. The blood samples underwent centrifugation at 10000 rpm for 5 minutes in order to obtain plasma. Harvested plasma was appropriately labeled and stored in a central repository at -80ºC until drug analysis.

3.2.1 Determination of terizidone and cycloserine concentrations in plasma

The method to analyse terizidone in human plasma was developed and validated using high performance liquid chromatography coupled with ultra-violet detection (HPLC-UV).

The method was validated using United States Food and Drugs Administration guidelines for analytical method validation and International Conference on Harmonisation for validation of analytical procedures (USFDA, 2013, ICH, 2005). The detailed procedure of the method is presented in Chapter four. The ultra-performance liquid chromatography tandem mass spectrometry method was developed and validated for determination of cycloserine in human plasma. The method was validated according to the international guidelines (ICH, 2005, USFDA, 2013) and details are presented in Chapter five.

3.3 COLLECTION OF CLINICAL DATA

3.3.1 Demographic data

Demographic data such as age, body weight, height and gender were obtained from each patient on the day of blood sampling. Body mass index (BMI) was calculated using height and body weight (Rolland-Cachera et al., 1991). Free fat mass was calculated for each gender using BMI and body weight (Janmahasatian et al., 2005).

3.3.2 Medical and treatment history

Medical history regarding HIV status and comorbidities was obtained from the patients' medical records. The dose of terizidone and the current anti-tuberculosis drug regimen were documented at the time of pharmacokinetic blood sampling. The antiretroviral treatment regimen, current anti-tuberculosis regimen and concurrent medications were recorded from the patients' medical records.

3.3.3 Liver function tests

An extra 5 mL of blood was collected from each patient for the determination of alanine aminotransferase (ALT), gamma-glutamyl transferase (GGT) and aspartate aminotransferase (AST). Other liver function markers such as albumin, unconjugated bilirubin, conjugated bilirubin and total bilirubin were analysed as well.

3.3.4 Kidney function tests

Creatinine clearance (Cockcroft, Gault, 1976) and estimated glomerular filtration rate (Levey et al., 2007) were calculated using serum creatinine concentration from each patient.

3.3.5 Inclusion criteria

The inclusion criteria in this study were the following:

1. Male and female with confirmed drug-resistant tuberculosis with or without HIV infection,
2. Age ranging from 17 to 65 years,
3. Intensive phase treatment of drug-resistant tuberculosis for at least two weeks,
4. Being treated with second-line anti-tuberculosis drugs (kanamycin or amikacin, moxifloxacin, ethionamide, terizidone, ethambutol, isoniazid and pyrazinamide),
5. Informed signed consent form.

3.3.6 Exclusion criteria

If the patients had the following characteristics, they were excluded from the study:

1. Paediatric patients,
2. Pregnant patients,
3. Patients with unstable haemodynamics,
4. Extensively drug-resistant tuberculosis,
5. Absence of informed signed consent form.

3.4 DETERMINATION OF PHARMACOKINETIC PARAMETERS

The population pharmacokinetics analysis approach was employed (Sun et al., 1999). The population pharmacokinetic model consists of three sub models namely; (1) structural model (which describes the overall pattern of data using fixed effects parameters), (2) statistical or stochastic model (which accounts for variability by using a hierarchy of random effects e.g. inter-individual variability, inter-occasion variability and residual variability) and (3) covariate model (which expresses relationships

between model parameters and covariates). The population pharmacokinetic model is the set of models for individual observation and generally represented as equation (1):

$$\boldsymbol{y_{ij}} = \boldsymbol{f_{ij}}\left(\boldsymbol{\Phi_j}, \boldsymbol{x_{ij}}\right) + \boldsymbol{\varepsilon_{ij}} \qquad (\mathbf{1})$$

Where $\boldsymbol{y_{ij}}$ is the vector of observed data like drug plasma concentrations at respective time points $\boldsymbol{x_{ij}}$ of the jth subject. $\boldsymbol{f_{ij}}$ is the function for the prediction of the ith response in the jth subject. $\boldsymbol{\Phi_j}$ represents the vector of estimable pharmacokinetic parameters of the jth subject and finally $\boldsymbol{\varepsilon_{ij}}$ is the ith measurement error in the jth subject. Furthermore $\boldsymbol{\Phi_j}$ is represented as equation (2):

$$\boldsymbol{\Phi_j} = \boldsymbol{g}\left(\boldsymbol{\theta}, \boldsymbol{z_j}\right) + \boldsymbol{\eta_j} \qquad (\mathbf{2})$$

Where $\boldsymbol{g}$ is a function describing the expected value of $\boldsymbol{\Phi_j}$ as a function of known individual specific covariates $\boldsymbol{z_j}$ and the vector of population parameters $\boldsymbol{\theta}$. $\boldsymbol{\eta_j}$ represents the random variation of individual parameters around the (mean) population prediction (Ette, Williams, 2004).

The concentration-time data for terizidone and cycloserine was analysed by employing the non-linear mixed-effects (NLME) modelling implemented in Monolix 2018R1 software (Lixoft, 2018). The software implements a stochastic approximation of the standard expectation maximization (SAEM) algorithm (Kuhn, Lavielle, 2005) for NLME models without approximations. The primary pharmacokinetic parameters such as absorption rate constant (ka), apparent volume of distribution (V/F) and apparent clearance (Cl/F) were estimated.

The secondary pharmacokinetic parameters such as area under the curve ($AUC_{0\text{-}24}$) and half-life were estimated using the final validated model without covariates. The Monolix MlxTran model file used to execute the estimation of secondary pharmacokinetic parameters is included in this thesis as Appendix 1. The values for the maximum concentration (C_{max}) and the time at which concentration is at C_{max} (T_{max}) were obtained from the file output of the individual predicted concentrations.

3.5 PHARMACOKINETIC MODEL BUILDING STRATEGY

3.5.1 Selection of the base and error model

The data set was explored using DatXplore interface in Monolix and relationships among the covariates were established. Selection of the pharmacokinetic structural or base model was aided by graphical exploration of concentration-time data for terizidone and cycloserine using ggplot2 package in R statistical software (R Core Team, 2018) in addition to DatXplore. The overall pattern of the time versus log-transformed concentration of terizidone and cycloserine profile determined the pharmacokinetic structural model to be initially selected. The concentration-time data for terizidone and cycloserine were modelled simultaneously using Monolix. One-compartment model was selected initially with constant or additive error model ($\boldsymbol{y} = \boldsymbol{f} + \boldsymbol{a}\,\boldsymbol{\varepsilon}$) without inclusion of covariates. Several error models such as proportional ($\boldsymbol{y} = \boldsymbol{f} + \boldsymbol{b}\,\boldsymbol{f}\,\boldsymbol{\varepsilon}$), combined1 ($\boldsymbol{y} = \boldsymbol{f} + (\boldsymbol{a} + \boldsymbol{b}\,\boldsymbol{f})\,\boldsymbol{\varepsilon}$), combined2, ($\boldsymbol{y} = \boldsymbol{f} + \sqrt{(\boldsymbol{a^2} + \boldsymbol{b^2}\,\boldsymbol{f^2}\,\boldsymbol{\varepsilon}}$)), and exponential ($\mathbf{log}(\boldsymbol{y}) = \mathbf{log}(\boldsymbol{f}) + \boldsymbol{a}\,\boldsymbol{\varepsilon}$) were tried as well iteratively in order to get the best error model. The error model associated with the lowest Objective Function Value (OFV) or Bayesian Information Criterion–BIC was selected (Schwarz, 1978). The two-compartment model was tried with several error models as for the one-compartment model.

After performing several runs, the selection of the best base model (structural) was based on visual inspection of individual fit plots as well as observed versus individual predicted concentration (IPRED) plots.

Selection of best error model was based on goodness-of-fit plots that included individual weighted residuals (IWRES) versus time plot and a plot of absolute values of IWRES (|IWRES|) versus IPRED. A lack of visible trend in both plots was the criteria for the appropriate error model. Additionally, the inspection of parameter distribution (Empirical Bayes Estimates–EBEs) plots of the simulated rather than the empirical distribution of the estimated individual parameters (Lavielle, Ribba, 2016) was performed. At every modelling stage, the structural and error models were assessed for any mis-specification.

3.5.2 Random effects

The distribution of standardised random effects associated with each estimated pharmacokinetic parameter (fixed effects) were assessed for normality by visual inspection of boxplots generated in Monolix. The model for random effects was thus accepted if the boxplots were in agreement or did not deviate from the expected standard normal distribution.

3.5.3 Covariate modelling

The covariate modelling was performed in a stepwise manner (Mandema, Verotta & Sheiner, 1992) and iteratively. The basic population pharmacokinetic model without any covariates, developed from the above described procedure was the starting point in the covariate modelling. Allometric scaling of all disposition parameters was applied in order to adjust for the expected effect of body size (Anderson, Holford, 2009) using body size descriptors like free fat mass (Janmahasatian et al., 2005), total body weight and body mass index. The correlations among the covariates were analysed in order to identify the possibility of collinearity. Pharmacological or biological plausible covariates were selected for covariate model building. In the case of collinearity, only one of the correlated covariates was included in the model.

3.5.4 Screening for covariate effects

Selection of covariate significance (pharmacologically or biologically plausible) was achieved through graphical observation of correlation trends between random effects of individual predicted pharmacokinetic parameters and continuous covariates in Monolix interface. In the case of categorical covariates, the boxplots of random effects of individual predicted pharmacokinetic parameters were assessed for any visible differences in the medians as the criteria for inclusion in the covariate model. Therefore, any visible trend between random effects and individual predicted parameters indicated eligibility of a covariate for inclusion in the covariate model.

Covariate modelling included the determination of relationship between various covariates (patient demographic variables, HIV status, liver and renal function markers and co-morbidities) and fixed parameters using Wald test integrated in Monolix. All continuous covariates were centered around the median value (typical median value).

The covariates that met the inclusion criteria as stated above were tested one at a time in a stepwise forward inclusion manner. A p-value of ≤ 0.05 (Wald test) was considered significant provided the OFV ($-2 * loglikelihood$) decreased by at least 3.84 points (goodness-of-fit criteria). After inclusion of all the significant covariates (full model), a backward deletion step was performed at the significance level of ≤ 0.01 provided there was a decrease in OFV by at least 6.63 points. The final population pharmacokinetic model (reduced model) consisted of relevant covariates of interest that were evaluated and retained.

3.5.5 Model diagnosis

The final population pharmacokinetic model was subjected to four types of model diagnoses in order to evaluate its validity. These were individual parameter estimates based diagnosis, residual type diagnosis, simulation based diagnosis and numerical diagnosis (Karlsson, Savic, 2007).

The individual parameter estimates based diagnosis involved the plot of the distribution of individual predicted parameters and the shrinkage. The low value of shrinkage and approximate normal distribution observed through histograms and QQ-plots of the EBEs was desirable. The other diagnostic test performed was plot of observed concentrations versus predicted concentrations (IPRED) in order to test if there was agreement.

Residual type diagnosis involved the plot of IWRES and time in order to identify any trend in the IWRES over time (independent variable). Model misspecification was assumed if there was a clear trend in the IWRES.

Simulation based model diagnosis involved the visual predictive checks (VPC) (Wang, Zhang, 2012). The VPC evaluated the predictability of final pharmacokinetic model by making graphical comparison between the multiple model-simulated predictions at each time point from the data set and observed data. The simulation of the single data set was also performed and compared with the observed data set (Karlsson et al., 1998).

Numerical diagnostics involved comparison of the OFV ($\boldsymbol{-2 * loglikelihood}$) between nested models or comparison of BIC between non-nested models. The

smaller the OFV or BIC value the better was the model. Model over fit was also assessed by the magnitude of relative standard errors of the pharmacokinetic parameters (Karlsson, Savic, 2007). High values indicated possible over fit. Finally, a bootstrap procedure was performed in Monolix aided by Rsmlx R package (Lavielle, Chauvin & Tran, 2018) in order to assess the precision of the parameters and robustness of the proposed joint population pharmacokinetic model.

3.6 Monte Carlo simulation

The final joint population pharmacokinetic model was used to simulate terizidone and cycloserine exposure ($AUC_{0\text{-}24}$ and C_{max}) at 750 mg terizidone daily dose across the three weight bands, 33 – 50, 51 – 70 and > 70 kg. This was done in order to assess whether similar exposure was achieved across these weight bands. In patients weighing 51 – 70 and > 70 kg, dosage optimisations were performed to achieve exposures similar to those in 33 – 50 kg weight band and taking 750 mg of terizidone daily.

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Chapter Four

In this chapter, a research paper entitled:

"Analysis of Terizidone in Plasma using HPLC-UV method and its Application in a Pharmacokinetic Study of Patients with Drug-resistant Tuberculosis",

is presented. It was published as a research article in the *Biomedical Chromatography*, 2018, 32(11): e4325. https://doi.org/10.1002/bmc.4325. It has been re-used in this thesis with permission from the publisher (John Wiley & Sons, Inc. license number 4476471000780). The Copyright license agreement is included as Appendix 2.

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RESEARCH ARTICLE

WILEY Biomedical Chromatography

Analysis of terizidone in plasma using HPLC-UV method and its application in a pharmacokinetic study of patients with drug-resistant tuberculosis

Mwila Mulubwa | Pierre Mugabo

School of Pharmacy, University of the Western Cape, Bellville, Cape Town, South Africa

Correspondence
Mwila Mulubwa, University of the Western Cape, Private bag X17, Bellville 7535, Cape Town, South Africa.
Email: 3579753@myuwc.ac.za; mwila.mulubwa@gmail.com

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Abstract

A chromatographic method has been developed and validated for the first time for analysis of terizidone in plasma. Terizidone was extracted from plasma by protein precipitation using a mixture of acetonitrile and methanol (1:1, v/v). The chromatographic separation was achieved with a gradient of acetonitrile and water both containing 0.1% formic acid on a Supelco Discovery® HS C_{18} (150 × 4.6 mm, 5 µm) reversed-phase column. Propranolol was used as the internal standard. The total run-time was 18 min. The calibration standard concentrations ranged between 3.125 and 200 µg/mL and calibration curves were linear with coefficient of determination values in the range of 0.9988–0.9999. The inter- and intra-day assay precision (percentage relative standard error) was <15% while mean accuracy was 107%. The mean extraction efficiencies of terizidone and IS were 76 and 89%, respectively. The validation results demonstrated that the method was selective and sensitive, and that terizidone was stable under the studied conditions. The method was successfully applied in a population pharmacokinetic study. The mean plasma concentration of terizidone in patients at all sampling time points was 51.8 ± 28 µg/mL. The method was simple, cheap and hence suitable for therapeutic drug monitoring of terizidone.

KEYWORDS

HPLC-UV, multidrug-resistant tuberculosis, plasma, Terizidone

1 | INTRODUCTION

Tuberculosis remains a major world health problem and ranks as the ninth leading cause of death in the world (WHO, 2017). If not properly managed, drug-resistant tuberculosis (DR-TB) emerges as a complication of tuberculosis (Zumla et al., 2012). Consequently, DR-TB necessitates the use of a second-line anti-tuberculosis drugs regimen, which usually contains five to seven drugs (WHO, 2014).

Terizidone is one of the second-line drugs used in both intensive and continuous phases of DR-TB treatment in South Africa (NDoH, 2013). It is made up of two molecules of cycloserine that are joined by a molecule of terephtalaldehyde and reported to have fewer adverse effects than cycloserine (Bartmann et al., 2013; Kottász & Babics, 1972; Vora, 2010). For DR-TB treatment to be successful, the plasma concentration of each drug in the regimen must reach the effective threshold necessary to kill the bacteria. In fact, studies have confirmed that variability in the plasma exposure of anti-tuberculosis drugs is one of the main causes of emergence of DR-TB or poor treatment outcomes (Chideya et al., 2009; Pasipanodya, Srivastava, & Gumbo, 2012; Srivastava, Pasipanodya, Meek, Leff, & Gumbo, 2011). Adequate plasma drug exposure and an optimal regimen not only prevent the emergence of DR-TB but also promote early desired treatment outcomes (Pasipanodya et al., 2013; Yuen et al., 2015). Therefore, therapeutic drug monitoring, which involves the measuring of plasma concentration of drugs at specific times after drug administration, is of utmost importance (Alsultan & Peloquin, 2014). Furthermore, the knowledge of the pharmacokinetic properties of drugs used in DR-TB aid in determining appropriate doses should drug

plasma concentrations be found to be lower or higher than the expected threshold (Reynolds & Heysell, 2014).

Terizidone has scarce information about its pharmacokinetics in the literature (WHO, 2015). In one pharmacokinetic study (Zitkova & Toušek, 1974), terizidone plasma concentration was determined using the calorimetric method (Jones, 1956), which is neither specific nor currently used in determination of plasma drug concentrations. However, literature from the World Health Organization (WHO, 2015) states that terizidone is not measurable in plasma and thus is thought to undergo complete pre-systemic hydrolysis in the gut wall and liver.

The objective of the current study was to develop and validate the first chromatographic method to determine terizidone concentrations in plasma and verify its presence in patient plasma samples for a population pharmacokinetic study.

2 | EXPERIMENTAL

2.1 | Chemicals and reagents

Terizidone powder, analytical grade (catalog no. T115500, Figure 1), was obtained from Toronto Research Chemicals, Canada. Propranolol powder (lot no. 29H4016), used as the internal standard (IS), was purchased from Sigma-Aldrich, Germany. HPLC-grade acetonitrile and methanol were sourced from Sigma-Aldrich and Merck (South Africa), respectively. Dimethylsulfoxide (DMSO) and formic acid were sourced from Merck, South Africa. Ten other drugs (ethionamide, ethambutol, kanamycin, isoniazid, moxifloxacin, stavudine, lamivudine, tenofovir, pyrazinamide and efavirenz) that are usually co-administered with terizidone were obtained from Western Cape Provincial Pharmaceutical Services (South Africa). Cycloserine powder (CAS no. 68-41-7) was purchased from Sigma-Aldrich, Germany. The HPLC-grade water (18 mΩ) was obtained from a Direct-Q3 (Mmillipore) water purification system. Drug-free pooled plasma was purchased from Sigma-Aldrich.

2.2 | Instrumentation

The analysis of terizidone was performed on a Waters® 1525 HPLC system (Milford, MA, USA) equipped with binary pumps with maximum operating pressure of 6000 psi and a programmable range of flow-rate between 0.00 to 10.00 mL/min in 0.01 mL increments. The system comprised an integral vacuum degasser for removal of dissolved gases from the mobile phase. Linked to the system was a Waters® 717 plus autosampler and Waters® 2487 (dual absorbance) UV detector, which was set at a sensitivity of 2.0000 AUFS. The handling of data was managed by Empower pro software.

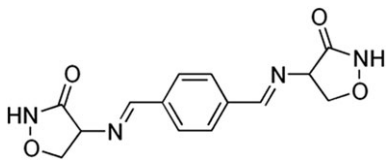

FIGURE 1 Chemical structure of terizidone

2.3 | Chromatographic and analytical conditions

The chromatographic separation of terizidone and IS was achieved using a reversed-phase Supelco Discovery® HS C_{18} column (150 × 4.6 mm, 5 µm). The mobile phase used was water containing 0.1% formic acid (A) and acetonitrile containing 0.1% formic acid (B). Gradient elution was carried out using the steps shown in Table 1. Terizidone and IS were both monitored with the Waters® 2487 UV detector set at 260 nM. The sample injection volume was 20 µL. The HPLC analysis was performed at a flow rate and temperature, 1.2 mL/min and 20°C, respectively.

2.4 | Preparation of stock solutions, calibration standards and quality controls

Stock solution of terizidone at 2 mg/mL (2000 µg/mL) was prepared by dissolving accurately weighed 20.0 mg of terizidone powder in 2 mL of DMSO and made up to 10 mL mark with methanol in a 10 mL volumetric flask. Stock quality control (QC) solution of terizidone was prepared at concentration 1.5 mg/mL (1500 µg/mL) by dissolving 15.0 mg of terizidone powder in 2 mL of DMSO and made up to 10 mL with methanol in a 10 mL volumetric flask. The IS stock solution was made at 0.4 mg/mL (400 µg/mL) by dissolving 20.0 mg of propranolol in methanol using a 50 mL volumetric flask. These solutions were kept at −20°C.

Seven standards for the calibration curve were prepared in the range 3.125–200 µg/mL by spiking 0.3 mL of terizidone stock solution in 2.7 mL of drug-free plasma and diluting serially with drug-free plasma. Drug-free plasma (1.8 mL) was spiked with 0.2 mL of terizidone QC stock solution and appropriately diluted with drug-free plasma. The QC samples, low (LQC), medium (MQC) and high (HQC), were thus prepared at 10, 37.5 and 150 µg/mL, respectively.

2.5 | Plasma sample treatment

The IS stock solution (in methanol) was diluted with equal volume of acetonitrile to 200 µg/mL. To every 200 µL of spiked plasma (calibration standards and QC samples), 800 µL of IS solution (200 µg/mL) was added and vortex mixed for 1 min. The protein precipitate was then separated through centrifugation (10,000 rpm) at 2°C for 10 min. The supernatant (800 µL) was pipetted into a clean vial and 20 µL was injected onto HPLC system.

TABLE 1 Gradient elution steps

Time	Flow rate (mL/min)	A (%)	B (%)
0.0	1.2	90.0	10.0
1.0	1.2	90.0	10.0
10.0	1.2	0.0	100.0
13.0	1.2	0.0	100.0
13.1	1.2	90.0	10.0
18.0	1.2	90.0	10.0

2.6 | Method validation

The validation of the method was performed according to the international guidelines (ICH, 2005; USFDA, 2013) for quantification of the limit of detection (LOD), lower limit of quantification (LLOQ) or sensitivity, selectivity, linearity, accuracy, precision, carry-over, recovery and stability.

2.7 | Analysis of patients' plasma samples

Patients' plasma samples kept at −80°C were left to thaw at room temperature. To each 200 µL of patient plasma, 800 µL of IS was added. The rest of the sample preparation process was the same as for the calibration standards. The analytical run consisted of blank sample (processed plasma without IS and terizidone), zero blank (processed plasma with IS only), calibration standards, patient samples and QC samples.

3 | RESULTS AND DISCUSSION

3.1 | Optimization of chromatographic conditions

The use of a mixture of methanol and acetonitrile (1:1, v/v) as precipitating solvent improved the extraction of the analyte and IS from the plasma. Other ISs tested apart from propranolol were acyclovir and reserpine. The separation of the analyte and IS was tried on three different columns, namely a Kinetex XB C_{18} (150 × 2.1 mm, 2.6 µm), a Zorbax Eclipse XDB C_8 (150 × 4.6 mm, 5 µm) and a Luna C_8 (250 × 4.6 mm, 5 µm). The organic mobile phases tested were methanol and acetonitrile. A buffer solution of octane sodium sulfonic acid and sodium bicarbonate at pH of 8 and 9 was tried too. The optimization objective was to achieve well-separated sharp peaks of both analyte and IS as well as a short run-time. Isocratic separation was tried first followed by gradient elution. In all of the chromatographic trial runs,

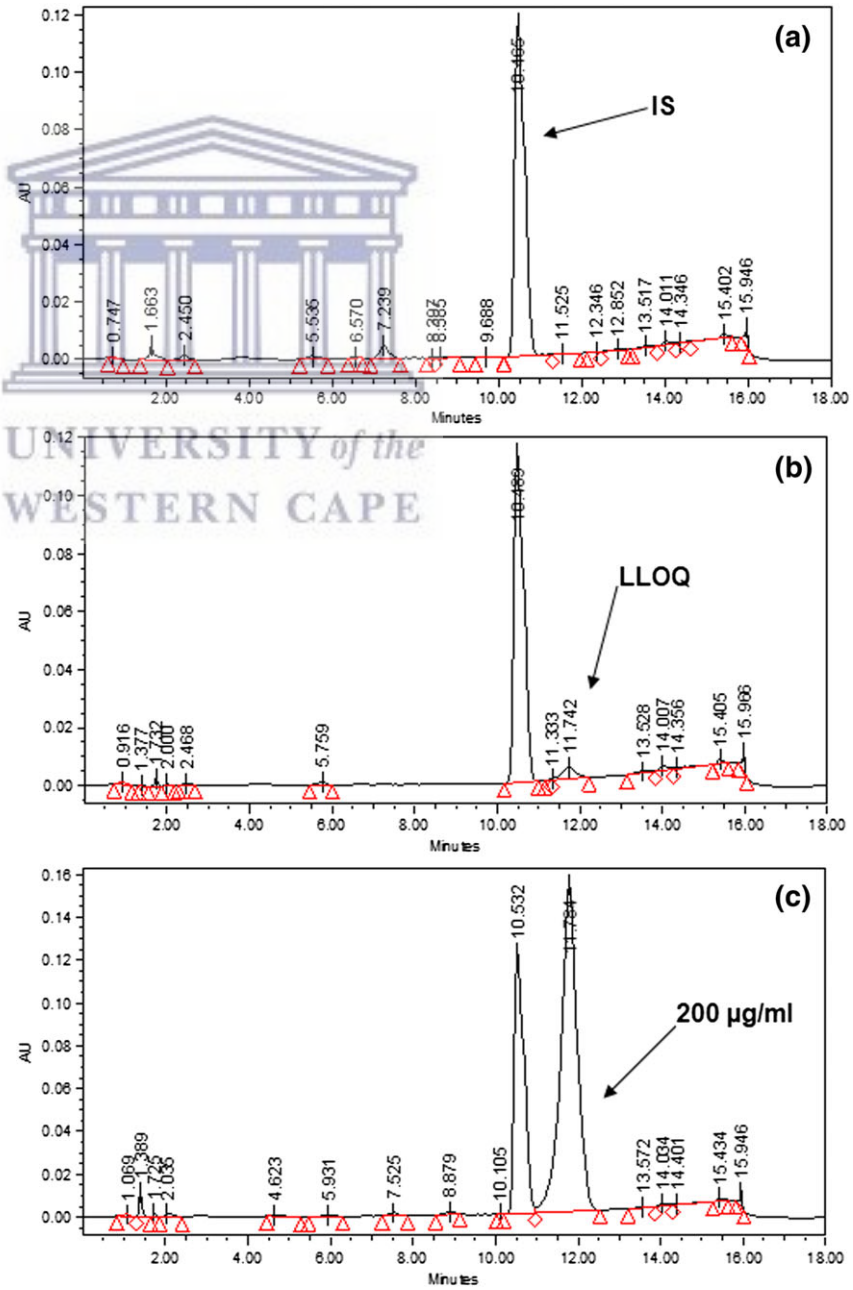

FIGURE 2 Chromatograms of IS (a), LLOQ (b) and highest calibration standard (c)

isocratic elution was found to be unsuitable. Hence, more time was spent in determining the best gradient steps at different flow rates.

Terizidone's chemical structure comprises two cycloserine structures joined by terephthalaldehyde moiety (Figure 1). The terephthalaldehyde moiety renders terizidone less polar than cycloserine. Hence, terizidone was retained more on a high-surface-area (HS C_{18}) column than cycloserine. Increasing the water content (polar mobile phase) in the first step of the gradient insured the repulsion of tezidone from the mobile phase and onto the nonpolar stationary phase (HS C_{18}), leading to a longer retention time, while increasing the organic mobile phase (acetonitrile) in the second step of the gradient speeded up the elution of terizidone. Acetonitrile was preferred to methanol because it has a higher elution strength than methanol on C_{18} columns.

The mobile phase consisting of acetonitrile and water with 0.1% formic acid generally produced good peaks of both IS and analyte. Addition of formic acid to acetonitrile (0.1%) produced sharper peaks than acetonitrile without formic acid. Therefore, the mobile phases found to be optimal were 0.1% formic acid in acetonitrile and 0.1% formic acid in water with gradient elution steps and flow rate as shown in Table 1. The column, an HS C_{18} column (150 × 4.6 mm, 5 µm) best separated the IS and analyte with a run-time of 18 min, as shown in Figure 2. The LC/MS method was initially tried but terizidone had problems with ionization. Therefore, it was difficult to detect its ion.

3.2 | Method validation

3.2.1 | Selectivity

Selectivity of the analytical method was tested by assessing possible interference by co-administered drugs and cycloserine (analyte metabolite). The drugs—ethionamide, ethambutol, kanamycin, isoniazid, moxifloxacin, stavudine, lamivudine, tenofovir, pyrazinamide, efavirenz and cycloserine—were each and in combination tested at 10 µg/mL. These drugs did not show any interference or co-elution at the retention times of the IS (propranolol) and the analyte (terizidone). The method was therefore able to differentiate the analyte and IS from co-administered drugs. The retention time for each drug is shown in Table 2. The interference from endogenous compounds was not assessed as pooled plasma was used. However, 5-fold dilution of plasma with IS solution (200 µL in 1000 µL) from the sample preparation procedure also resulted in dilution of the endogenous compounds. Hence, the interference of the endogenous compounds was assumed to be negligible.

3.2.2 | Sensitivity, LOD and linearity

The LLOQ or sensitivity was the lowest concentration of the calibration standards that was reliably quantified with accuracy within 20% of the nominal concentration and precision of ≤20% of the relative standard deviation (RSD) of the back-calculated concentration (USFDA, 2013). Each calibration standard was injected six times. The concentration of 3.125 µg/mL met the criterion for the LLOQ as it had an accuracy of 118.9%, which was within 20% of the nominal concentration with an RSD of 9.43%. Half of the concentration of the LLOQ (1.562 µg/mL) could not meet the criteria as it had an accuracy of 158% and an RSD of 25.1%, and hence was excluded from the calibration curve.

TABLE 2 Retention time for analyte, IS and other drugs

Drugs	Retention time (min)
Cycloserine	0.21*
Ethionamide	4.80
Ethambutol	1.43
Efavirenz	3.62
Isoniazid	1.55
Lamivudine	3.23
Kanamycin	1.90
Moxifloxacin	6.94
Tenofovir	8.06
Stavudine	5.87
Pyrazinamide	4.18
Propranolol (IS)	10.53 ± 0.024
Terizidone (analyte)	11.62 ± 0.029

*Cycloserine peak was in the void and very small.

The LOD was determined by visual evaluation of the analyte peak (ICH, 2005). The LLOQ sample was serially diluted with plasma to three concentrations levels: 1.56, 0.78 and 0.39 µg/mL. The response (peak) corresponding to the concentration of 0.78 µg/mL was reliably clear and hence considered as the LOD.

The calibration curve was constructed from the plot of chromatogram peak area ratios of the analyte and the IS as a linear function of the analyte nominal concentration. The average coefficient of determination (r^2) obtained from six replicate analytical runs of the seven calibration standards was 0.9997. The average of the residual sum of squares was 0.001. Table 3 shows the summary of linearity parameters. Therefore, the curve was linear over the concentration range 3.125–200 µg/mL.

3.2.3 | Precision and accuracy

The accuracy of the analytical method was determined by replicate analysis of LLOQ, LQC, MQC and HQC. The results are presented in Table 4. Accuracy was expressed as the percentage ratio of the mean back-calculated concentration and nominal concentration. It ranged between 99.7 and 118.9% for both within- and between-run accuracy and was within the acceptable limits (USFDA, 2013). Precision, expressed as percentage relative standard deviation (RSD), was determined from the replicate analysis of LLOQ, LQC, MQC and HQC within an analytical run (on one day) and between analytical runs (on

TABLE 3 Parameters describing the linearity of the calibration curve

Run number	r^2	Equation	Residual sum of squares
1	0.9999	$y = 0.0103x - 0.026$	0.00029
2	0.9998	$y = 0.011x - 0.0115$	0.00058
3	0.9999	$y = 0.0112x - 0.0074$	0.00019
4	0.9996	$y = 0.0107x - 0.0223$	0.00121
5	0.9999	$y = 0.0102x - 0.0192$	0.00034
6	0.9988	$y = 0.0104x - 0.0032$	0.00353

TABLE 4 Within-run and between-run precision and accuracy of the analytical assay for LLOQ, LQC, MQC and HQC

Nominal concentration (μg/mL)	Mean concentration, ± SD (μg/mL)	RSD (precision, %)	Accuracy (%)
Within-run analysis			
3.125[a]	3.7 ± 0.35	9.4	118.9
10[b]	11.3 ± 0.33	2.97	112.7
37.5[b]	38.1 ± 0.19	0.50	101.6
150[b]	149.6 ± 0.53	0.35	99.7
Between-run analysis			
3.125[b]	3.4 ± 0.58	17.35	107.2
10[b]	10.7 ± 0.73	6.79	107.4
37.5[b]	38.8 ± 0.98	2.52	103.5
150[b]	150.8 ± 2.23	1.48	100.5

[a]Six replicates.

[b]Five replicates.

SD, Standard deviation.

different days). As shown in Table 4, the within-run precision ranged between 0.35 and 9.4% while between-run precision ranged between 1.48 and 17.35%, which was within acceptable limits (USFDA, 2013).

3.2.4 | Carry-over and recovery

Carry-over was assessed by injecting a blank sample (without IS and analyte) after the highest concentration sample (200 μg/mL) of the calibration standard. There was no noticeable peak at the retention time for the analyte or IS. Hence, there was no carry-over observed. Recovery or extraction efficiency was evaluated by comparing the instrument response (peak area) of the analyte extracted from the plasma and peak area of the analyte prepared in the solvent (USFDA, 2013). The recoveries of the analyte at 3.125 and 200 μg/mL were 66.7–70.% and 79.8–80.2%, respectively. The overall mean extraction for the analyte was 75.6%. The extraction efficiency of the IS was 80.6–94.3% with a mean of 88.5%.

3.2.5 | Stability

The analyte stability assessment was carried out according to the situations likely to be encountered during patient plasma sample preparation and analysis (USFDA, 2013). These situations were the maximum number of freeze–thaw cycles and the maximum time the

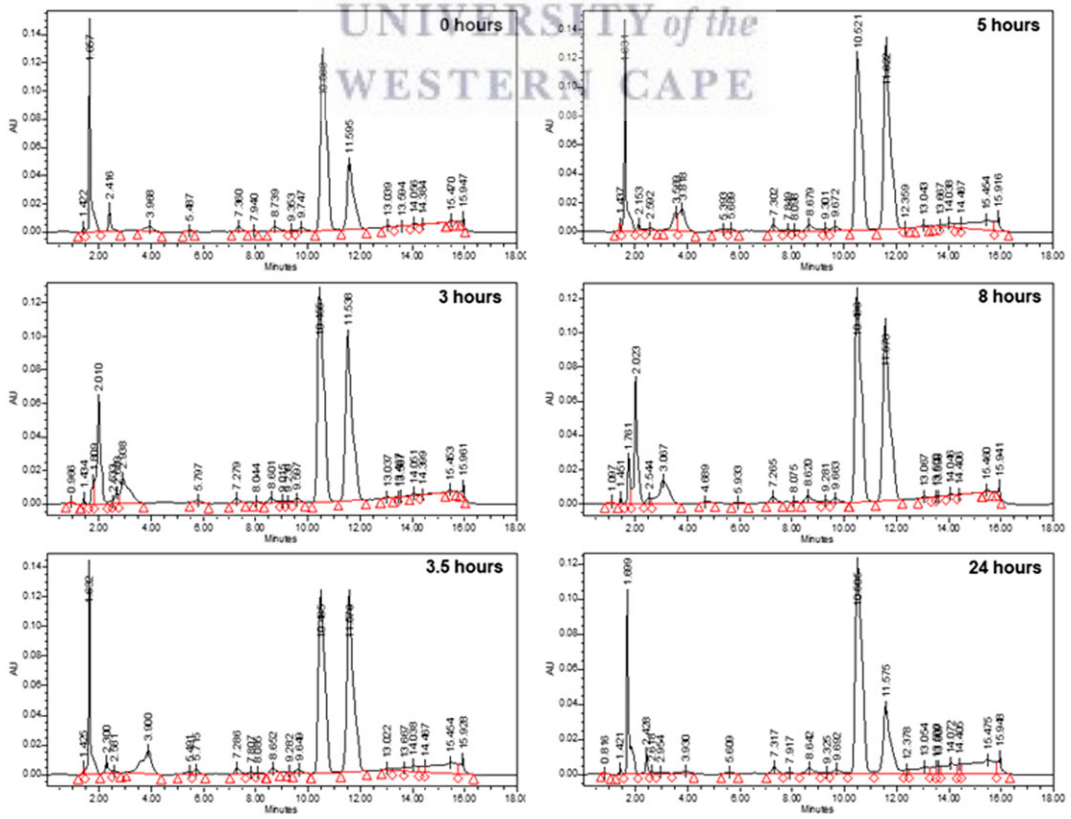

FIGURE 3 Chromatograms of terizidone from a patient at six sampling time points (0–24 h)

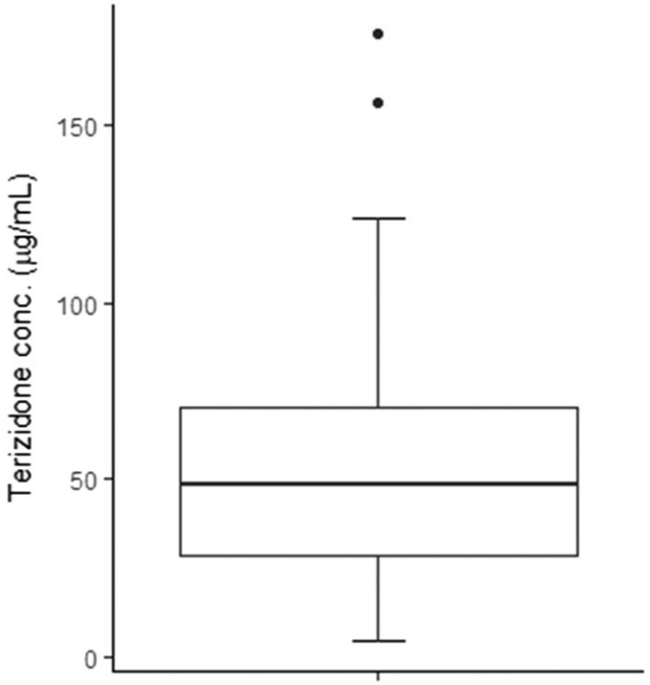

FIGURE 4 Boxplot of terizidone concentrations in patients with drug-resistant tuberculosis

samples remained in the autosampler at 20°C for each batch analysis. After three freeze–thaw cycles, the concentrations of the HQC and LQC samples dropped by 0.37% (0.74 µg/mL) and 1.9% (0.12 µg/mL), respectively. However, the concentrations of HQC and LQC samples after 24 h in the autosampler did not change. Therefore, the analyte was stable in all circumstances where patient plasma samples were prepared and analyzed.

3.3 | Method application in a pharmacokinetic study

The method developed was used to determine the concentration of terizidone in patient plasma samples for a population pharmacokinetic study. The study involved 78 DR-TB patients who were undergoing an intensive phase of treatment for at least 2 weeks (steady state). A total of 608 plasma samples were analyzed but terizidone was only found in 272 samples from 39 patients. There was no chromatographic peak seen at terizidone retention time for all samples that did not have terizidone. Figure 3 depicts a typical example of terizidone chromatograms from a patient with blood sampled at baseline (0), 3, 3.5, 5, 8 and 24 h. The median and mean concentrations from the 39 patients were 49.3 (4.3–176.1) and 51.8 ± 28.1 µg/mL, respectively. The summary distribution of the concentrations is shown in Figure 4. To our knowledge, this is the first chromatographic method developed to analyze terizidone in plasma.

4 | CONCLUSION

The HPLC-UV method was hereby developed and validated, and for the first time, successfully applied to determine plasma concentration of terizidone in patients with DR-TB. The method is simple and does not require expensive instrumentation or reagents. The validation results demonstrate unequivocally that the method is selective, sensitive, linear, accurate, precise and stable. The method is suitable for therapeutic drug monitoring of terizidone in DR-TB patients, unlike monitoring of its metabolite (cycloserine). As the literature on terizidone dose optimization is scarce, the current method is appropriate for pharmacokinetic studies in order to establish optimal doses.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

ORCID

Mwila Mulubwa http://orcid.org/0000-0003-2404-9717

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Chapter Five

Sensitive Ultra-performance Liquid Chromatography Tandem Mass Spectrometry Method for Determination of Cycloserine in Plasma for a Pharmacokinetics Study

This is a pre-copyedited, author-produced version of an article accepted for publication in *Journal of Chromatographic Science* following peer review. The version of record "*Sensitive ultra-performance liquid chromatography tandem mass spectrometry method for determination of cycloserine in plasma for a pharmacokinetics study"* is available online at https://academic.oup.com/chromsci/article/57/6/560/5423659, https://doi.org/10.1093/chromsci/bmz028. The Copyright license agreement is included as Appendix 3.

ABSTRACT

A simple and sensitive ultra-performance liquid chromatography tandem mass spectrometry method has been developed and validated for the analysis of cycloserine in patients' plasma. Using methanol, cyloserine and propranolol (internal standard–IS) was extracted from plasma by protein precipitation procedure. The chromatographic separation was successfully achieved on Phenomenex Kinetex™ PFP C_{18} (2.1 mm x 100 mm, 2.6 µm) reversed-phase column. Acidified with 0.1 % formic acid, water and acetonitrile were used as mobile phases for gradient elution. Cycloserine and IS were detected by Xevo® TQ MS triple quadrupole tandem mass spectrometer. The transition of protonated precursor to product ion were monitored at 103→75 *m/z* and 260.2→183 *m/z* for cycloserine and IS, respectively. The lower limit of quantification was 0.01 µg/mL. The method was linear over the concentration range 0.01–50 µg/mL with average coefficient of determination of 0.9994. The within-run and between-run precision and accuracy were in the range 3.7–19.3% (RSD) and 98.7–117.3%, respectively. Processed cycloserine sample was stable for 48 hours at 8 ºC and after three freeze-thaw cycles. The extraction efficiency ranged between 88.7 and 91.2%. The method was successfully applied in a pharmacokinetic study for the determination of cycloserine in plasma of patients with drug-resistant tuberculosis.

Keywords: Cycloserine, UPLC-MS/MS, plasma, drug-resistant tuberculosis.

5.0 INTRODUCTION

Cycloserine is a structural analogue of D-alanine, a broad-spectrum antibiotic, naturally produced from *Streptomyces lavendulae* and *Streptomyces garyphalus* (Batson et al., 2017). It is clinically used as the second-line drug for treatment of multidrug-resistant tuberculosis (World Health Organization, 2014). Cycloserine exerts bacteriostatic (Di Perri and Bonora, 2004) action by preventing the biosynthesis of peptidoglycan through inhibition of D-alanine:D-alanine ligase and alanine racemase of the *Mycobacterium tuberculosis* (Prosser and de Carvalho, 2013).

Cycloserine is water soluble and absorbed faster in healthy individuals than tuberculosis patients (Chang et al., 2017, Zhu et al., 2001). It distributes widely in body fluids and tissues. Primarily the kidneys excrete it mostly in unchanged form. The clearance is lower in healthy individuals than in tuberculosis patients (Brennan et al., 2008). Although cycloserine is effective in treating resistant strains of *Mycobacterium tuberculosis*, its use is limited due to severe toxicities on the kidney and neuropsychiatric adverse reactions (Batson et al., 2017, World Health Organization, 2014). Consequently, the World Health Organization (World Health Organization, 2014) recommends monitoring of cycloserine plasma concentration to ensure that the peak concentration is kept below 35 µg/mL.

Determination of plasma concentration of cycloserine or any drug requires a method that has less steps in sample preparation and better sensitivity. Several bioanalytical methods for cycloserine employing LC-MS/MS have been described in literature (Polagani et al., 2013, Patel et al., 2011, Mao et al., 2017, Yaroshenko et al., 2014) with sensitivities in the range 0.05 – 0.5 µg/mL. The other HPLC-MS and HPLC-MS/MS methods had sensitivities of 0.5 and 0.2 µg/mL, respectively (Stepanova et al., 2016, Supriya et al., 2012). Three of these methods employed solid phase extraction procedure, which is expensive and time consuming. The other methods made use of protein precipitation and derivatisation (Polagani et al., 2013, Mao et al., 2017, Stepanova et al., 2016) that involved several steps and used very small volumes of reagents. More errors are likely to be made if small volumes of reagents are used in sample processing, which eventually affect precision.

The only UPLC-MS/MS method (Han et al., 2013) for cycloserine analysis available has two shortcomings. Firstly, many steps are involved when mixing volumes of reagents. Moreover, the method required a sample to be centrifuged twice before it is ready for analysis. Secondly, as small as 4 and 5 µL volumes of reagents were used in sample preparation process. These pitfalls compromise the precision and accuracy especially if the method is adapted for routine batch analysis of clinical samples. The objective of this study was to develop a bioanalytical method that has less steps in sample preparation, simple as well as better sensitivity for analysis of cycloserine in plasma of patients.

Therefore, in the current study a simple, stable and sensitive UPLC-MS/MS method was developed and validated for the determination of cycloserine in plasma. The method was applied to quantify cycloserine as a metabolite of terizidone in pharmacokinetic study of patients with drug-resistant tuberculosis.

5.1 EXPERIMENTAL

5.1.1 Chemical reagents

The reference standard, cycloserine (Figure 1) powder, analytical grade (CAS: 68-41-7) and propranolol powder (internal standard–IS, Figure 1) analytical grade (Lot: 29H4016) were purchased from Sigma-Aldrich, Germany. The HPLC grade of methanol and acetonitrile were purchased from Merck (South Africa) and Sigma-Aldrich (Germany), respectively. Formic acid was acquired from Merck, South Africa. Water (18 mΩ) was obtained from a Direct-Q3 (Mmillipore) water purification system. Blank pooled plasma (for research purpose) was purchased from Sigma-Aldrich, Germany.

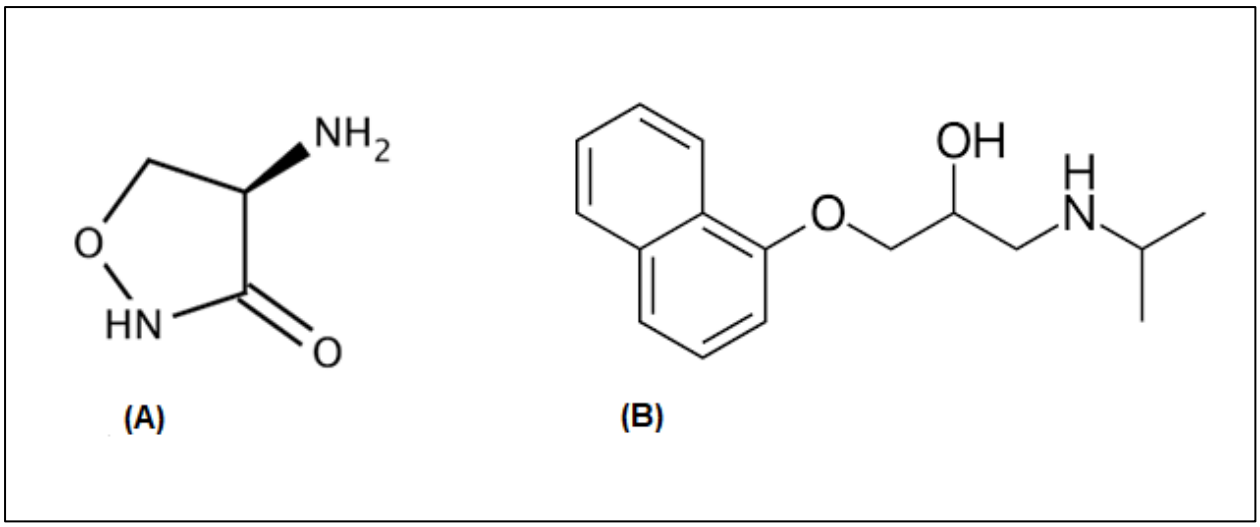

Figure 1. Chemical structure of cycloserine (A) and propranolol (B).

5.1.2 Instrumentation

The analytical equipment consisted of a Waters® Acquity (Waters, Milford, MA, USA) ultra-performance liquid chromatography (UPLC) system. It consisted of a binary solvent manager with two independent pump systems that pumped solvent through the system with a maximum pressure of 15000 psi. The Sample manager injected samples that were drawn from the vials located in the Sample organiser onto the chromatographic column. The system was also equipped with a column heater. Temperature in the Sample organiser was kept at 10 ºC. Coupled to the UPLC system was a Xevo® TQ MS triple quadrupole tandem mass spectrometer (atmospheric pressure ionisation) and was used for high-resolution UPLC-MS/MS analysis. The MassLynx™ software, acquired, managed and processed the mass spectrometry data and UPLC instrument control.

5.1.3 Chromatographic and mass spectrometry conditions

Separation of cycloserine and propranolol was carried out on Phenomenex Kinetex™ PFP C_{18} (2.1 mm x 100 mm, 2.6 µm) reversed-phase column. The column temperature was maintained at 45 ºC. The mobile phases consisted of water (A) and acetonitrile (B) both acidified with 0.1 % formic acid. The gradient steps in Table 1 were used to elute cycloserine and propranolol at flow rate of 0.4 mL/min. The total chromatographic run time was 7 minutes.

Table 1. Gradient elution steps

Time (minutes)	%Water (A)	%Acetonitrile (B)
0	100	0
0.5	100	0
4.5	90	10
6	0	100
6.5	0	100
6.51	100	0
7	100	0

Ionisation, detection and quantification of cycloserine and IS was achieved on Xevo® TQ MS triple quadrupole tandem mass spectrometer equipped with electrospray ionisation source operating in positive ion mode. The mass spectrometer performed the detection of cycloserine and IS using multiple reaction mode (MRM) with the transition (precursor → product) of 103 →75 *m/z* for cycloserine and 260.2 →183 *m/z* for IS. The operating parameters used were set as follows: capillary voltage, 3.5 V; cone voltage, 15 V; collision energy range, 7-15 eV; source temperature, 140 ºC; desolvation temperature, 400 ºC; desolvation gas, 800 L/h and cone gas, 50 L/h.

5.1.4 Preparation of stock solutions, quality controls and calibration standards

Stock solution of cycloserine (1000 µg/mL) was prepared by dissolving accurately weighed 10.0 mg cycloserine in 10 mL of methanol. The stock quality control (QC) cycloserine solution with concentration of 800 µg/mL was prepared by dissolving 8.0 mg of cycloserine in 10 mL of methanol. The IS stock solution with a concentration of 100 µg/mL was prepared by dissolving 10 mg of propranolol in 100 mL of methanol. These solutions were kept at -80 ºC.

The stock solution of cycloserine was appropriately diluted with methanol in order to make eight concentrations of standard working solution (500, 250, 50, 10, 2, 1, 0.5, 0.1 µg/mL). Calibration standards were prepared by spiking 100 µL of each standard working solution to 900 µL of blank plasma. In this way, eight concentrations (50, 25, 5, 1, 0.2, 0.1, 0.05, 0.01 µg/mL) for the calibration standard curve were prepared. The QC working standard solution was prepared by appropriate dilution of stock QC solution with methanol to 400, 100 and 5 µg/mL. The QC samples were prepared at

concentrations of 40, 10 and 0.5 µg/mL for high QC, medium QC and low QC, respectively, by spiking 100 µL of each QC working solution with 900 µL of blank plasma. The IS solution was further diluted with methanol to the concentration of 0.01 µg/mL.

5.1.5 Plasma sample pre-treatment

Extraction of cycloserine from the spiked plasma (calibration standard curve and QC samples) was achieved through plasma protein precipitation. To each 200 µL of spiked plasma, 800 µL of IS solution was added and vortex mixed for 1 minute. In order to separate the precipitate, the contents were centrifuged (13000rpm) for 10 minutes at 2 ºC. Five hundred micro-litres (500 µL) of the supernatant were pipetted into a clean vial. A further 500 µL of water was added to the supernatant and vortex mixed. Finally, 2 µL of this solution was injected onto the UPLC system.

5.1.6 Method validation

The bioanalytical method was validated according to the International Conference for Harmonisation and United States Foods and Drugs Administration guidelines for validation of analytical procedures (USFDA, 2013, ICH, 2005). The method was validated for sensitivity or limit of quantification, limit of detection, linearity, accuracy, precision, recovery, carry-over, matrix effect and stability.

5.1.7 Analysis of patient's plasma samples

Plasma cycloserine (terizidone metabolite) concentration was determined in 78 drug-resistant tuberculosis patients treated with 750 mg terizidone daily dose and other anti-tuberculosis drugs. The study was approved by the ethics committees of University of Cape Town (Ref: 777/2014) and University of the Western Cape (Ref: 07/6/12). Frozen patients' plasma samples were left to thaw at room temperature and vortex mixed before pipetting. To every 200 µL of plasma, 800 µL of IS were added and vortex mixed for 1 minute. The procedure for the rest of the patients' plasma sample preparation was same as the calibration standards. The bioanalytical run consisted of the processed sample plasma without IS and terizidone, processed plasma with IS only, calibration standards, processed patient samples and QC samples.

5.2 RESULTS

5.2.1 Chromatographic and mass spectrometry conditions optimisation

In order to optimise the separation efficiency and chromatographic peak shape of the analyte and the IS, several mobile phase compositions, the flow rate and different C_{18} columns were employed. Acetonitrile and water, both acidified with 0.1% formic acid were found to be the best mobile phases with gradient composition shown in Table 1. Additionally, the flow rate of 0.4 mL/min was found suitable for current method. The column, Acquity UPLC BEH C_{18} (2.1 mm x 100 mm, 1.7 µm), was tried but the analyte eluted quite early. Separation of cycloserine and IS was best achieved on a Phenomenex Kinetex^{TM} PFP C_{18} (2.1 mm x 100 mm, 2.6 µm) reversed-phase column as it is ideal for separation of polar compounds (Figure 2).

Using electrospray ionisation in positive mode, the protonated precursor ions [M+H]+ of cycloserine and propranolol that were dominant had *m/z* ratios of 103 and 260.2, respectively. After the conditions for fragmentation were optimised with collision energy in the range 7-15 eV, the most abundant and stable ions in the product spectra were observed at *m/z* ratios of 75 and 183 for cycloserine and propranolol, respectively. Other parameters for ionisation were optimised to obtain highest, stable and consistent signal intensity for cycloserine and propranolol.

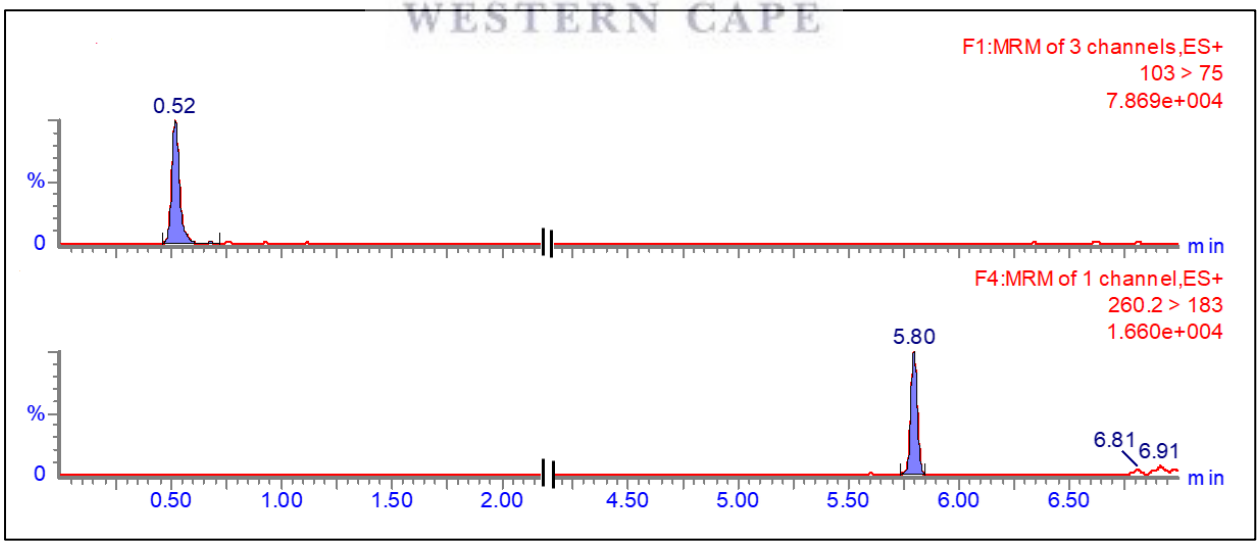

Figure 2. Chromatograms of cycloserine and propranolol with retention times at 0.52 minutes and 5.8 minutes, respectively.

5.2.2 Method validation

5.2.2.1 Sensitivity and limit of detection

Sensitivity was the lowest cycloserine concentration (lower limit of quantification–LLOQ) that was measured with acceptable precision and accuracy. The lowest concentration of the calibration standards was determined to be 0.01 µg/mL as it had a precision of 19.3% (percentage relative standard deviation–%RSD) and accuracy of 98.7% (Table 3). This was within the acceptable precision of not more than 20% and accuracy within 80 – 120% (USFDA, 2013).

The limit of detection (LOD) based on the calibration curve, was calculated using the formula (1):

$$LOD = 3.3\sigma/S \qquad (1)$$

where σ was the standard deviation of the Y-intercepts of the regression equations shown in Table 2. Similarly, S was the average of the slopes of the regression equations. The value of the LOD was 0.004 µg/mL.

5.2.2.2 Linearity

The eight-point standard calibration curve was constructed by ploting cycloserine nominal concentrations on the X-axis against peak area ratios of cycloserine and IS on the Y-axis (response). The weighting factor of 1/x improved the linear regression fit. Linearity was assessed statistically by fitting the data (nominal concentration and response) to a linear regression model by method of least squares. The regression equations constructed from six replicate bioanalytical runs of the eight calibration standards are shown in Table 2 together with the corresponding coefficient of determination values and the sum of residuals. The average coefficient of determination and correlation coefficient was 0.9994 and 0.9997, respectively.

Table 2. Bioanalytical method linearity parameters

Run number	R-square (r^2)	Linear equation	Residual sum of squares
1	0.9998	$Y = 4.202x + 0.0604$	0.00069
2	0.9998	$Y = 4.161x + 0.0511$	0.00107
3	0.9999	$Y = 4.244x + 0.0536$	0.00093
4	0.9996	$Y = 4.301x + 0.0489$	0.00141
5	0.9998	$Y = 4.215x + 0.0578$	0.00022
6	0.9989	$Y = 4.207x + 0.0621$	0.00209

5.2.2.3 Accuracy and precision

Five replicate values from the analyses of LLOQ, low QC, median QC and high QC were employed to calculate accuracy of the assay. The accuracy was estimated as the percentage ratio average (five replicates) of the back-calculated concentrations and the nominal concentration. The accuracy ranged from 98.7 – 116% and 99 – 117% for within-run analysis and between-run analysis, respectively (Table 3). Precision (%RSD) was calculated as the percentage ratio of the standard deviation and the mean of the replicates analysed on same day (within-run) and different days (between-run). The within-run precision ranged between 3.7 and 19.3% while between-run precision was in the range 2.6 – 15% (Table 3). The accuracy and precision was within the accepted range (USFDA, 2013).

Table 3. Within-run and between-run accuracy and precision of the LLOQ, low QC, median QC and high QC assay.

Nominal concentration (µg/mL)	Mean concentration ± SD (µg/mL)	Accuracy (%)	Precision (%RSD)
Within-run analysis			
0.01	0.0098 ± 0.002	98.7	19.3
0.5	0.58 ± 0.062	114.6	10.8
10	9.94 ± 0.697	99.4	7
40	40.48 ±1.48	101.2	3.7
Between-run analysis			
0.01	0.012 ± 0.002	117.3	15
0.5	0.57 ± 0.03	111	5.2
10	10.3 ± 0.674	103	6.5
40	39.6 ± 1.06	99	2.6

SD, Standard deviation

5.2.2.4 Carry-over and matrix effect

The carry-over was assessed by injecting the highest concentration of the calibration standard (50 µg/mL) followed by a blank sample. A response of 238.874 was read after injection of 50 µg/mL and 0.00677 after injection of blank sample. The blank sample response corresponded to cycloserine concentration of 0.0021 µg/mL and was below the LLOQ.

The matrix effect was evaluated by comparing the instrument response (peak area) of cycloserine and IS that was prepared in methanol and the one spiked in plasma. The average ratios of the peak area of cycloserine spiked in plasma to the one prepared in methanol were 0.9927 and 1.003 for low QC and high QC, respectively. Similarly, the average IS ratios were 0.9961 and 1.01. These results show that the effect of plasma (matrix) on the ionization of both cycloserine and IS was not significant.

5.2.2.5 Stability and recovery

The analyte stability was evaluated according to the situations that were expected to be experienced during patient plasma sample processing and analysis (USFDA, 2013). These situations were freeze-thaw of at least one cycle and stability of processed samples in the autosampler for maximum of 24 hours. The mean cycloserine concentration for the low QC and high QC after three freeze-thaw cycles was almost close to the nominal concentration. Nevertheless, the mean concentration of the processed samples that were stored at 2 – 8 ºC dropped by 11.9 and 1.75% for low QC and high QC, respectively, after 48 hours. The mean accuracy was 88.1 and 98.3% while precision was 9.1 and 3.7% (RSD) the low QC and high QC, respectively. Recovery was determined by comparing instrument response of the analyte that was prepared in the solvent with analyte extracted from plasma (USFDA, 2013). The percentage recovery was in the range 88.7 – 91.2 for both low and high QC.

5.2.3 Application of bioanalytical method in pharmacokinetic study

Seventy-eight patients participated in the study after signing informed consent form. They provided 608 plasma samples, which were analysed for cycloserine as a metabolite of terizidone. The mean and median concentrations were 2.1 ±1.5 µg/mL and 1.9 (0.01 – 8.2) µg/mL, respectively. The distribution of the cycloserine concentration in shown in Figure 3.

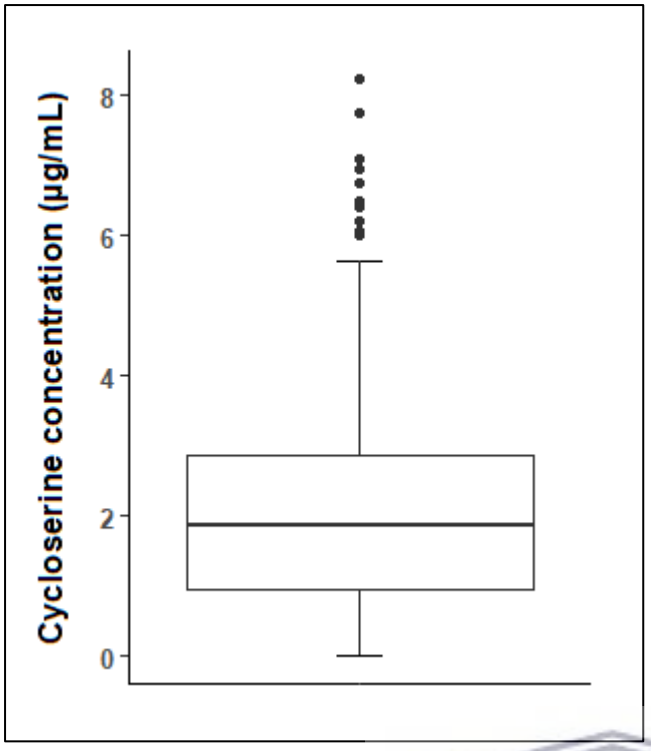

Figure 3. Distribution of cycloserine concentrations in drug-resistant tuberculosis patients.

5.3 DISCUSSION

Protein precipitation was the preferred method of analyte (cycloserine) extraction from plasma as it is simple, fast and inexpensive (Sargent, 2013). The two-fold dilution of the supernatant with water and five-fold in protein precipitation procedure ensured also the dilution of interfering compounds. This procedure reduced the matrix effect (Mao et al., 2017).

In the chromatographic separation, the initial 100% aqueous phase favoured the elution of cycloserine first as it is more polar than the IS. Subsequently, the gradual reduction of the aqueous phase or gradual increase of the organic phase in the second step of the gradient favoured the elution of the IS. The excellent peak shape, better separation and reproducibility (Figure 2) resulted from the combined C_{18} and pentafluoropheny (PFP) functionality through pi-pi, hydrogen bonding, dipole-dipole and hydrophobic interactions. Hence, the PFP C_{18} (2.1 mm x 100 mm, 2.6 µm) reversed-phase column was the best of the columns that were tried.

The carry-over effect (0.0021 µg/mL), although minimal, was rather expected because cycloserine molecule is polar and has affinity for pentafluoropheny moiety (stationary

phase component). Hence, it remained stuck in the column but was gradually eluting with each run. On the other hand, the calibration curve was linear (ICH, 2005) over the concentration range 0.01 – 50 µg/mL as the coefficient of determination value and the sum of the residuals was approximatelty one and zero, respectively.

Furthermore, our method had a better sensitivity of 0.01 µg/mL than a previously reported UPLC-MS/MS method (Han et al., 2013) with 0.5 µg/mL. Hence, it was suitable to measure low concentrations of cycloserine as a metabolite of terizidone in patients who were slow metabolisers. The method was successfully used to determine plasma concentrations of cycloserine in a population pharmacokinetic study of patients with drug-resistant tuberculosis hospitalised for intensive phase of treatment.

5.4 CONCLUSION

The simple, sensitive and reliable UPLC-MS/MS method was developed and validated for analysis of cycloserine in plasma of patients with drug-resistant tuberculosis. The validation parameters indicated that the method was linear, sensitive, stable, precise and accurate. The method is suitable for pharmacokinetic studies as sample processing is relatively simple and does not require use of expensive chemicals.

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Chapter Six

In this chapter, a research paper entitled:

"Steady-state Population Pharmacokinetics of Terizidone and its Metabolite Cycloserine in Patients with Drug-resistant Tuberculosis"

is presented. It was published as an original article in the *British Journal of Clinical Pharmacology*, 2019: 85:1946–1956. https://doi.org/10.1111/bcp.13975. It has been re-used in this thesis with permission from the publisher (John Wiley & Sons, Inc. license number 4632450146888). The Copyright license agreement is included as Appendix 4.

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ORIGINAL ARTICLE

BJCP BRITISH PHARMACOLOGICAL SOCIETY

Steady-state population pharmacokinetics of terizidone and its metabolite cycloserine in patients with drug-resistant tuberculosis

Mwila Mulubwa | Pierre Mugabo

School of Pharmacy, University of the Western Cape, Bellville, Cape Town, South Africa

Correspondence
Mwila Mulubwa, University of the Western Cape, Private bag X17, Bellville 7535, Cape Town, South Africa.
Email: 3579753@myuwc.ac.za; mwila.mulubwa@gmail.com

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Aims: Despite terizidone being part of the second-line recommended drugs for treatment of drug-resistant tuberculosis (DR-TB), information on its pharmacokinetics is scarce. The aim of this study was to describe the steady-state population pharmacokinetics (PPK) of terizidone and its primary metabolite cycloserine in patients with DR-TB and determine the effect of patient characteristics.

Methods: This clinical study involved 39 adult DR-TB patients admitted to Brewelskloof Hospital in Cape Town, South Africa for intensive treatment phase. Blood samples were collected at predose and 0.5, 1, 2, 3, 3.5, 4, 8, 16 and 24 hours after drug administration. The estimation of PPK parameters was performed using nonlinear mixed-effects modelling software Monolix 2018R1. Free-fat mass was used to perform allometric scaling on disposition parameters.

Results: A 1-compartment model best described the pharmacokinetics of terizidone and cycloserine. A modified transit compartment model described the absorption of terizidone. The parameters of terizidone model were mean transit time (1.7 h), absorption rate constant (2.97 h^{-1}), apparent volume of distribution (*Vp/F*: 13.4 L) and apparent total clearance (0.51 L h^{-1}). In the joint model, apparent fraction of terizidone converted to cycloserine was 0.29 while apparent clearance of terizidone via other routes and apparent cycloserine clearance was 0.1 L h^{-1} and 2.94 L h^{-1}, respectively. Serum albumin had significant effect on *Vp/F*.

Conclusions: The developed PPK model described well the concentration–time profile for terizidone and cycloserine in DR-TB patients. High albumin concentration was associated with low *Vp/F*.

KEYWORDS

cycloserine, drug-resistant tuberculosis, population pharmacokinetics, terizidone

1 | INTRODUCTION

Drug-resistant tuberculosis, which includes rifampicin mono-resistant and multidrug-resistant tuberculosis, is a persistent global threat and is linked to inadequate tuberculosis treatment.[1,2] Usually, the suboptimal drug treatment influences spontaneous mutations in the *Mycobacterium tuberculosis* chromosomal genes, which lead to the emergence of resistant strains.[3] To prevent further drug resistance, a multidrug treatment regimen consisting of 5–7 drugs is used in the treatment of drug-resistant tuberculosis.[4]

The authors confirm that the PI for this paper is Prof. Pierre Mugabo and that he had direct clinical responsibility for patients.

Terizidone, a condensation product of two cycloserine molecules, is one of the medicines used to treat drug-resistant tuberculosis.[5,6] Information on how terizidone is metabolised into cycloserine and the enzymes involved is not available in literature. Nevertheless, it seems to undergo hydrolysis into cycloserine presystemically.[7] Terizidone and cycloserine exert their respective antibacterial effect by disrupting the synthesis of peptidoglycan needed for bacterial cell wall formation through inhibition of D-alanine ligase and L-alanine racemase.[8] Terizidone is a potential drug for treatment of extra-pulmonary tuberculosis[9] and has been reported to have fewer central nervous system side effects than cycloserine and well tolerated in patients on dialysis.[10,11] A recent study indicated that both terizidone and cycloserine were clinically effective in the intensive treatment phase of drug-resistant tuberculosis.[12]

Despite terizidone being recommended and currently used in the treatment of drug-resistant tuberculosis,[4] information on its pharmacokinetics in literature is hardly found or poorly described. There appears to be only one study published in which terizidone and cycloserine pharmacokinetics were compared after a single-dose administration of each drug in tuberculosis patients.[13] After oral administration of 250–750 mg, terizidone reaches maximum concentration within 3 hours with absorption rate constant (*ka*) in the range 1.17–1.36 h^{-1}. The distribution volume is high, ranging between 113 and 246 L, while clearance is in the range 2.49–6.4 L h^{-1}. Thirty-nine percent of the administered dose is excreted in urine after 30 hours.[13] Terizidone plasma and urine concentrations in this study were not measured but estimated based on cycloserine using colorimetric method.[13] By contrast, the population pharmacokinetics of cycloserine in multidrug-resistant tuberculosis patients have been described[14] as well as noncompartmental pharmacokinetics in which cycloserine was measured as terizidone metabolite.[15] Cycloserine reaches maximum concentration within 2–3 hours with *ka* of 0.135 h^{-1} after oral daily dose of 500–1000 mg. It is widely distributed in most body fluids and tissues with distribution volume of 10.5 L. Its clearance is 1.38 L h^{-1} and primarily eliminated via renal route with 50–70% excreted unchanged within 12–24 hours.[14,16] The primary and secondary pharmacokinetic parameters of terizidone at steady state in drug-resistant tuberculosis patients are still unknown.

The objective of this study was to describe the population pharmacokinetics of terizidone and cycloserine at steady state in patients with drug-resistant tuberculosis and assess the influence of patient characteristics on pharmacokinetic parameters. We also estimated the associated secondary pharmacokinetic parameters.

What is already known about this subject

- Single-dose pharmacokinetics of terizidone.

What this study adds

- The study outlines the first description of population pharmacokinetics of terizidone and cycloserine at steady state in drug-resistant tuberculosis patients with and without human immunodeficiency virus infection.
- The study characterises for the first time the secondary pharmacokinetic parameters of terizidone at steady state.
- The first description of terizidone fraction undergoing biotransformation into cycloserine and the influence of serum albumin on apparent volume of distribution of terizidone.

2 | METHODS

2.1 | Study design

This was a non-randomised observational clinical study involving adult patients admitted for intensive treatment phase of drug-resistant tuberculosis at Brewelskloof Hospital, Western Cape province, South Africa. All patients were taking second-line anti-tuberculosis drugs such as pyrazinamide, ethionamide, kanamycin, moxifloxacin or ofloxacin, and ethambutol in addition to terizidone. In patients with tuberculosis monoresistant to rifampicin, isoniazid was added to their treatment. The doses were administered according to the local treatment guideline for management of drug-resistant tuberculosis.[17]

The patients included in this study were on anti-tuberculosis treatment for at least 2 weeks and had consented to participate in the study. Patients were excluded from the study if they requested so, were pregnant, breast-feeding, severely dehydrated or intolerant to terizidone. The demographic information was captured from patients on the day of the study while medical and treatment history was obtained from patients' folders. The ethics committees of the University of the Western Cape (Ref: 07/6/12) and University of Cape Town (Ref: 777/2014) approved this study. The patients' information was treated with confidentiality and the study was conducted in conformity with the principles outlined in the declaration of Helsinki.[18]

2.2 | Pharmacokinetic blood sampling

The patients were in fasting state from 22:00 hours prior to the morning of the blood-sampling day. Using an intravenous catheter placed in a vein of the forearm, 5 mL of blood from each patient was collected in heparinised tubes at baseline (predose) and at 0.5, 1, 2, 3, 3.5, 4, 8, 16 and 24 hours after drug administration. After centrifugation, the plasma was stored at −80°C until the date of analysis. Other blood samples were also collected for renal and liver function tests, virological and immunological tests. Patients then took their usual dose of anti-tuberculosis medications, including terizidone, and the time was noted. Human immunodeficiency virus (HIV) coinfected patients also received antiretroviral drugs as prescribed. Patients were then allowed to eat and drink as usual.

2.3 | Plasma quantification of terizidone and cycloserine

Plasma concentration of terizidone was analysed using high-performance liquid chromatography–UV method. It was extracted from plasma using protein precipitation method. The average inter- and intraday precision was 3.3 and 7%, respectively, while the mean accuracy was 107%. Calibration curves were linear with coefficient of determination ranging between 0.9988 and 0.9999. The lower limit of quantification and limit of detection was 3.125 and 0.78 μg mL^{-1}, respectively.[19] Cycloserine concentrations were analysed using ultra-performance liquid chromatography–tandem mass spectrometry method and validated according to the international guidelines.[20] Extraction was achieved through plasma protein precipitation with propranolol as internal standard. The mean inter- and intraday precision was 10.2 and 7.3%, respectively. The inter- and intraday accuracy was 103.8 and 108.7%, respectively. The curves were linear over the concentration range of 0.01–50 μg mL^{-1} with mean coefficient of determination of 0.9994. The quantification and detection limits were 0.01 and 0.004 μg mL^{-1}, respectively, while the carry-over was 0.0021 μg mL^{-1}. The matrix effect was insignificant. Cycloserine was stable after 3 freeze–thaw cycles and extraction efficiency ranged between 68.7 and 71.2%.

2.4 | Pharmacokinetic modelling

At the time of pharmacokinetic blood sampling, all patients had already achieved steady-state concentration for terizidone. The previous dose of terizidone was administered 24 hours before the sampling day. Therefore, the predose sampling time of 0 hours was set to 23.9 hours for modelling purposes. Conversion of concentration from μg mL^{-1} to μmol L^{-1} (molar units) was performed by using molar mass of 302.3 g mol^{-1} and 102.1 g mol^{-1} for terizidone and cycloserine, respectively. The doses for terizidone were also converted from mg to μmol.

The population pharmacokinetic parameters were estimated using nonlinear mixed-effects modelling in Monolix 2018R1 software.[21] The software utilises stochastic approximation expectation maximization algorithm[22] to carry out parameter estimations. The likelihood and Fisher information matrix were computed using importance sampling and stochastic approximation, respectively. Selection of the base model was guided by the visual inspection of diagnostic plots, change in the objective function value (OFV: −2 * *loglikelihood*), plausibility and precision of the parameter estimates (percentage relative standard error–%RSE).

We performed modelling in 2 parts. In the first part, only terizidone concentration–time profile was modelled. Based on the visual inspection of terizidone concentration–time profile using Datxplore interface of Monolix 2018R1, 1- and 2-compartment models were fit to the data. Absorption process was modelled using lag-time or transit compartment model[23] and assessed if it improved the model fit. In the case of the transit compartment being over-parameterised, modification was performed by setting *ka* equal to transit rate constant (*Ktr*; http://mlxtran.lixoft.com/examples/transit-compartments-weibull-absorption/).

In the second part, terizidone and cycloserine concentration–time profiles were modelled jointly. Terizidone model was modified in order to link it to cycloserine compartment. We assumed that terizidone did not undergo first-pass metabolism but was eliminated by biotransformation into cycloserine and other routes. Additionally, oral bioavailability (F) was assumed to be one. The fraction (*Fm*) of terizidone that is converted into cycloserine is unknown and unidentifiable. The clearance of terizidone by other routes is also unidentifiable. Similarly, the apparent volume of distribution of cycloserine (*Vm/F*) is unidentifiable, as the dose was not administered directly. To circumvent this problem, we decided to fix *Vm/F* to the literature value of 10.5 L.[14] This decision allows *Fm* to be identified and to distinguish between terizidone clearance via biotransformation and other routes.[24,25] It is worth noting that *Fm* is not the true fraction but apparent fraction.[26] Another way of dealing with parameter identifiability problem is to set the volume of the parent drug equal to metabolite volume, fixing metabolite volume equal to 1 or fixing *Fm* to any value between zero and 1. However, we chose not to undertake these options.

Proportional and combined (additive and proportional) error models were explored to model residual unexplained variability. The between-subject variability (BSV) model described the random variation in population pharmacokinetic parameters. We assumed that these parameters were log-normally distributed.

2.5 | Covariate model

After the base model was selected using the criteria in previous section, we investigated the effects of covariates on pharmacokinetic model parameters. The total body weight (TBW), free-fat mass[27] (FFM) or body mass index was used as body size descriptor. In order to adjust for the expected effect of body size, allometric scaling was performed on clearance and volume parameters. The exponents were either fixed to 0.75 for clearance and 1 for volume[28] or estimated. Other covariates explored were age, sex, HIV status, alanine aminotransferase, aspartate transaminase, total bilirubin, TBW creatinine clearance and FFM creatinine clearance. Creatinine clearance was calculated using Cockcroft–Gault formula.[29] A covariate was selected if it was pharmacologically plausible; a correlation existed between a covariate and the random effects of the predicted individual parameters. Retention of the covariate in the model was based on statistical significance ($P \leq .05$ using Wald test), a decrease in OFV and BSV. Covariates, normalised by the typical population median value, were added in the model one at a time in log-linear fashion. The relationship between a pharmacokinetic value (θ_i) and continuous covariate (cov_i) and of an i-th individual was expressed as shown in equation 1:

$$\theta_i = \theta_{pop} * \left(\frac{cov_i}{COV_{median}}\right)^{\beta_{\theta i}} * e^{\eta_i} \quad (1)$$

where θ_{pop} represented the typical population pharmacokinetic value and COV_{median} the population median value of the continuous covariates. The random effect associated with the *i*-th individual was denoted by η_i where $\eta_i \approx N(0, \omega^2)$. The factor describing the effect of a continuous covariate on θ_i was denoted by $\beta_{\theta i}$. The relationship, in the case of the categorical covariates was expressed as shown in equation 2:

$$\theta_i = \theta_{pop} * e^{\beta_{\theta i,cat}[cat=n]} * e^{\eta_i} \quad (2)$$

where $\beta_{\theta i,cat}[cat = n]$ denoted the difference in parameter (θ_i) between an individual belonging to group n and the reference group. Correlations among random effects of estimated individual parameters were then investigated and significant ones were estimated as population parameters.

2.6 | Model evaluation

The model was evaluated visually by inspection of the diagnostic plots such as individual- and population predicted *vs* observed concentrations, individual-weighted residuals *vs* time and predicted concentrations. The visual predictive checks for both terizidone and cycloserine were inspected for possible model misspecification. The distribution of parameter estimates randomly sampled from the conditional distribution[30] were evaluated in order to ensure that the assumption of normality was met. We also performed a bootstrap procedure of 250 runs using Monolix software aided by Rsmlx (R Speaks 'Monolix') R package (http://rsmlx.webpopix.org), in order to evaluate the robustness of the final joint model.

2.7 | Secondary pharmacokinetic parameters

The other pharmacokinetic parameters for terizidone and cycloserine were calculated using MLXTRAN coded formulae in Monolix as shown in Appendix A. The area under the concentration–time curve up to 24 hours ($AUC_{0\text{-}24h}$) and half-life were calculated by integration of the concentrations predicted from the final joint pharmacokinetic model without covariates and formulae, respectively. The maximum plasma concentration (C_{max}) and time to reach C_{max} (T_{max}) were obtained from the output file of the predicted individual concentrations. Finally, the clearance of terizidone resulting from biotransformation into cycloserine was calculated from the individual predicted estimates of terizidone apparent volume of distribution (V_p/F), *Fm* and biotransformation rate constant. Model-based simulations of the distributions of $AUC_{0\text{-}24h}$ and C_{max} were performed across 3 weight bands specified in the local treatment guideline[17] using current dose (750 mg daily). A dosing schedule was proposed that would normalise exposure across weight bands using Monte-Carlo simulations.

3 | RESULTS

Thirty-nine patients including 27 HIV infected and 20 females participated in this study. Thirty-eight patients received a daily dose of 750 mg of terizidone while 1 patient received 500 mg. In total, they provided 571 plasma concentrations, of which 272 were for terizidone. The average number of plasma samples per patient was 7 and 8 for terizidone and cycloserine, respectively. The summary of patients' demographic characteristics are displayed in Table 1. The plot of the concentration *vs* time of the original data for terizidone and cycloserine is shown in Figure 1.

3.1 | Terizidone pharmacokinetic model

The base model consisted of a 1-compartment pharmacokinetic model with first-order absorption and linear elimination. A combined additive and proportional error model best modelled the residual unexplained variability in terizidone concentration. A lag-time parameter was added to describe absorption delay but did not improve the model fit (OFV = +4). When a transit compartment model was used instead, resulted in worse fit (OFV = +8.52) than the base model. Additionally, the *ka* was overestimated and thus not plausible besides %RSE could not be estimated. The transit compartment model was then modified by setting *Ktr* equal to *ka*. This modification resulted in improved model fit (OFV = −39.1) and good parameter precision (Table 2). Therefore, the final terizidone model had the following parameters: mean transit time (*Mtt*), *ka*, *Vp/F* and apparent total clearance (Cl_{tot}/F).

TABLE 1 Summary of patients' demographic characteristics

Variable	Value
Sample size (*n*)	39
Sex	
Female (*n*)	20
Male (*n*)	19
Age (years)	32 (17–56)[a]
Total body weight–TBW (kg)	51.4 (32.4–71)[a]
Free fat mass–FFM (kg)	39.8 (24.8–51)[a]
Body mass index (kg m^{-2})	18.4 (12.4–26.1)[a]
Alanine aminotransferase (IU L^{-1})	11 (4–46)[a]
Aspartate transaminase (IU L^{-1})	33 (17–109)[a]
Albumin (g L^{-1})	32 (15–48)[a]
TBW creatinine clearance (ml min^{-1})	83 (34.5–128)[a]
FFM creatinine clearance (ml min^{-1})	60.4 (26.8–106)[a]
Total bilirubin (μmol L^{-1})	7 (2–24)[a]
Human immunodeficiency virus status	
Infected (n)	27
Uninfected (n)	12
CD4 count (cells μl^{-3})	227 (9–1243)[a]
Viral load (copies mL^{-1})	3279 (42–4 331 310)[b]

[a]Median and range.

[b]Median and range from 19 patients who had their viral load above 40 copies mL^{-1} while the viral load from 8 patients was below 40 copies mL^{-1}.

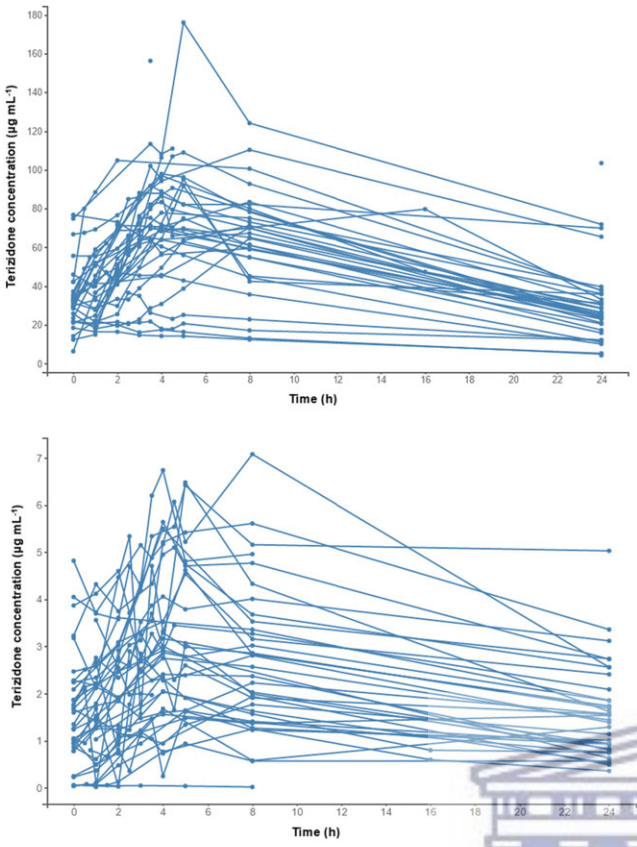

FIGURE 1 Observed concentration–time profiles for terizidone and cycloserine

TABLE 2 Population pharmacokinetic parameters from terizidone only model

Parameter	Estimate	%RSE
Mtt (h)	1.6	17.1
ka (h^{-1})	2.97	19.1
Vp/F [a] (L)	13.4	4.8
Cl_{tot}/F [a] (L h^{-1})	0.51	10.9
Coefficient (effect) of albumin on *Vp/F*	−0.61	30.3
Between-subject variability (CV%)[b]		
Mtt	82	18.3
ka	36.1	53.1
Vp/F	16	35
Cl_{tot}/F	64	13
Residual error		
Additive (µmol L^{-1})	13.6	31
Proportional	0.13	20

[a]Allometrically scaled parameters using FFM by fixing exponents to 1 and 0.75 on *Vp/F* and Cl_{tot}/F, respectively.

[b]Coefficient of variation percentage calculated as $\left(\sqrt{e^{(SD)^2}-1}\right)*100$ where SD is the estimated standard deviation.

%RSE, percentage relative standard error; *Mtt*, mean transit time; ka, absorption rate constant; *Vp/F*, apparent volume of distribution; Cl_{tot}/F, apparent total clearance of terizidone.

Allometric scaling using FFM, TBW and body mass index was performed on *Vp/F* and Cl_{tot}/F. The FFM was found to be the best descriptor of body size as it was associated with the lowest OFV. Allometric scaling using FFM after fixing exponent to 1 and 0.75 on *Vp/F* and Cl_{tot}/F resulted in model improvement (OFV = −90.04) and this explained 13.5 and 5% of the variation in *Vp/F* and Cl_{tot}/F, respectively. The covariates, sex, FFM creatinine clearance and HIV status had significant effect on Cl_{tot}/F, while albumin had significant effect on *Vp/F*. However, when all significant covariates were included in the model, only albumin remained significant on *Vp/F* and led to improved model fit (OFV = −11.9; $p = 0.0062$). The variation explained by albumin in *Vp/F* was 43.2%. There were no significant correlations found among parameter random effects. The summary of the population pharmacokinetic parameters of terizidone are shown in Table 2.

3.2 | Joint terizidone and cycloserine model

Terizidone final model was modified in order to incorporate a cycloserine compartment as illustrated in Figure 2. The Cl_{tot}/F was divided into apparent clearance of terizidone due to biotransformation into cycloserine (*Clpm* = Cl_{tot}/Fm) and apparent clearance of terizidone via other routes (*Clp* = $Cl_{tot}/(1\text{-}Fm)$). A 1-compartment model with first-order elimination best characterised cycloserine disposition. The combined additive and proportional error model best described the

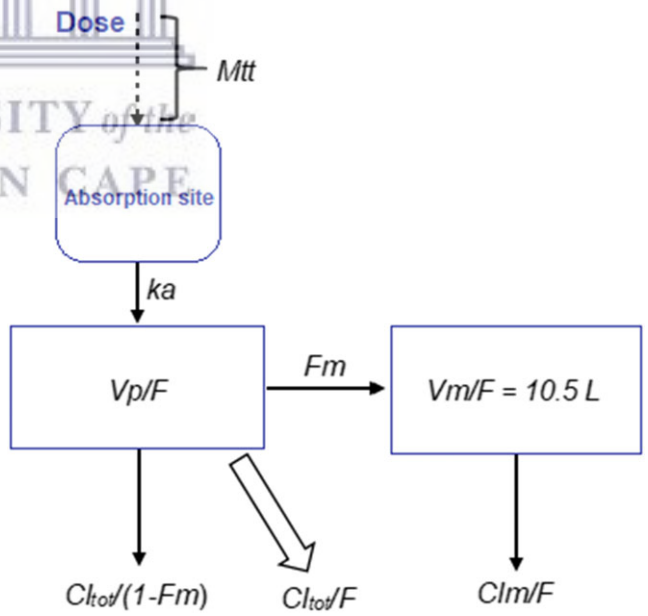

FIGURE 2 Schematic diagram for the joint pharmacokinetics model of terizidone and cycloserine. *Mtt*, mean transit time of terizidone from ingestion to its absorption; *Ad*, amount of terizidone at the absorption site; *ka*, absorption rate constant of terizidone; *Vp/F*, apparent volume of distribution of terizidone; *Clp* = $Cl_{tot}/(1\text{-}Fm)$, clearance of terizidone via other routes; *Fm*, apparent fraction of terizidone converted to cycloserine; Cl_{tot}/F, apparent total clearance of terizidone (*Clp* + *Clpm*), *Vm/F* (10.5 L), apparent volume of distribution of cycloserine; *Clm/F*, apparent clearance of cycloserine

residual unexplained variability in cycloserine concentration. Allometric scaling on apparent clearance of cycloserine (*Clm/F*) using FFM and fixing the exponent to 0.75 slightly improved the joint model with OFV change of −0.82. The variation in *Clm/F* explained by this scaling was 17%. After scaling of *Clm/F* with FFM, no covariates were found significant on *Fm*, *Clp* and *Clm/F*. The values for *Mtt*, *ka*, and *Vp/F* in the terizidone and joint model were not exactly the same but similar.

The joint model was described by the following system of ordinary differential equations 3–5:

$$dAd/dt = -ka * Ad \tag{3}$$

$$dApc/dt = ka * Ad - (Cl_{tot}/(1 - Fm))/Vp * Apc - (Cl_{tot}/Fm)/Vp * Apc \tag{4}$$

$$dAm/dt = ((Cl_{tot}/Fm)/Vp) * Apc - (Clm/F)/10.5 * Am \tag{5}$$

The amount of terizidone at the absorption site and central compartment was denoted by *Ad* and *Apc*, respectively, with *ka* as the absorption rate constant. The amount of cycloserine formed from terizidone metabolism was denoted as *Am*.

The final joint model had the parameters: *Mtt*, *ka*, *Vp/F*, *Clp*, *Fm and Clm/F* (Table 3). The final individual models of *Vp/F*, *Clp* and *Clm/F* belonging to an *i*-th individual were described as follows 6–8:

$$\frac{Vp}{F_i} = 14 * \left(\frac{FFM_i}{39.8}\right)^{1} * \left(\frac{Albumin_i}{32}\right)^{-0.51} * e^{\eta_i} \tag{6}$$

$$Clp = 0.1 * \left(\frac{FFM_i}{39.8}\right)^{0.75} * e^{\eta_i} \tag{7}$$

$$\frac{Clm}{F_i} = 2.94 * \left(\frac{FFM_i}{39.8}\right)^{0.75} * e^{\eta_i} \tag{8}$$

3.3 | Model evaluation

The plots of individual predicted *vs* observed concentration for both terizidone and cycloserine indicated that there was a good agreement

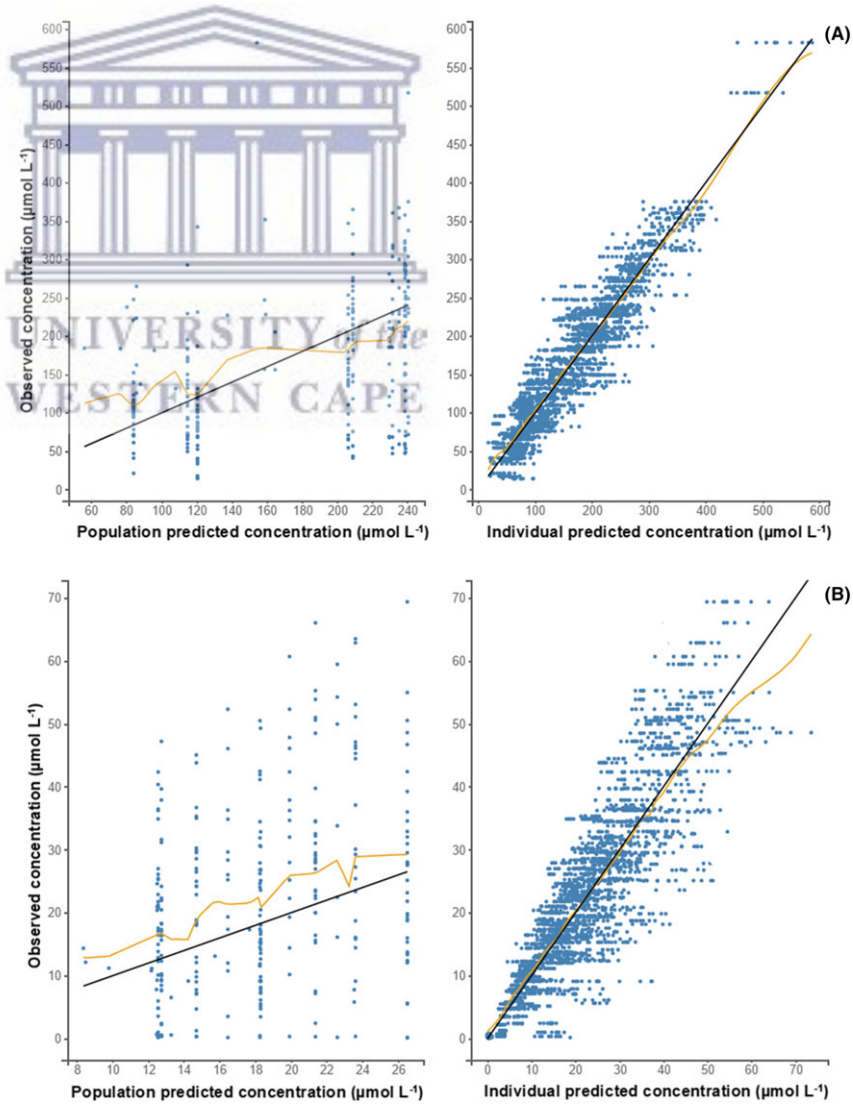

FIGURE 3 Population and individual predicted *vs* observed concentrations for terizidone A, and cycloserine B

between predicted and observed concentration (Figure 3). Similarly, Figure 4 showed that there was no bias observed in the plots of individual-weighted residuals *vs* time and predicted concentration. The visual predictive checks for the joint model are shown in Figure 5. Most of the observed data points lay within 90% prediction interval (generated from 1000 simulations) except for the 95th percentile of cycloserine visual predictive checks that showed some over prediction in variability. The bootstrap parameters in Table 3 were similar to the ones estimated from the original data set. Therefore, the developed joint model described fairly the observed plasma concentration–time profiles for terizidone and cycloserine.

3.4 | Secondary pharmacokinetic parameters

The secondary pharmacokinetic parameters for terizidone were C_{max}, T_{max}, $AUC_{0\text{-}24h}$, half-life and *Clpm* and for cycloserine were C_{max}, T_{max}, $AUC_{0\text{-}24h}$ and half-life (Table 4). The MLXTRAN model file for Monolix in Appendix A gives the code of how these parameters were calculated. The simulated distributions of $AUC_{0\text{-}24h}$ and C_{max} (Figure 6) show a decreasing trend in the median value across the 3 weight bands, 33–50 kg, 51–70 kg and > 70 kg, respectively. Monte-Carlo simulations showed that a terizidone daily (every 24 hours) dose of 750, 900 and 1200 mg achieved similar exposure across the weight bands 33–50, 51–70 and > 70 kg, respectively. The proposed dosing schedule is shown in Table 5.

4 | DISCUSSION

There is scarce information in the literature on pharmacokinetics of terizidone although it is an old drug. In this study, we, for the first, time developed a population pharmacokinetic model of terizidone and a joint model (with cycloserine) at steady state in patients with drug-resistant tuberculosis. A 1-compartment model with first-order absorption and elimination best described terizidone pharmacokinetics. A modified transit compartment model described better the absorption delay than the lag time, as the precision in the former

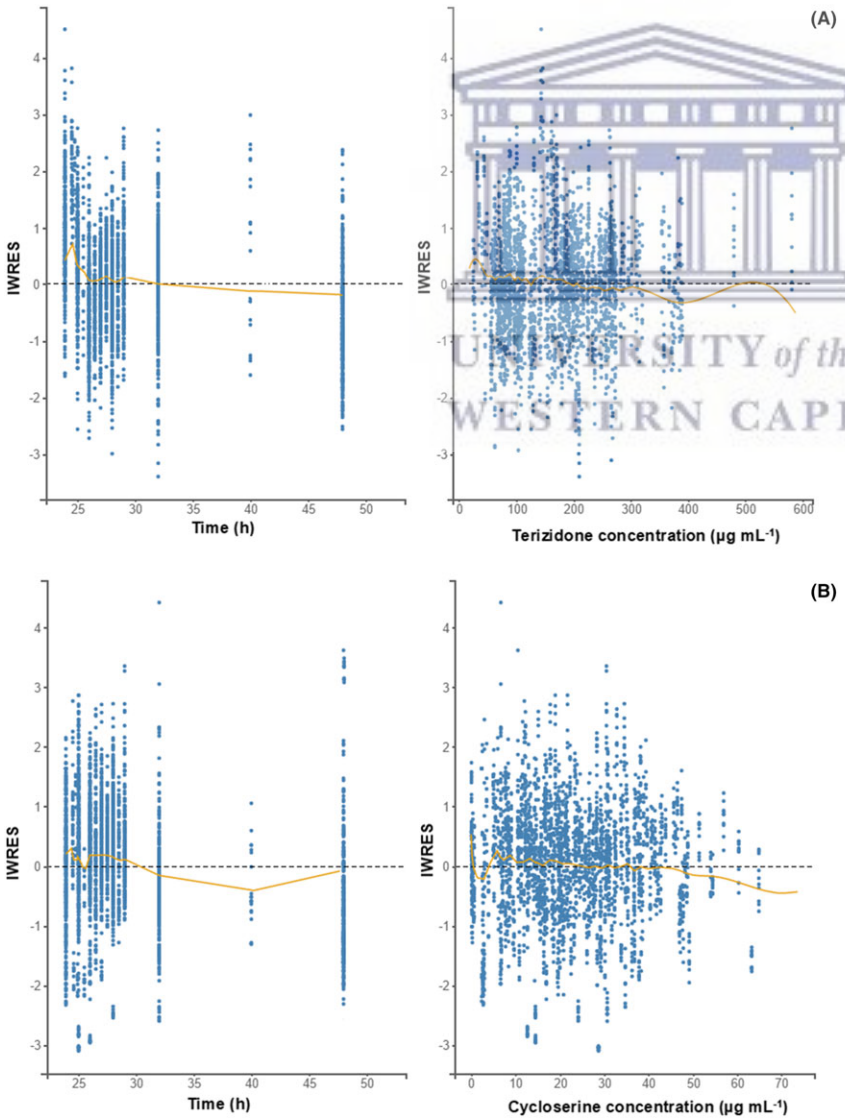

FIGURE 4 Individual-weighted residuals *vs* time and predicted concentrations for terizidone A, and cycloserine B

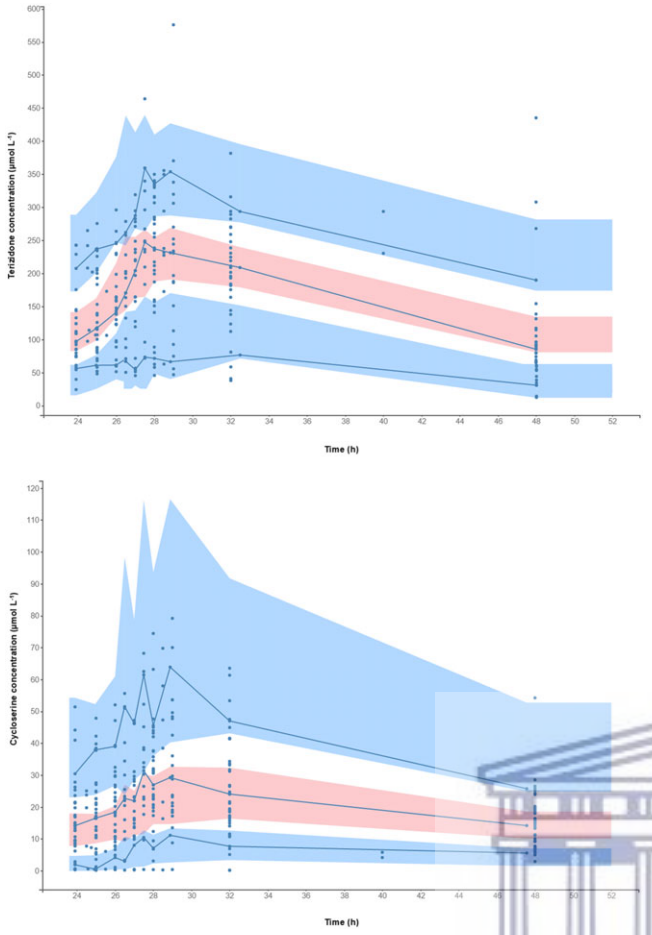

FIGURE 5 Visual predictive checks of terizidone and cycloserine generated from 1000 simulations. The shaded areas represent the prediction interval at 5th, 50th and 95th percentiles (95% confidence interval). The solid lines represent the empirical median of the 5th, 50th and 95th percentiles. The observed data is represented by dots

TABLE 3 Terizidone and cycloserine population pharmacokinetic parameters from a joint model

	Model estimate		Bootstrap	
Parameter	Estimate	%RSE	Median	95% CI (lower, upper)
Mtt (h)	1.43	14	1.01	0.92, 1.75
ka (h^{-1})	3.2	10.5	3.1	2.69, 3.4
Vp/F [a] (L)	14	11	13.4	12.4, 14.6
Clp [a] (L h^{-1})	0.1	12	0.08	0.05, 0.09
Fm	0.29	10	0.24	0.19, 0.33
Clm/F [a] (L h^{-1})	2.94	20.2	2.7	2.44, 3.01
Coefficient (effect) of albumin on *Vp/F*	−0.51	54.9	−0.50	−0.73, −0.47
Between-subject variability (CV%)[b]				
Mtt	75	10	83	71.2, 96
Ka	46	28	48	43, 56
Vp/F	22	15	25	91, 27
Clp	52	16.1	46.2	44, 51
Fm	27	31.2	21.8	19, 29
Clm/F	189	11	171	162, 226
Residual error				
Additive, terizidone (µmol L^{-1})	25	17.9	21	20, 24.4
Proportional, terizidone	0.04	63.1	0.04	0.04, 0.05
Additive, cycloserine (µmol L^{-1})	0.29	33.6	0.3	0.22, 0.3
Proportional, cycloserine	0.32	6.14	0.3	0.24, 0.31

[a]Allometrically scaled parameters using FFM by fixing exponents to 1 on *Vp/F* and 0.75 on *Clp* and *Clm/F*.

[b]Coefficient of variation percentage calculated as $\left(\sqrt{e^{(SD)^2}-1}\right)*100$ where SD is the estimated standard deviation.

%RSE, percentage relative standard error; CI, confidence interval; *Mtt*, mean transit time; ka, absorption rate constant; *Vp/F*, apparent volume of distribution; Cl_{tot}/F, apparent total clearance of terizidone; *Fm*, fraction of terizidone that is converted into cycloserine; *Clm/F*, apparent clearance of cycloserine.

was better than the latter. Initially, addition of a lag time or transit compartment to model the delayed absorption did not improve the model fit. This indicated a possibility of over parameterisation for the transit compartment model. When the number of parameters were reduced from 3 to 2 by setting *ka* equal to *Ktr*, this resulted in improved model fit. The average time (*Mtt*) it took from the ingestion of terizidone to its absorption was about 102 minutes (1.7 hours). It then underwent fast absorption with *ka* of 2.97 h^{-1}. Within a median T_{max} of 4 hours, terizidone reached a C_{max} of 239 µmol L^{-1}.

Adjusting for the effect of body size (allometric scaling) on *Vp/F* and Cl_{tot}/F using FFM and fixing exponents, resulted in model improvement. The FFM was the best descriptor of body size as it was associated with the lowest OFV. As a result of this scaling, the BSV in *Vp/F* and Cl_{tot}/F decreased by 13.5 and 5%, respectively. The population estimates of the *Vp/F* and Cl_{tot}/F were thus estimated with good precision. Serum albumin had significant effect on the *Vp/F* and accounted for 43.2% of the variation in the *Vp/F*. Additionally, this improved the model fit. The effect was in such a way that high serum albumin concentration was significantly associated with low values of *Vp/F* or vice versa. This phenomenon is well known as increased drug binding resulting from increased serum albumin concentration tend to decrease the volume of distribution.[31,32] This effect of albumin on *Vp/F* may have potential clinical impact on patients with hepatic impairment.

The pharmacokinetic parameters (*Mtt*, *ka* and *Vp/F*) of the joint model, although not exactly the same, were similar to those of terizidone model. Additionally, these parameters were estimated with good precision. In the joint model, it was clearly observed that the

TABLE 4 Summary of the secondary pharmacokinetic parameters of terizidone and cycloserine

Pharmacokinetic parameters	Median and range
Terizidone	
C_{max} (μmol L^{-1})	239 (64.2–520)
T_{max} (h)	4 (2–8)
$AUC_{0\text{-}24h}$ (μmol h L^{-1})	1635 (483–8954)
Half-life (h)	17.8 (9–45)
Clpm (L h^{-1})	0.47 (0.05–1.88)
Cycloserine	
C_{max} (μmol L^{-1})	24.1 (0.54–63.5)
T_{max} (h)	8 (3–8)
$AUC_{0\text{-}24h}$ (μmol h L^{-1})	203 (3.6–99)
Half-life (h)	2.49 (0.32–11.9)

C_{max}, maximum plasma concentration; T_{max}, time to reach C_{max}; $AUC_{0\text{-}24h}$, area under the concentration–time curve up to 24 hours; *Clpm*, apparent clearance of terizidone due to biotransformation into cycloserine.

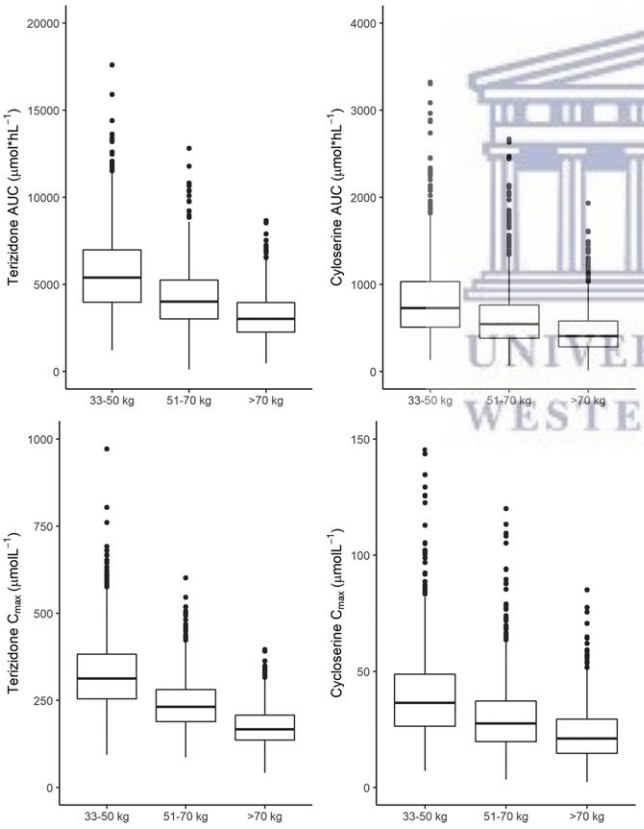

FIGURE 6 Box plots of simulated area under the concentration–time curve up to 24 hours (AUC) and maximum plasma concentration (C_{max}) for the current recommended terizidone daily dose of 750 mg stratified by weight band

estimated *Clp* (0.1 L h^{-1}) was lower than *Clpm* (0.47 L h^{-1}). This implied that terizidone clearance via biotransformation into cycloserine was higher than clearance via other routes. The closeness of the sum value

TABLE 5 Proposed dosing schedule of terizidone across 3 weight bands

Weight band (kg)	Dose (mg)[a]	Simulated exposure	
		$AUC_{0\text{-}24h}$ (μmol h L^{-1})[b]	C_{max} (μmol L^{-1})[b]
33–50	750	4929 (3694–6317)	278 (228–336)
51–70	900	5045 (3929–6392)	278 (227–335)
> 70	1200	5047 (3926–6381)	279 (228–335)

[a]Dosed every 24 hours.

[b]Values expressed as median and interquartile range.

of *Clp* and *Clpm* (0.57 L h^{-1}) and Cl_{tot}/F (0.51 L h^{-1}) indicated that the joint model was able to discriminate well between the *Clpm* driven by *Fm* and *Clp*. On average, 29% of the total amount of terizidone in the body was being converted to cycloserine, suggesting that 71% of the remaining was eliminated via other routes. However, this was not the absolute but apparent fraction, owing to the unavailability of urine data. No covariates tested had significant effect on *Clp*, *Fm* and *Clm/F*. Meanwhile, there was high BSV observed in *Clm/F* and *Mtt*, which could not be explained by the covariates tested. The pharmacokinetic parameters of terizidone in our study are different from the previously reported,[13] where blood was sampled after a single dose or before steady state. In the previous study,[13] *Vp/F* and Cl_{tot}/F were higher (245.6 L, 6.4 L h^{-1}) than in our study (13.4 L, 0.51 L h^{-1}). Meanwhile, in the study by Zitkova and Toušek,[13] the observed average C_{max} resulting from 750 mg dose of terizidone was very different from the C_{max} that we simulated using their model (approximately 19 *vs* 6.1 μg mL^{-1}, respectively). It is noteworthy that the method[33] Zitkova and Toušek[13] used to determine terizidone concentrations was validated for cycloserine and not terizidone. In their study,[13] terizidone was not determined directly but estimated based on the cycloserine concentration. Therefore, concentrations might have been inaccurately estimated and lead to incorrect estimation of terizidone pharmacokinetic parameters. These shortcomings associated with determination of terizidone in Zitkova and Toušek's study[13] may explain the differences in *Vp/F* and Cl_{tot}/F in our study.

The precision at which pharmacokinetic parameters were estimated in the joint and terizidone model was generally good. However, the %RSE in the estimation of BSV in *ka*, proportional error estimate and coefficient of albumin on *Vp/F* were slightly above 50%. There was good agreement between observed and predicted concentrations and the model predicted well the concentrations across all time points as no bias was seen in the residual plots. The visual predictive checks indicated that the joint model fitted well the observed terizidone and cycloserine concentration–time data, as most of the observed data overlapped with the simulated percentiles. Although the model showed slight over prediction in the absorption phase, the bootstrap parameters were comparable with those estimated from the original data set. Therefore, the final joint model without covariates that was used to estimate the C_{max}, T_{max}, $AUC_{0\text{-}24h}$, half-life and *Clpm* was appropriate.

The local treatment guideline for the management of drug-resistant tuberculosis[17] recommends terizidone daily dose of 750 mg for patients in the weight bands 33–50 and 51–70 kg and 750–1000 mg for patients >70 kg. Although no information on the target $AUC_{0\text{-}24h}$ or C_{max} for terizidone is available from the literature, model-based simulations show that a 750 mg daily does not achieve similar $AUC_{0\text{-}24h}$ or C_{max} across the 3 weight bands. Therefore, we propose a terizidone daily dose of 900 and 1200 mg for patients in the weight bands 51–70 and > 70 kg, respectively. This would ensure the achievement of a similar exposure in patients weighing 33–50 kg and taking 750 mg of the drug.

In conclusion, we report, for the first time the population pharmacokinetics of terizidone and its metabolite cycloserine in patients with drug-resistant tuberculosis. We characterised the secondary pharmacokinetic parameters of terizidone and cycloserine. High serum albumin concentration was significantly associated with low *Vp/F* in this patient population. The FFM was found to be the best descriptor of body size and most ideal for body size effect adjustment. On average, 29% of the terizidone amount in the body was converted into cycloserine. The low Cl_{tot}/F or long half-life supports once daily dosing of terizidone in drug-resistant tuberculosis patients.

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COMPETING INTERESTS

There are no competing interests to declare.

CONTRIBUTORS

M.M. did the modelling, interpreted pharmacokinetic data, and drafted the manuscript. P.M. conceived and designed the project, collected blood samples and patient information, conducted laboratory tests, and co-drafted the manuscript. Both authors approved the final draft.

ORCID

Mwila Mulubwa https://orcid.org/0000-0003-2404-9717

Pierre Mugabo https://orcid.org/0000-0001-5653-0551

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APPENDIX A

AUC computation of parent and metabolite for 1-compartment model with first-order absorption and elimination with parameters Mtt, ka, Vp, Cl, Fm and Clm using ODE.

```
[LONGITUDINAL]
input = {Mtt, ka, Vp, Cl, Fm, Clm}

PK:
depot (target = Ad, Mtt, Ktr = ka, ka)

EQUATION:
odeType = stiff
Vm = 10.5
kp = Cl/(Vp*(1-Fm))
kt = Cl/(Vp*Fm)
km = Clm/Vm

;Initial conditions
t_0 = -120
Ad_0 = 0
Apc_0 = 0
Am_0 = 0

;Differential equations
ddt_Ad = -ka*Ad
ddt_Apc = ka*Ad - kp*Apc - kt*Apc
ddt_Am = kt*Apc - km*Am
ddt_AUCp = 1/Vp * Apc
ddt_AUCm = 1/Vm * Am
if(t < 24)
   AUC24p = AUCp
end
if(t < 48)
   AUC48p = AUCp
end
AUC24_48p = AUC48p - AUC24p
if(t < 24)
   AUC24m = AUCm
end
if(t < 48)
   AUC48m = AUCm
end
AUC24_48m = AUC48m - AUC24m

;other PK parameters
T_HalfTZ = log(2)/(kp + kt)
T_HalfCS = log(2)/km
Cl_TZtrans = kt*Vp

Cp = Apc/Vp
Cm = Am/Vm

OUTPUT:
output = {Cp, Cm}
table = {AUC24_48p, AUC24_48m, T_HalfTZ, T_HalfCS, Cl_TZtrans}
```

Chapter Seven

In this chapter, a research paper entitled:

"Amount of Cycloserine Emanating from Terizidone Metabolism and Relationship with Hepatic Function in Patients with Drug-Resistant Tuberculosis"

is presented. It was published as an original research article in the *Drugs in R&D*, 2019; 19:289–296. https://doi.org/10.1007/s40268-019-00281-4. It has been re-used in this thesis under the terms of the Creative Commons Attribution-NonCommercial 4.0 International License (http://creativecommons.org/licenses/by-nc/4.0/), which permits any noncommercial use, distribution, and reproduction in any medium. The Creative Commons license statement from Springer Nature (Publisher) is included as Appendix 5.

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ORIGINAL RESEARCH ARTICLE

Amount of Cycloserine Emanating from Terizidone Metabolism and Relationship with Hepatic Function in Patients with Drug-Resistant Tuberculosis

Mwila Mulubwa[1] · **Pierre Mugabo**[1]

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Abstract

Background and objectives The dosing of cycloserine and terizidone is the same, as both drugs are considered equivalent or used interchangeably. Nevertheless, it is not certain from the literature that these drugs are interchangeable. Therefore, the amount of cycloserine resulting from the metabolism of terizidone and the relationship with hepatic function were determined.

Methods This prospective clinical study involved 39 patients with drug-resistant tuberculosis admitted for an intensive phase of treatment. Cycloserine pharmacokinetic parameters for individual patients, like area under the curve (AUC), clearance (CLm/F), peak concentration (C_{max}) and trough concentration (C_{min}), were calculated from a previously validated joint population pharmacokinetic model of terizidone and cycloserine. Correlation and regression analyses were performed for pharmacokinetic parameters and unconjugated bilirubin (UB), conjugated bilirubin (CB), albumin, the ratio of aspartate transaminase to alanine aminotransferase (AST/ALT), or binding affinity of UB to albumin (K_{af}), using R statistical software version 3.5.3.

Results Thirty-eight patients took a daily dose of 750 mg terizidone, while one took 500 mg. The amount of cycloserine [median (range)] that emanated from terizidone metabolism was 51.6 (0.64–374) mg. C_{max} ($R^2 = 22\%$, $p = 0.003$) and C_{min} ($R^2 = 10.6\%$, $p = 0.044$) were significantly associated with increased CB concentration. C_{max} was significantly associated with increased K_{af} ($R^2 = 10.1\%$, $p = 0.048$), while high CLm/F was significantly associated with decreased AST/ALT ($R^2 = 21\%$, $p = 0.003$).

Conclusions Cycloserine is not interchangeable with terizidone, as amounts are lower than expected. Cycloserine may be a predisposing factor to the development of hyperbilirubinaemia, as CLm/F is affected by hepatic function.

Key Points

Terizidone is not completely metabolised into cycloserine in patients with drug-resistant tuberculosis.

Cycloserine and terizidone cannot be used interchangeably.

Cycloserine and terizidone exposure may be a predisposing factor to the development of jaundice in patients with drug-resistant tuberculosis.

✉ Mwila Mulubwa
3579753@myuwc.ac.za; mwila.mulubwa@gmail.com

[1] School of Pharmacy, University of the Western Cape, Private Bag X17, Bellville, Cape Town 7535, South Africa

1 Introduction

Drug-resistant tuberculosis remains a public health crisis, and treatment success continues to be low, at 55% worldwide [1]. Cycloserine is among the recommended group C second-line drugs for treatment of drug-resistant tuberculosis [2]. One of its advantages is that it does not share cross-resistance with other anti-tuberculosis drugs in the regimen, although it is associated with neurological side effects [3]. Its antimycobacterial bacteriostatic effect is achieved through inhibition of the enzymes D-alanine ligase and alanine racemase, which are both essential for the biosynthesis of peptidoglycan, a bacterial cell wall component [4]. The susceptibility breakpoint is 64 µg/mL, and efficacy is driven by the percentage of time the plasma concentration is above the minimum inhibitory concentration. Nevertheless, the doses likely to achieve bactericidal effect in patients could be neurotoxic, as they are high [5].

Terizidone, which is a condensation product of two molecules of cycloserine joined by a terephthalaldehyde moiety, is also part of the recommended group C second-line drugs for treatment of drug-resistant tuberculosis [2, 6]. The metabolism of terizidone has not been characterised. However, it is thought to convert into cycloserine and para-phthalate, likely by hydrolysis of imine groups pre-systemically [7, 8]. Terephthalaldehyde in humans has been shown to undergo quick metabolism into terephalic acid, which is a more stable metabolite and readily excreted in urine [9]. However, the enzymes involved in these metabolic processes are not known. The in vivo metabolism of cycloserine, a structural analogue of amino acid D-alanine [6], has not been characterised in humans. Meanwhile, in vitro studies show that D- or L-cycloserine in the presence of alanine racemase undergo racemisation followed by transamination to form a stable isoxazole by irreversible tautomerisation of the intermediate substrate, ketimine [10].

In patients with drug-resistant tuberculosis, a dose of 250–500 mg of cycloserine administered orally twice daily is slowly absorbed, with an absorption rate constant of 0.135 h^{-1}, and reaches the average maximum plasma concentration of 22 µg/mL within 4 h [11]. It is widely distributed to most body fluids (bile, synovial fluid, breast milk, sputum) and tissues (lymphatic tissues and lungs) and crosses the placenta, with the apparent distribution of 10.5 L. Cycloserine apparent clearance is 1.38 L/h. It is primarily excreted in urine, with 50 and 70% excreted unchanged in 12 and 24 h, respectively. The elimination half-life is 5.27 h. The between-subject variation in apparent clearance and distribution volume is 22.3 and 35.1%, respectively [11, 12].

Treatment of drug-resistant tuberculosis with a multidrug regimen consisting of cycloserine/terizidone and other second-line drugs is associated with adverse reactions [13]. Hepatotoxicity, nephrotoxicity and hypokalaemia are the most possible life-threatening adverse reactions that require alteration of the drug regimen or temporal withdraw [14]. Furthermore, mortality is high when hepatotoxicity is accompanied by jaundice, encephalopathy and ascites [15].

Cycloserine and terizidone are considered equivalent, and doses are currently used interchangeably [7, 8]. Owing to the unavailability of bioequivalence or mass balance studies of terizidone, the equivalence of dosing has not yet been established [8]. The objective of this study was to determine the average amount of cycloserine that results from the metabolism of terizidone in patients with drug-resistant tuberculosis. We also assessed the potential effect of cycloserine and terizidone exposure on the incidence of hepatotoxicity or nephrotoxicity in these patients.

2 Methods

The details of the study design, including the study population, drug administration, ethics, inclusion and exclusion criteria, and population pharmacokinetic modelling, have been described elsewhere [16]. Briefly, this was a prospective clinical study of patients admitted for intensive phase treatment of drug-resistant tuberculosis. They were taking 500–750 mg of terizidone once daily, and other second-line anti-tuberculosis drugs (ethionamide, pyrazinamide, moxifloxacin, ethambutol, isoniazid and kanamycin) were administered according to the local treatment guideline [17]. Blood for pharmacokinetics study was sampled at baseline and 0.5, 1, 2, 3, 3.5, 4, 8, 16 and 24 h after drug administration. Separate blood samples were also drawn for liver [total bilirubin, conjugated bilirubin, unconjugated bilirubin, albumin, alanine aminotransferase (ALT), aspartate transaminase (AST)] and renal function markers (serum creatinine). Estimated glomerular filtration rate (eGFR) [18] and creatinine clearance (CrCL) [19] were calculated, and demographic information including HIV status was obtained from each patient.

2.1 Determination of Cycloserine and Terizidone in Plasma

Cycloserine concentration in plasma was determined using an ultra-performance liquid chromatography tandem mass spectrometry method. Methanol was used to extract cycloserine and propranolol (internal standard) from plasma by a protein precipitation method. Acidified acetonitrile with 0.1% formic acid was used as the mobile phase, and chromatographic separation was achieved on a Phenomenex PFP reversed phase column. The lower limit of quantification and limit of detection were 0.01 µg/mL and 0.004 µg/mL, respectively. The average intra-day precision was 10.2%, while inter-day was 7.3%. Accuracy ranged between 98.7 and 117.3%, and the coefficient of determination was 0.9994. Cycloserine was stable after three freeze–thaw cycles, and extraction efficiency was in the range 88.7–91.2% [20]. Terizidone was analysed using a high-performance liquid chromatography with UV detector (HPLC-UV) method. The within- and between-run accuracy was in the range 99.7–112.7% and 100.5–107.4%, respectively. The intra- and inter-day precision, measured as percentage relative standard deviation, ranged between 0.35 and 9.4% and 1.48 and 6.79%, respectively. The lower limit of quantification was 3.125 µg/mL, while the limit of detection was 0.78 µg/mL. The coefficient of determination value ranged between 0.9988 and 0.9999, and calibration curves were linear [21].

2.2 Pharmacokinetic Modelling

The concentration–time profiles of terizidone and its metabolite cycloserine were jointly modelled using non-linear mixed effects implemented in Monolix 2018R1 software [22]. The final joint model without covariates was used to estimate the biotransformation rate constant (ktr) describing the metabolism of terizidone into cycloserine and cycloserine clearance (CLm/F). The model and the validation details have been described elsewhere [16].

2.3 Calculation of the Amount of Cycloserine and Other Pharmacokinetic Parameters

The following formulae were coded and added to the Monolix MLXTRAN model script of the final joint model for the computation of parameters. The amount of cycloserine was calculated as shown in Eq. (1):

$$\text{Dose} = \text{AUC} \times \text{Clm/F}, \tag{1}$$

where dose is the amount of cycloserine, AUC is the area under the concentration–time profile of cycloserine from 0 to 24 h, and CLm/F is the apparent clearance of cycloserine.

The AUC was calculated by integrating the concentration from 0 to 24 h according to Eq. (2):

$$\text{AUC} = \int_0^{24} C(t)\mathrm{d}t, \tag{2}$$

where C is cycloserine concentration.

Cycloserine half-life was calculated according to Eq. (3):

$$\text{Cycloserine half life} = \frac{\ln(2)}{\text{ke}}, \tag{3}$$

where ke = (Clm/F)/10.5.

The distribution volume of cycloserine was fixed to a literature value of 10.5 L [11] when estimating CLm/F. This was done in order to overcome the non-identifiability parameter problem encountered in parent–metabolite pharmacokinetic models of orally administered drugs [23].

The biotransformation half-life was calculated as shown in Eq. (4):

$$\text{Biotransformation half-life} = \frac{\ln(2)}{\text{ktr}}. \tag{4}$$

The peak (C_{max}) and trough (C_{min}) concentrations were obtained from the observed data for both cycloserine and terizidone concentration–time profiles. The individual predicted values for terizidone clearance due to biotransformation (CLtm/F) and other routes (CLto/F) were computed in Monolix and extracted from the output file.

2.4 Calculation of Binding Affinity of Unconjugated Bilirubin to Albumin and AST/ALT Ratio

The strength of unconjugated bilirubin binding to albumin was calculated according to Eq. (5) [24]:

$$K_{af} = \frac{\text{TB} - \text{UB}}{\text{UB} \times \text{albumin} - \text{TB} + \text{UB}}, \tag{5}$$

where K_{af} is the binding affinity, with the units L/μmol, and TB and UB are total bilirubin and unconjugated bilirubin, respectively. The AST/ALT ratio [25], a marker reflecting alterations in hepatic function, was calculated by dividing the AST value by the ALT value for each patient.

2.5 Statistical Analysis

Mann–Whitney U test was performed to determine differences in the demographic information stratified by HIV status. We performed Spearman's correlations between hepatic or renal function (eGFR/CrCL) markers and pharmacokinetic parameters (C_{min}, C_{max}, CLm/F, CLtm/F and CLto/F). This was done in order to determine whether a statistically significant linear relationship existed, as cycloserine or terizidone could bind to albumin or affect the binding affinity of unconjugated bilirubin to albumin. Furthermore, cycloserine concentration may be affected by alterations in hepatic or renal function. If the correlations were significant, linear stepwise regression analysis was performed in order to determine if pharmacokinetic parameters were significantly predicting hepatic or renal function markers. A two-tailed p value of ≤ 0.05 was considered statistically significant. The analysis was performed in the R version 3.5.3 statistical environment [26].

3 Results

Thirty-nine drug-resistant tuberculosis patients (20 females and 19 males) participated in this study. Out of the seven patients with comorbidities, three had cryptococcal meningitis, while each of the remaining four had deep venous thrombosis, hyperlipidaemia, gastric acid or epilepsy. Thirty-eight out of 39 patients took a daily dose of 750 mg terizidone, while one patient took 500 mg. Table 1 shows the summary of demographic information. The HIV-infected patients had significantly lower albumin than their HIV-uninfected counterparts (30 vs. 35.5 g/L; $p = 0.041$). However, the rest of the demographic variables were similar in HIV-infected and HIV-uninfected groups.

Table 1 Patients' demographic information

Variables	HIV infected ($n=27$)[a]	HIV uninfected ($n=12$)[a]	*P* value
Age (years)	31 (17–44)	34 (20–56)	0.46
Weight (kg)	51.4 (32.4–64)	50 (39.8–71)	0.69
BMI (kg/m^2)	18.5 (12.4–23.5)	17.9 (15–26.1)	0.8
Albumin (g/L)	30 (15–45)	35.5 (26–48)	0.041*
ALT (IU/L)	11 (4–46)	10.5 (4–23)	0.47
AST (IU/L)	36 (20–109)	26.6 (17–76)	0.053
Conjugated bilirubin (μmol/L)	1 (1–8)	1 (1–9)	0.86
Unconjugated bilirubin (μmol/L)	5 (1–17)	7 (2–11)	0.42
Total bilirubin (μmol/L)	6 (2–24)	8 (3–20)	0.69
AST/ALT ratio	3.2 (0.8–8.7)	2.7 (1.9–5.9)	0.39
K_{af} (L/μmol)	146 (70.9–710)	128 (75.5–377)	0.84
CrCL (mL/min)	86.4 (34.4–128)	77.8 (55.4–113)	0.41
eGFR (mL/min/1.73 m^2)	118 (46–228)	104 (74.9–192)	0.57

ALT alanine aminotransferase, *AST* aspartate transaminase, *BMI* body mass index, *CrCL* creatinine clearance, *eGFR* estimated glomerular filtration rate, K_{af} binding affinity

*Statistically significant

[a]The values in the column are reported as median and range

3.1 Amount of Cycloserine and Pharmacokinetic Parameters

The summary of the calculated amount of cycloserine or dose resulting from terizidone metabolism and the values for pharmacokinetic parameters is shown in Table 2. There was wide variation in cycloserine dose and also in the pharmacokinetic parameters, except for cycloserine half-life, for which the variation was relatively narrow. Table 3 shows the summary of terizidone pharmacokinetic parameters.

Table 3 Pharmacokinetic parameters for terizidone

Parameter	Median (range)
C_{min} (μmol/L)	88 (14.3–307)
C_{max} (μmol/L)	247 (61–583)
CLtm/F (L/h)	0.29 (0.05–1.14)
CLto/F (L/h)	0.11 (0.035–0.31)

CLtm/F terizidone clearance due to biotransformation, *CLto/F* terizidone clearance via other routes, C_{max} peak concentration, C_{min} trough concentration

3.2 Correlations Between Hepatic Function Markers and Pharmacokinetic Parameters of Cycloserine and Terizidone

Significant positive correlations existed between AST, ALT, conjugated bilirubin, K_{af} and cycloserine pharmacokinetic parameters (C_{min}, C_{max} or CLm/F), as shown in Table 4.

Table 2 Cycloserine dose and pharmacokinetic parameters

Parameter	Median (range)
Cycloserine dose (mg)	51.6 (0.64–374)
C_{max} (μmol/L)	28.6 (0.49–69.4)
C_{min} (μmol/L)	9.1 (0.15–36.3)
AUC (μmol h/L)	201 (3.07–983)
Biotransformation half-life (h)	22.6 (10.2–216)
CLm/F (L/h)	2.6 (0.64–21)
Cycloserine half-life (h)	2.78 (0.34–11.3)

AUC area under the curve, *CLm/F* apparent clearance of cycloserine, C_{max} peak concentration, C_{min} trough concentration

There was significant negative correlation between AST/ALT ratio and CLm/F ($p=0.016$). Meanwhile, there was no significant correlation between albumin or unconjugated bilirubin and the pharmacokinetic parameters ($p>0.05$). Similarly, no significant correlations existed between renal function markers (CrCL and eGFR) and CLm/F ($p>0.05$). Significant positive correlations existed between terizidone C_{max} and conjugated or unconjugated bilirubin ($p=0.038$ and 0.02, respectively), as shown in Table 5.

3.3 Pharmacokinetic Parameters Predictive of Hepatic Function Markers

The cycloserine C_{max} was found to be a significant predictor of both conjugated bilirubin and K_{af}, as shown by regression plots b and c of Fig. 1. The associations were in such a way that an increase in C_{max} resulted in a significant increase in conjugated bilirubin and K_{af} (plot b, $\beta=0.06$, $R^2=22\%$ and $p=0.003$; plot c, $\beta=2.3$, $R^2=10.1\%$ and $p=0.048$). C_{min}

Table 4 Correlations between cycloserine pharmacokinetic parameters and liver function markers

	C_{min} (µmol/L)	C_{max} (µmol/L)	CLm/F (L/h)
AST (IU/L)	$r=0.26$	$r=0.34$*	$r=-0.06$
ALT (IU/L)	$r=0.15$	$r=0.11$	$r=0.35$*
Conjugated bilirubin (µmol/L)	$r=0.36$*	$r=0.47$**	$r=0.07$
Unconjugated bilirubin (µmol/L)	$r=0.12$	$r=0.15$	$r=0.09$
K_{af} (L/µmol)	$r=0.38$*	$r=0.46$**	$r=-0.02$
AST/ALT ratio	$r=0.1$	$r=0.2$	$r=-0.38$*

ALT alanine aminotransferase, *AST* aspartate transaminase, *CLm/F* apparent clearance of cycloserine, C_{max} peak concentration, C_{min} trough concentration, K_{af} binding affinity, *r* Spearman's ρ correlation coefficient
*$p<0.05$, **$p<0.001$

Table 5 Correlations between terizidone pharmacokinetic parameters and liver function markers

	C_{min} (µmol/L)	C_{max} (µmol/L)	CLtm/F (L/h)	CLto/F (L/h)
AST (IU/L)	$r=0.06$	$r=0.15$	$r=-0.13$	$r=-0.06$
ALT (IU/L)	$r=0.16$	$r=0.14$	$r=0.1$	$r=0.05$
Conjugated bilirubin (µmol/L)	$r=0.22$	$r=0.33$*	$r=0.13$	$r=0.06$
Unconjugated bilirubin (µmol/L)	$r=0.25$	$r=0.37$*	$r=-0.01$	$r=-0.01$
K_{af} (L/µmol)	$r=0.07$	$r=0.1$	$r=-0.07$	$r=-0.15$
AST/ALT ratio	$r=-0.1$	$r=-0.06$	$r=0.16$	$r=-0.17$

ALT alanine aminotransferase, *AST* aspartate transaminase, *CLtm/F* terizidone clearance due to biotransformation, *CLto/F* terizidone clearance via other routes, C_{max} peak concentration, C_{min} trough concentration, K_{af} binding affinity, *r* Spearman's ρ correlation coefficient
*$p<0.05$

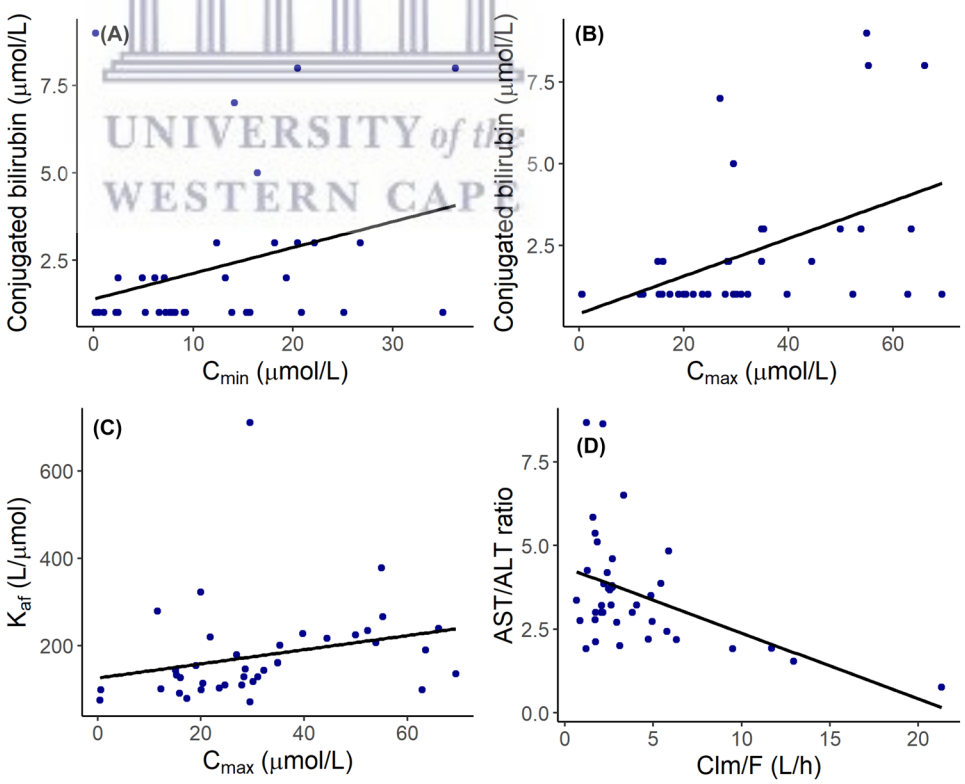

Fig. 1 Regression plots of cycloserine pharmacokinetic parameters predictive of conjugated bilirubin, AST/ALT ratio and K_{af}. *ALT* alanine aminotransferase, *AST* aspartate transaminase, K_{af} binding affinity, *CLm/F* apparent clearance of cycloserine, C_{max} peak concentration, C_{min} trough concentration

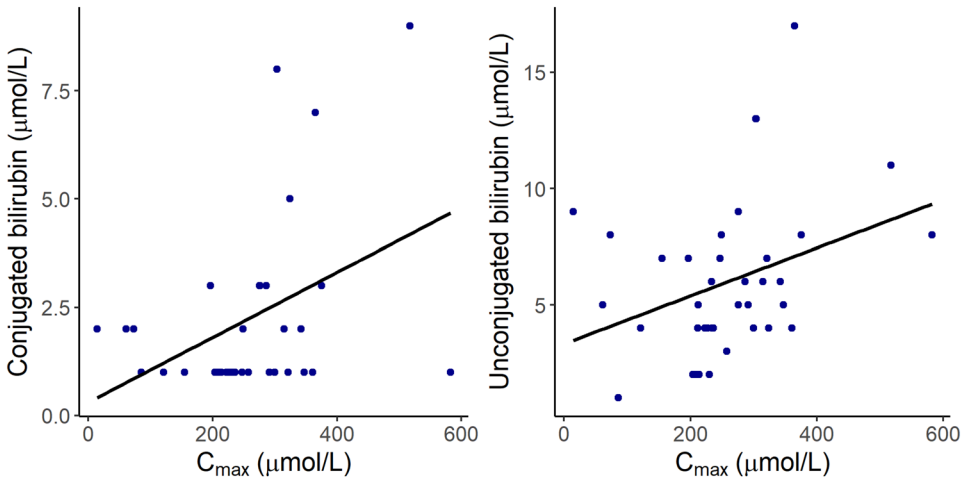

Fig. 2 Regression plots of terizidone pharmacokinetic parameters predictive of conjugated and unconjugated bilirubin. C_{max} peak concentration

could not significantly predict K_{af} in the regression analysis, although a significant correlation existed between C_{min} and K_{af} in correlation analysis. Meanwhile, increase in C_{min} was significantly associated with an increase in conjugated bilirubin (plot a, $\beta = 0.1$, $R^2 = 10.6\%$ and $p = 0.044$). The CLm/F significantly predicted AST/ALT ratio, as shown in plot d of Fig. 1. Increase in CLm/F was significantly associated with a decrease in AST/ALT ratio ($\beta = -0.2$, $R^2 = 21\%$ and $p = 0.003$). Terizidone C_{max} significantly predicted concentration of conjugated bilirubin ($\beta = 0.008$, $R^2 = 14.2\%$ and $p = 0.018$) as well as unconjugated bilirubin ($\beta = 0.012$, $R^2 = 11.4\%$ and $p = 0.035$). The regression plot is shown in Fig. 2.

4 Discussion

This study aimed to determine the amount of cycloserine that results from the metabolism of terizidone and the relationship between cycloserine exposure parameters and hepatic or renal function markers in patients receiving treatment for drug-resistant tuberculosis. There appears to be no studies in the literature that have quantified the amount of cycloserine as a terizidone metabolite in drug-resistant tuberculosis patients. Terizidone is thought to undergo complete hydrolysis into cycloserine pre-systemically [7]. Since one mole of terizidone has two moles of cycloserine and a mole of terephthalaldehyde moiety, it is expected for a 750-mg daily dose of terizidone to produce 507 mg of cycloserine, with an assumption that bioavailability is near 100%. In the current study, the median dose of cycloserine produced from a 750-mg dose of terizidone was 51.6 mg, which was lower than expected. This observation clearly suggests that terizidone is not completely hydrolysed to cycloserine pre-systemically. Furthermore, terizidone was detected systemically over a period of 24 h after its administration, and on average, only 29% of the total dose was metabolised to cycloserine [16].

The clinical implication of this finding in the current study is that cycloserine and terizidone should not be used interchangeably, as exposure parameters (C_{max}, C_{min} and AUC) may be significantly lower in patients taking terizidone than those taking cycloserine. Owing to the low amount of cycloserine produced from terizidone, dose optimisation or therapeutic drug monitoring, contrary to what some authors imply [27], should be based on terizidone and not cycloserine concentration. Additionally, in patients treated with terizidone, Court et al. [28] reported higher cycloserine C_{max} than in the current study. The difference could have been that in the Court et al. study [28], there were patients who had renal insufficiency, which could have led to accumulation of cycloserine, as it primarily undergoes renal elimination [6]. Furthermore, the bioavailability and biotransformation rate of terizidone into cycloserine in the current study could have been lower than in the Court et al. [28] study. We cannot overlook the possibility of pharmacokinetic interactions with co-administered drugs in the current study.

Bilirubin, a pigment derived from the breakdown of haemoglobin, increases in blood because of an imbalance between its production and excretion. Since unconjugated bilirubin is not water soluble, it is bound to albumin and transported to the liver, where it is made soluble or conjugated with glucuronic acid and subsequently undergoes biliary excretion into the gastrointestinal tract [29]. Increased serum levels of conjugated bilirubin are an indication of hepatic dysfunction or liver disease, clinically manifested as jaundice [25]. In the current study, cycloserine C_{min} and C_{max} as well as terizidone C_{max} were significantly associated with increased concentration of conjugated bilirubin.

Although the concentration of conjugated bilirubin in most of the patients was within the normal range, this observation implies that high plasma cycloserine or terizidone concentrations may be associated with hyperbilirubinaemia in some patients.

Unconjugated bilirubin is insoluble in water and hence cannot be excreted via the kidney. It is instead bound to albumin and transported to the liver for conjugation. Increased serum levels of unconjugated bilirubin indicate that there is an accumulation in the bloodstream due to haemolysis, which may be caused by haemolytic anaemia, hepatocellular dysfunction (e.g. hepatitis) and cirrhosis in which the excretory function of the hepatocytes is impaired [25]. Some drugs have been shown to compete with unconjugated bilirubin for binding to albumin, which leads to increased concentration of unconjugated bilirubin and causes encephalopathy in neonates [30–33]. Therefore, the binding affinity of unconjugated bilirubin to albumin is of clinical importance. Cycloserine C_{max} in the current study was associated with increased K_{af}, while C_{min} results were insignificant. This means that high cycloserine concentrations improve the binding affinity of unconjugated bilirubin to albumin, which is potentially a desired characteristic in bilirubin metabolism. The observed relationship between K_{af} and C_{max} may also imply that cycloserine is less bound to albumin.

The AST/ALT ratio is a non-invasive diagnostic index used to predict liver cirrhosis or fibrosis, and a value higher than 1 is indicative of non-alcoholic fatty liver disease [34, 35]. Cycloserine is primarily renally cleared, with 70% excreted unchanged [12], while the other portion is metabolised in the liver. However, the enzymes specifically involved in the catabolism of cycloserine in vivo are not known. In the current study, higher CLm/F was significantly associated with low AST/ALT ratio. Additionally, the median value of the AST/ALT ratio was more than 2. This was an obvious indication that liver disease affects hepatic function, which leads to low activity or production of cycloserine-metabolising enzymes and eventually results in low CLm/F. Meanwhile, the non-correlation between CLm/F and CrCL or eGFR in the current study was unexpected, as cycloserine is also cleared via the renal route. This observation could suggest a possibility of more cycloserine undergoing active tubular secretion than glomerular filtration, as seen in other drugs [36].

This study has some limitations to consider. In vitro drug plasma binding studies were not performed due to ethical issues as we were supposed to use drug-free human plasma. Cycloserine metabolites were not measured in plasma; this could have helped to determine the rate of cycloserine metabolism in vivo. Furthermore, cycloserine was not measured in patients' urine for determination of the fraction of the dose that is excreted unchanged. We only measured cycloserine and terizidone in plasma. Since patients were on a multidrug treatment regimen, other anti-tuberculosis drugs could have shown similar results as cycloserine and terizidone. The study, however, is important as it gives an insight into how cycloserine and terizidone exposure might affect hepatic function. This can guide the clinical decision regarding whether or not to withdraw terizidone in the case of hepatotoxicity.

5 Conclusions

In conclusion, the amount of cycloserine resulting from the metabolism of terizidone in drug-resistant tuberculosis patients was lower than expected. High concentrations of cycloserine could potentially favour bilirubin disposition, albeit be a predisposing factor to the development of jaundice in drug-resistant tuberculosis patients taking terizidone. Cycloserine clearance is reduced in hepatic dysfunction.

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Compliance with Ethical Standards

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Conflict of interest The authors, Mwila Mulubwa and Pierre Mugabo, declare that they have no competing interests.

Ethical approval This study was approved by the ethics committee of the University of the Western Cape (Ref: 07/6/12) and the University of Cape Town (Ref: 777/2014). The study was conducted according to the principles outlined in the declaration of Helsinki.

Informed consent Written informed consent was obtained from all individual participants included in this study.

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Chapter Eight

8.0 INTRODUCTION

In this chapter, a brief general discussion of important findings from chapter four, five, six and seven are presented. These findings are with regard to the developed methods for bioanalysis of terizidone and cycloserine in plasma. Other findings concern the population pharmacokinetics of terizidone and cycloserine as well as the use of cyloserine and terizidone interchangeably. Otherwise, the detailed discussion has already been outlined in these chapters.

8.1 Quantification of terizidone in plasma

There appears to be only one method in literature in which terizidone was quantified in plasma (Zitkova and Toušek, 1974) using colorimetric method (Jones, 1956). In this method, terizidone was not measured directly but estimated based on its metabolite cycloserine. The principle behind colorimetric method is the measurement of optical absorbance of a coloured reaction product between analyte and suitable reagents using light (Benhabib and Li, 2013). Colorimetric method of analysis is not suitable for pharmacokinetic studies as interfering substances can produce similar colours and cause errors in the results (Armstrong and Kuder, 1935). Furthermore, colorimetric method is outdated (World Health Organization, 2018). Additionally, in colorimetry, different compounds exhibit variances that can affect the accuracy of the results (Scott, 2017). Chromatographic methods are commonly used in pharmacokinetic studies as they are the most versatile bioanalytical techniques and able to produce highly accurate and precise results (USFDA, 1999, ICH). In Chapter four (Mulubwa and Mugabo, 2018), a chromatographic method (HPLC-UV) for determination of terizidone in plasma was developed and validated. This was the first-ever chromatographic method to be developed for analysis of terizidone. In this developed method (Mulubwa and Mugabo, 2018), terizidone was accurately quantified unlike in the colorimetric method where it was estimated based on cycloserine (Jones, 1956).

In Chapter four (Mulubwa and Mugabo, 2018), terizidone was detected and quantified in patients' plasma samples. However, this was contrary to the proposition by the World Health Organization that terizidone is not measurable in plasma as it is thought to hydrolyse completely into cycloserine pre-systemically (World Health Organization, 2015). The proposition was based on the fact that there were no analytical methods in literature in which terizidone was directly measured in plasma and not estimated based on its primary metabolite cycloserine. Therefore, this proposition no longer holds because terizidone is measurable in plasma.

8.2 Quantification of cycloserine in plasma

There are several bioanalytical methods that have been described in literature for the determination of cycloserine in plasma using liquid chromatograph coupled to mass spectrometer (Polagani et al., 2013, Patel et al., 2011, Mao et al., 2017, Yaroshenko et al., 2014, Han et al., 2013). The aim of Chapter five was to develop a method that involved less steps in sample preparation and sensitive or ability to measure lower concentrations than those reported in literature with good accuracy and precision. The developed method in Chapter five (Mulubwa and Mugabo, 2019a) had relatively less steps in sample preparation than the previously reported methods (Han et al., 2013, Supriya et al., 2012). Furthermore, the method (Mulubwa and Mugabo, 2019a) was more sensitive (0.01 µg/mL vs. 0.05 µg/mL) than the previously reported (Polagani et al., 2013). The method was suitable for bioanalysis especially where cycloserine is expected to be in very low concentrations as terizidone metabolite.

8.3 Pharmacokinetics of terizidone and cycloserine

The aim of Chapter six (Mulubwa and Mugabo, 2019b) was to model the pharmacokinetics of terizidone and cycloserine and to determine the factors that affect the pharmacokinetics of these drugs. A one-compartment model with first-order elimination best described the pharmacokinetics of terizidone. A modified transit compartment model characterised well the absorption of terizidone. Cycloserine underwent first order elimination with a shorter half-life than terizidone. Terizidone's long half-life implies that it can be dosed once daily and still achieve the desired plasma concentration although the target C_{max} or AUC is still unknown.

The clearance of terizidone via biotransformation is higher than clearance via other routes. This finding has clinical implications in patients who may have reduced hepatic function due to liver disease. Although the enzymes involved in terizidone metabolism are not known, patients with liver disease may experience toxic effects as a result of accumulation of terizidone in plasma due to reduced hepatic clearance. The inverse relationship between terizidone distribution volume and albumin may have clinical implications. It may imply that in patients with low albumin concentration due to a liver disease, the exposure pharmacokinetic parameters such as C_{max} and C_{min} may reduce due to an increase in terizidone distribution volume. This has potential to cause resistance owing to low plasma drug exposure.

The fraction of the total amount of terizidone in the body that was converted to cycloserine, on average was 29% (Mulubwa and Mugabo, 2019b). This observation clarifies the contrary view of the World Health Organization that terizidone is near complete (World Health Organization, 2018) or completely converted into cycloserine (World Health Organization, 2015).

8.4 Cycloserine amount resulting from terizidone metabolism

The objective of Chapter seven was first to determine the amount of cycloserine that emanates from the metabolism of terizidone in patients with drug-resistant tuberculosis. The second objective was to determine the relationship between the cycloserine pharmacokinetic parameters and markers of hepatic or renal function. The average amount of cycloserine that emanated from terizidone metabolism was far less than the expected theoretical amount. This means that terizidone and cycloserine are not equivalent. The clinical implication is that they should not be used interchangeably as exposure parameters such as C_{max}, C_{min} and AUC may be lower in patients taking terizidone than those taking cycloserine.

The ratio of aspartate transaminase to alanine aminotransferase, which relates to non-alcoholic fatty liver disease (Alexopoulou et al., 2019, Angulo, 2002), was inversely related to cycloserine clearance. This indicated that cycloserine was affected by hepatic function and implied that it was cleared by the liver in addition to the known renal clearance (Brennan et al., 2008). Meanwhile, the non-significant relationship between exposure parameters (C_{max} and C_{min}) and markers of renal function

(estimated glomerular filtration rate and creatinine clearance) implied that cycloserine was not freely filtered but secreted in renal tubules. High cycloserine concentrations promoted disposition of unconjugated bilirubin by increasing its binding affinity to albumin in order to transport it to the liver for conjugation and subsequent elimination into the bile. However, cycloserine concentration was significantly associated with increased concentration of conjugated bilirubin. The clinical implication of this observation is that cycloserine may be associated with hyperbilirubinemia as a side effect in some patients as conjugated bilirubin is what is responsible for the clinical signs of jaundice (Longo et al., 2013).

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Chapter Nine

9.0 STUDY OBJECTIVES ACCOMPLISHED

In this non-randomised clinical study, 39 patients with drug-resistant tuberculosis and in intensive treatment phase were prospectively enrolled at Brewelskloof Hospital in South Africa. All of the objectives set out in Chapter one were successfully achieved. These objectives included:

i. the development and validation of a high-performance liquid chromatography method coupled with ultraviolet detection (HPLC-UV) for determination of terizidone in plasma,
ii. the development and validation of a sensitive ultra-performance liquid chromatography tandem mass spectrometry (UPLC-MS/MS) method for determination of cycloserine in plasma,
iii. the determination of plasma concentrations of terizidone and cycloserine in patients with drug-resistant tuberculosis,
iv. modelling population pharmacokinetics of terizidone and its metabolite cycloserine in patients with drug-resistant tuberculosis,
v. the determination of the factors influencing pharmacokinetics of terizidone and cycloserine in patients with drug-resistant tuberculosis,
vi. the determination of the amount of cycloserine emanating from terizidone metabolism in patients with drug-resistant tuberculosis.

9.1 OVERALL CONCLUSIONS

The bioanalytical method (HPLC-UV) for the determination of terizidone in plasma for pharmacokinetic study was successfully developed, validated and applied in the analysis of patients' plasma samples. The method was simple, accurate and precise making it also suitable for therapeutic drug monitoring of terizidone. The UPLC-MS/MS bioanalytical method for determination of cycloserine in plasma was developed and validated. The method was sensitive or able to measure very low concentrations of

cycloserine with acceptable accuracy and precision. The method was successfully used to measure cycloserine as terizidone metabolite in patients' plasma samples.

The population pharmacokinetic of terizidone and cycloserine, has for the first time, been described in patients with drug-resistant tuberculosis. A one-compartment model with first-order elimination process best describes the pharmacokinetics of terizidone in drug-resistant tuberculosis patients. A modified transit model best characterises its absorption process. About 29% of the total amount of terizidone in the body is metabolised into cycloserine and undergoes first-order elimination process. The apparent distribution volume of terizidone in drug-resistant patients is influenced by albumin concentration, which may affect drug concentration in patients with hepatic impairment. Terizidone and cycloserine cannot be used interchangeably as the amount of cycloserine emanating from metabolism of terizidone is far lower than expected. Cycloserine plasma exposure may be a predisposing factor to development of jaundice because of its relationship with conjugated bilirubin and its apparent clearance is reduced in patients with non-alcoholic fat liver disease.

9.2 STUDY LIMITATIONS

This study addressed only the pharmacokinetics of terizidone and its metabolite cycloserine. The pharmacodynamics part, which needed to establish the minimum inhibitory concentration (MIC) of terizidone, was not performed. Consequently, the Monte Carlo simulations for terizidone dose optimization in this population of patients were not carried out.

The concentrations of both terizidone and cycloserine were not measured in urine. This information would have helped to determine the amount of each drug eliminated in unchanged form. The sample size in this study was not calculated due to lack of a population pharmacokinetic model of terizidone in literature, which could have been used to simulate and estimate the required patient sample size.

9.3 RECOMMENDATIONS

The MIC of terizidone in resistant strains of *Mycobacteria tuberculosis* needs to be determined in order to establish pharmacodynamic index for dosage optimisation in drug-resistant tuberculosis patients. Furthermore, suitable pharmacodynamic indices

such as C_{max}/MIC (Blaser et al., 1987, Preston et al., 1998), AUC/MIC (Holmes et al., 2013, Ramos-Martin et al., 2017, Zinner et al., 2001) or percentage of time that plasma concentration remains above MIC (%T> MIC) (Andes et al., 2003, McKinnon et al., 2008) for terizidone need to be determined.

Based on low clearance and long half-life, terizidone should be dosed once daily. The dosing should not be same as that of cycloserine but should be based on the appropriate pharmacodynamics index. Although albumin was significantly affecting the distribution volume of terizidone, the clinical significance of this needs to be evaluated and establish whether dose adjustment is necessary. Future studies should determine the amount of terizidone and cycloserine excreted in patients' urine. Finally, the sample size for future terizidone pharmacokinetics studies can be estimated from the model developed in this thesis.

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APPENDIX 1

MlxTran Model file

```
DESCRIPTION:
AUC computation of parent and metabolite for 1-compartment
model with first-order absorption and elimination with
parameters Mtt, ka, Vp, Cl, Fm and Clm using Ordinary
Differential Equations.

[LONGITUDINAL]
input = {Mtt, ka, Vp, Cl, Fm, Clm}

PK:
depot(target = Ad, Mtt, Ktr = ka, ka)

EQUATION:
odeType = stiff
Vm = 10.5
kp = Cl/(Vp*(1-Fm))
kt = Cl/(Vp*Fm)
km = Clm/Vm

;Initial conditions
t_0 = -120
Ad_0 = 0
Apc_0 = 0
Am_0 = 0

;Differential equations
ddt_Ad = -ka*Ad
ddt_Apc = ka*Ad - kp*Apc - kt*Apc
ddt_Am = kt*Apc - km*Am
ddt_AUCp = 1/Vp * Apc
ddt_AUCm = 1/Vm * Am


if(t < 24)
```

```
     AUC24p = AUCp
end
if(t < 48)
     AUC48p = AUCp
end
AUC24_48p = AUC48p - AUC24p
if(t < 24)
     AUC24m = AUCm
end
if(t < 48)
     AUC48m = AUCm
end
AUC24_48m = AUC48m - AUC24m

;other PK parameters
T_HalfTZ = log(2)/(kp + kt)
T_HalfCS = log(2)/km
Cl_TZtrans = kt*Vp
Cp = Apc/Vp
Cm = Am/Vm

OUTPUT:
output = {Cp, Cm}
table = {AUC24_48p, AUC24_48m, T_HalfTZ, T_HalfCS, Cl_TZtrans}
```

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Title: Amount of Cycloserine Emanating from Terizidone Metabolism and Relationship with Hepatic Function in Patients with Drug-Resistant Tuberculosis

Author: Mwila Mulubwa, Pierre Mugabo

Publication: Drugs in R&D

Publisher: Springer Nature

Date: Jan 1, 2019

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