

**PHARMACOKINETICS AND DOSING RATIONALE OF PARA-AMINOSALICYLIC
ACID IN CHILDREN AND THE EVALUATION OF THE IN VITRO METABOLISM OF
ETHIONAMIDE, TERIZIDONE AND PARA-AMINOSALICYLIC ACID**

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**Thesis presented in fulfillment of the requirements for the degree of Masters of
Science in Medical Sciences (Pharmacology) at the University of Stellenbosch.**



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DECLARATION

I, the undersigned, hereby declare that the work contained in this thesis is my original work and that I have not previously submitted it, in its entirety or in part, at any University for a degree.

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ABSTRACT

BACKGROUND: The emergence of *mycobacterium tuberculosis* resistance to first line drugs has renewed interest in second-line anti-tuberculosis drugs. Generally, Para-aminosalicylic acid (PAS) is less potent and frequently more toxic than the first line drugs. Furthermore, the pharmacokinetics of PAS in children has not been well characterized.

AIMS: The aims of the present study were (1) to determine the pharmacokinetics of PAS in pediatric patients, (2) to describe the discrepancy between children and adult pharmacokinetics and the appropriate dosing regimen of PAS and (3) to investigate the potential of the second-line anti-tuberculosis drugs PAS, terizidone and ethionamide (often used as first-line drug in children) to inhibit the catalytic activities of CYP450 1A2 and 2C9.

PATIENTS: Twenty two patients with drug resistant tuberculosis were included in the study. Ten patients were children with mean age of 4.2 years (range: 1 to 12 years). Twelve patients were adults with mean age of 31.3 years (range: 18 to 53). 4 children (40%) and 4 adults (33.3%) were HIV positive and were on ART.

METHODS: Children received 75 mg/kg twice daily on the first visit and after two weeks they received 150 mg/kg once. Adults received a standard 4 g twice daily. Blood samples were taken at different time points after the dose. In the additional study, the inhibitory effects of PAS, ethionamide and terizidone on phenacetin O-deethylation, a marker substrate of CYP1A2 and diclofenac 4'-hydroxylation, a marker substrate of CYP2C9, were studied using human liver microsomes.

RESULTS: For the 75 mg/kg dose, the mean AUC was 233.3 $\mu\text{g}\cdot\text{h}/\text{ml}$ and the mean CL was 10.4 l/h/kg. The mean of the observed C_{max} of the drug was 45.4 $\mu\text{g}/\text{ml}$ and the mean T_{max} was 4.8 hrs. For the 150 mg/kg dose, the mean AUC of PAS was 277.9 $\mu\text{g}\cdot\text{h}/\text{ml}$ and the mean CL was 47.1 l/h/kg. The mean of the observed C_{max} of the drug was 56.5 $\mu\text{g}/\text{ml}$ and the mean T_{max} was 4.8 hrs. On the first visit the mean AUC was 368 $\mu\text{g}\cdot\text{h}/\text{ml}$ and the mean CL was 13.2 l/h/kg. The mean of the observed C_{max} of PAS was 51.3 $\mu\text{g}/\text{ml}$ and the mean T_{max} was 5.2 hrs. On the second visit the mean AUC was 230

$\mu\text{g}\cdot\text{h}/\text{ml}$ and the mean CL was 23.9 l/h/kg. The mean of the observed C_{max} of PAS was 37.6 $\mu\text{g}/\text{ml}$ and the mean T_{max} was 5.2 hrs. The comparisons between pharmacokinetics profile of PAS and patients characteristics e.g. age, indicated no statistically significant differences between children (both treatment regimens) and adult patients as well as HIV positive and negative patients. In the *in vitro* study, all drugs demonstrated no inhibition potency towards the investigated CYP450 enzymes.

CONCLUSIONS: The dose of 75 mg/kg twice daily in children appears to be appropriate to achieve serum concentration above the PAS minimum inhibitory concentration of approximately 1 $\mu\text{g}/\text{ml}$. PAS, ethionamide and terizidone are unlikely to affect the metabolism of concomitantly administered medications that are metabolized by either CYP450 1A2 and/or 2C9 isoenzymes.

OPSOMMING

AGTERGROND: Die opkoming van eerstelinie-middel-weerstandige mycobacterium tuberculosis het opnuut belangstelling in tweedelinie-antituberkulosemiddels aangewakker. Oor die algemeen is para-aminosalisielsuur (PAS) minder kragtig en dikwels ook meer toksies. Verder is die farmakokinetika van PAS in kinders nog nie goed vasgestel nie.

DOELSTELLINGS: Die doelstellings van hierdie studie was (1) om die farmakokinetika van PAS in pediatriese pasiënte vas te stel, (2) om die diskrepansie tussen kinder- en volwasse-farmakokinetika, sowel as die toepaslike doseringskedule, van PAS te beskryf en (3) om die potensiaal van die tweedelinie-antituberkulosemiddels PAS, terisidoon en etioonamied (gereeld gebruik as eerste linie middels in kinders) te ondersoek wat betref hul vermoë om die katalitiese werking van CYP450 1A2 en 2C9 te inhibeer.

PASIËNTE: Twee-en-twintig pasiënte met middelweerstandige tuberkulose is in hierdie studie ingesluit. Tien pasiënte was kinders met 'n gemiddelde ouderdom van 4.2 jaar (reeks: 1 tot 12 jaar). Twaalf pasiënte was volwassenes met 'n gemiddelde ouderdom van 31.3 jaar (reeks: 18 tot 53 jaar). 4 kinders (40%) en 4 volwassenes (33.3%) was MIV positief en was op TRM's.

METODES: Kinders het 75 mg/kg twee maal daaliks gedurende die eerste besoek ontvang en 150 mg/kg een maal ná twee weke ontvang. Volwassenes het 'n standaarddosering van 4 g twee maal daaglik ontvang. Bloedmonsters is op verskillende tye ná die dosering geneem. In die addisionele studie is in die inhiberende effekte van PAS, etioonamied en terisidoon op fenasetien-O-deëtilering, 'n merkersubstraat van CYP1A2 en diklofenak-4'-hidroksilasie, 'n merkersubstraat van CYP2C9, ondersoek deur gebruik te maak van menslike lewermikrosome.

RESULTATE: Vir die 75 mg/kg dosering was die gemiddelde area-onder-die-kurve (AOK) 233.3 $\mu\text{g}\cdot\text{h}/\text{ml}$ en die gemiddelde middelopruiming (CL) 10.4 l/h/kg. Die gemiddelde geobserveerde C_{maks} van die middel was 45.4 $\mu\text{g}/\text{ml}$ en die gemiddelde T_{maks} was 4.8 h. Vir die 150 mg/kg dosering was die gemiddelde AOK van PAS 277.9 $\mu\text{g}\cdot\text{h}/\text{ml}$ en die gemiddelde CL 47.1 l/h/kg. Die gemiddelde geobserveerde C_{maks} van die middel was

56.5 $\mu\text{g/ml}$ en die gemiddelde T_{maks} was 4.8 h. Gedurende die eerste besoek was die AOK 368 $\mu\text{g}\cdot\text{h/ml}$ en die gemiddelde CL was 13.2 l/h/kg. Die gemiddelde geobserveerde C_{maks} van PAS was 51.3 $\mu\text{g/ml}$ en die gemiddelde T_{maks} was 5.2 h. Gedurende die tweede besoek was die gemiddelde AOK 230 $\mu\text{g}\cdot\text{h/ml}$ en die gemiddelde CL 23.9 l/h/kg. Die gemiddelde geobserveerde C_{maks} van PAS was 37.6 $\mu\text{g/ml}$ en die gemiddelde T_{maks} was 5.2 h. Die vergelyking van PAS-farmakokinetika en eienskappe van die pasiënte het geen statisties beduidende verskille in die gemiddelde AOK tussen kinders (op albei doserings) en volwassenes getoon nie. Met die *in vitro*-studie het geen van die middels inhibisie-werking teenoor die CYP450-ensieme wat ondersoek is, getoon nie.

GEVOLGTREKKINGS: Die gevolgtrekking kan gemaak word dat die dosering van 75 mg/kg twee maal daaglik voldoende is om serumkonsentrasies wat bo PAS se minimum inhiberende konsentrasie van 1 $\mu\text{g/ml}$ te bereik. Dit is onwaarskynlik dat PAS, etioonamied en terisidoon die metabolisme van gelyktydig-toegediende medikasies, wat op hul beurt deur die CYP240-isoënsieme 1A2 en/of 2C9 gemetaboliseer word, sal affekteer.

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ABBREVIATIONS

ADME	Absorption, Distribution, Metabolism, Elimination
APAS	Acetyl-p-aminosalicylate
ART	Antiretroviral therapy
ARV	Antiretroviral
AUC	Area under the curve
CM	Capreomycin
C _{max}	Maximum concentration
CS	Cycloserine
CSF	Cerebrospinal fluid
CYP	Cytochrome P450 enzyme
CV	Coefficient of variation
DDI	Drug-drug interaction
DMSO	Dimethyl sulfoxide
DST	Drug susceptibility test
ETH	Ethionamide
FA	Formic acid
FDA	Food and Drug Administration
FMO	Flavin dependent mono-oxygenase
HIV	Human immunodeficiency virus
HLMs	Human liver microsomes

HMG CoA	Hydroxymethyl glutaryl co-enzyme A
HPLC	High performance liquid chromatography
IS	Internal standard
K _i	Inhibition constant
K _m	Michaelis-Menten constant
MAO	Monoamine oxidase
MRC	Medical research council
MDR-TB	Multi drug resistant tuberculosis
MS	Mass spectrometer
<i>MTB</i>	<i>Mycobacterium tuberculosis</i>
NADPH	β-nicotinamide adenine dinucleotide phosphate - reduced form
NAT-1	N-acetyltransferase-1
NJ	New Jersey
NSAIDS	Non steroidal anti-inflammatory drugs
PAA	Para-aminosalicylic acid
PAS	Para-aminosalicylic acid
PK	Pharmacokinetics
QC	Quality control
SD	Standard deviation
SE	Standard error
SLDs	Second line drugs

TB	Tuberculosis
TBM	Tuberculosis meningitis
TCH	Tygerberg Children Hospital
T_{\max}	Time taken for the drug to reach maximum concentration
USA	United States of America
V_{\max}	Maximum reaction velocity
WHO	World Health Organization
XDR-TB	Extensively drug resistant tuberculosis

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CHAPTER ONE

INTRODUCTION

Tuberculosis (TB), an ubiquitous, highly contagious chronic granulomatous bacterial infection, is still a leading killer of young adults worldwide. TB has returned with a new face and the global scourge of multi-drug resistant TB (MDR-TB) is reaching epidemic proportions. It is endemic in most developing countries and resurgent in developed and developing countries with high rates of human immunodeficiency virus (HIV) infection. (Du Toit *et al* 2006)

Over the past two decades, two major obstacles to global TB control have emerged. The first is the high prevalence of HIV among TB patients and the second is the growing problem of anti-TB drug resistance. Treatment for MDR-TB is longer (LoBue 2009) and includes the use of second-line drugs (SLDs). (Raviglione *et al* 2007) These drugs are generally less potent and frequently more toxic than isoniazid and rifampin. (Peloquin 1993)

Para-aminosalicylic acid (PAS), one of the SLDs was found to be effective in the treatment of TB in the 1940s. (Lehmann 1946) PAS was widely used in combination chemotherapy against *Mycobacterium tuberculosis* (*MTB*). As better tolerated antibiotics became available, PAS usage has diminished considerably in the past. However, the appearance of the widespread epidemic of MDR-TB has necessitated the addition of PAS to the SLDs. Hence, PAS has become one of the principle SLDs for the treatment of MDR-TB. (Rengarajan *et al* 2004) Despite the long history of PAS usage it has received less attention, and little information is available on its disposition and other factors that may influence it, especially in children. Appropriate dose adjustment of PAS in children using pharmacokinetic (PK) data is important in order to avoid either too high serum concentrations which could be toxic or too low concentrations to complete eradication of *MTB*.

The work presented in this thesis addresses two closely related questions in order to determine the appropriate dose of PAS in children and the potential for drug-drug interactions (DDIs) with PAS and two other drugs often used in patients with MDR/XDR-TB, terizidone and ethionamide. The main clinical part of this project was conducted in

order to extend our understanding of the PK of PAS in children. The PK data in children were compared to the data obtained in adults who served as a reference population. In addition, an exploratory study was performed to determine the potential of PAS, ethionamide and terizidone to cause DDIs via selected CYP isoenzymes. For this purpose, the methodology of *in vitro* testing of drug metabolism by the use of human liver microsomes (HLMs) was established. The *in vitro* metabolism study was conducted with the assumption that legacy drugs such as PAS may be substrates or inhibitors of the CYP enzyme system and may affect the pharmacokinetics of co-administered drugs metabolized by this system. Patients with TB have a high prevalence of co-morbid conditions that require other medications. In order to determine the potential for DDIs, knowledge of clearance mechanisms, enzymes responsible for drug elimination and modulating capabilities of enzyme activities are essential. *In vitro* data can guide clinical strategies for complex pharmacotherapy regimens. (Hyland *et al* 2008) Techniques for the study of drug metabolism *in vitro* are extensively utilized to provide presumptive answers to fundamental clinical questions regarding drug metabolism and drug interactions. (Greenblatt *et al* 2002)

CHAPTER TWO

LITERATURE REVIEW

2.1 Burden of TB

The estimates of the global burden of disease caused by TB in 2009 are as follows: 9.4 million incident cases (range, 8.9 million-9.9 million). (WHO 2010) HIV infection has been described as a driving force of the TB epidemic in the most affected populations. A study conducted in peri-urban communities in South Africa has shown that TB notification rates have increased 2.5-fold, reaching a rate of 1468 cases per 100,000 persons in 2004. The estimated population prevalence of HIV infection increased from 6% to 22% during the same period. After stabilization of prevalence of the HIV infection, the TB notification rate continued to increase steeply, indicating ongoing amplification of the TB epidemic. In 2004, at least 50% of children aged 0-9 years who developed TB were HIV infected. Annual TB notification rates among adolescents increased from 0 cases in 1996-1997 to 436 cases per 100,000 persons in 2003-2004, and these increases were predominantly among females. However, 20-39 year-old persons were affected most, with TB notification rates increasing from 706 to 2600 cases per 100,000 persons among subjects in their 30s. In contrast, TB rates among persons aged >50 years did not change. In this study 98 (59%) of 166 persons who provided TB notifications were HIV positive. On the basis of these data, the TB notification rate among HIV-infected individuals was calculated to be 4381 cases per 100,000 persons (95% CI, 3570-5313 cases per 100,000 persons. (Lawn *et al* 2006)

Children are mainly infected by adult pulmonary TB source cases, and childhood TB therefore reflects the intensity of ongoing transmission of *MTB* within a community. (Schaaf *et al* 2005) Since most children acquire the organisms from adults in their surroundings, the epidemiology of childhood TB follows that in adults. An estimated 10% of the 2.9 million new cases of TB in sub-Saharan Africa during 2007 occurred in children: 38% of all incident TB cases in sub-Saharan Africa (regardless of age group) were HIV-infected in 2007. (WHO 2009) TB case load studies in children suggest an exponential rise in the proportion of the TB case load in children as the prevalence of TB rises, nearly 40 per cent of the case load in certain high incidence communities.

(Donald 2002) In Cape Town, South Africa, children suffer considerable morbidity: 207/439 (47.1%) had disease manifestations other than uncomplicated lymph node disease, while 26/439 (5.9%) were diagnosed with disseminated (miliary) disease and/or TB meningitis (TBM). (Marais *et al* 2004)

2.2 Drug-resistant TB

The treatment and control of TB is more complex when the organism is resistant to the action of anti-TB drugs. MDR-TB is associated with mortality as high as 43-93% in adults. Despite limited information in children, resistance patterns in children have generally been found to be similar to those of adults from the same areas and similar backgrounds. (Nelson *et al* 2004) Traditionally, patients with drug-resistant TB are classified as having acquired or primary drug resistance on the basis of a history of previous treatment. In a survey in 35 countries, the median prevalence of primary resistance to any anti-TB drug was 9.9% (range 2-41) and that of acquired resistance was 36%. (Van Rie *et al* 2000) In a study conducted at Tygerberg Children Hospital (TCH), Cape Town, among 313 children (range 2 weeks-12.9 years) in whom drug susceptibility test (DST) results were available, 40 (12.8%) and 17 (5.4%) were infected with strains resistant to isoniazid and rifampicin, respectively. All who had rifampicin-resistant strains had co-existent resistance to isoniazid (MDR-TB). Only 1/40 (2.5%) children with resistance to isoniazid and/or rifampicin were also resistant to ethambutol (Schaaf *et al* 2007). In a study conducted in Johannesburg, South Africa, the MDR-TB prevalence rate of 8.5% was higher than in previous studies (2.3-6.7%). These results suggest a high prevalence of MDR-TB. (Fairlie *et al* 2011)

XDR-TB has now been reported from 45 countries, though this almost certainly underestimates its true extent as many countries lack laboratory facilities to detect resistance to SLDs. The outbreak in South Africa was particularly alarming because most patients with XDR-TB had no history of TB treatment, implying person to person transmission of XDR-TB, and because of evidence of transmission in healthcare settings. (Grant *et al* 2008)

2.3 Pharmacokinetics

PK is the discipline that applies mathematical models to describe and predict the time course of drug concentrations in body fluids. (Greenblatt *et al* 2002) PK measures, such as area under the curve (AUC), maximum concentration (C_{max}) and parameters calculated from those measures, such as clearance, half life, and volume of distribution, reflect the absorption, distribution and elimination of a drug from the body. A drug can be eliminated by metabolism to one or more active or inactive metabolites and by excretion of the unchanged drug. The overall set of processes is often referred to as ADME (Absorption, Distribution, Metabolism and Excretion), which ultimately controls systemic exposure to a drug and its metabolites after drug administration. Systemic exposure reflected in plasma drug and/or metabolite concentrations is generally used to relate dose to both beneficial and adverse effects. All drugs show inter- and intra-individual variance in PK measures and/or parameters. Variance can sometimes be substantial. In the pediatric population, growth and developmental changes in factors influencing ADME also lead to changes in PK measures and/or parameters. (Gilman 1992, Butler *et al* 1994)

PK studies have led to the appreciation of the large degree of variability in PK parameter estimates that exist across individuals; many studies have quantified the effect of factors such as age, gender, disease states and concomitant drug therapy on the PK of drugs, with the purpose of accounting for individual variability. (Atkinson Jr *et al* 2007)

PK of xenobiotics can differ widely between children and adults due to physiological differences and the immaturity of enzyme systems and clearance mechanisms. This makes extrapolation of adult dosimetry estimates to children uncertain, especially at early postnatal ages. Once exposure has occurred, the PK handling of xenobiotics is likely to differ from that in adults with respect to their metabolism, clearance, protein binding and volume of distribution. (Ginsberg *et al* 2001) Key factors explaining differences in drug distribution between the pediatric population and adults are organ size, membrane stability, plasma protein concentration and characteristics, endogenous substances in plasma, total body and extracellular water, fat content, regional blood flow and transporters such as P-gp, which is present not only in the gut, but also in liver,

kidney, brain and other tissues. (Benedetti *et al* 2005) The changes in body size and maturation during development of the children affect PK concentration-time profiles. (Anderson *et al* 2006)

Developmental changes are responsible for differences in drug disposition seen throughout childhood; therefore the weight-adjusted drug dose may not be the same for different age groups. During a period of latent or rapid growth, some drugs that may cause severe or protracted toxicity can alter the final mature expression of a system. These concepts need to be appreciated for appropriate use of drugs in children. Age-related differences in PK, in addition to those of body size, can be used to guide calculations. Altered absorption, distribution, and elimination are most marked in the newborn, but for many drugs, disposition processes may equal or exceed the adult capacity by late infancy and/or childhood. (Pradhan *et al* 1986) Areas of importance in pediatric PK are as follows:

Absorption: Developmental changes in the pediatric population that can affect absorption include effects on gastric acidity, rates of gastric and intestinal emptying, surface area of the absorption site, gastrointestinal enzyme systems for drugs that are actively transported across the gastrointestinal mucosa, gastrointestinal permeability, and biliary function. Similarly, developmental changes in skin, muscle and fat including changes in water content and degree of vascularization, can affect absorption patterns of drugs delivered via intramuscular, subcutaneous or percutaneous routes. (Yaffe 1992)

Distribution: Distribution of a drug may be affected by changes in body composition, such as changes in total body water and adipose tissue that are not necessarily proportional to changes in total body weight. Plasma protein binding and tissue binding changes arising from changes in body composition with growth and development may also influence distribution. (Gilman 1990)

Metabolism: Important differences have been found in the pediatric population compared to adults both for phase I and II enzymes, reductive and hydrolytic enzymes. Generally, the major enzyme differences observed in comparison with the adult age are in newborn infants, although for some enzymes e.g. glucuronosyl-transferases important differences still exist between infants and toddlers and adults. (Benedetti *et al*

2005) A study that investigated the role of the NAT2 genotype and enzyme maturation on isoniazid PK showed that improved phenotypic expression of NAT2 with age extends beyond the neonatal period. (Zhu *et al* 2011)

Excretion: Drug elimination clearance may increase with weight, height, age, body surface area and creatinine clearance. (Anderson and Holford 2008) Because these processes mature at different rates in the pediatric population, age can affect systemic exposure for drugs where renal excretion is a dominant pathway of elimination. Consideration should also be given to the maturation of other excretory pathways, including biliary and pulmonary routes of excretion. (Brown 1989)

2.4 Drug biotransformation (Metabolism)

Drug biotransformation is one of the most important factors that can affect the overall therapeutic and toxic profile of a drug. It can lead to detoxification and excretion of the drug, but also to bioactivation. (Brandon *et al* 2003)

A typical chemical metabolism pathway involves the oxidation of the parent substance (phase I oxidation), followed by conjugation of the oxidised moiety with highly polar molecules, such as glucose, sulphate, methionine, cysteine, glutathione or glucuronic acid (phase II conjugation) (Xu *et al* 2005).

The key enzymes for phase I oxidation are the isoforms of the CYP family of enzymes. Regulated by nuclear receptors (Honkakoshi and Negishi 2000), the superfamily of CYPs are heme containing enzymes with very wide substrate specificities by virtue of their existence in a large number of isoforms or isozymes. While the liver is the main site of xenobiotic metabolism, it is important to note that phase I and II metabolism also occurs in most tissues and the gut microflora. (Nishimura *et al* 2003) Usually, these conversions result in a decrease in toxicity and/or an increased excretion of the chemical. However, metabolic activation is also possible, which can also be inhibited or induced by pharmaceutical, environmental and/or dietary chemicals. (Nakajima *et al* 2001)

Several factors can alter drug metabolism, including the presence or absence of disease and/or concomitant medications. While most of these factors are usually relatively stable over time, concomitant medications can alter metabolism abruptly and

are of particular concern. The influence of concomitant medications on metabolism becomes more complicated when a drug is metabolized to one or more active metabolites. In this case, the safety and efficacy of the drug are determined not only by exposure to the parent drug but by exposure to the active metabolites. (Huang *et al* 2007) The activity of many CYP isoforms and a single glucuronosyltransferase (UGT) isoform is markedly diminished during the first two months of life. In addition, the acquisition of adult activity over time is enzyme and isoform-specific. (Kearns *et al* 2003)

Although several enzyme systems participate in phase I metabolism of xenobiotics, perhaps the most notable pathway in this scheme is the monooxygenation function catalyzed by the cytochrome P450s (CYPs; P450s) as discussed in detail below. The CYPs detoxify and/or bioactivate a vast number of xenobiotic chemicals and conduct functionalization reactions that include N- and O-dealkylation, aliphatic and aromatic hydroxylation, N- and S-oxidation, and deamination. Examples of toxicants metabolized by this system include nicotine and acetaminophen, as well as the procarcinogenic substances, benzene and polyaromatic hydrocarbons. (Omiecinski *et al* 2011)

2.5 *In vitro* models of drug metabolism

In vitro systems are well established as valuable tools for studying various aspects of drug metabolism; in particular, kinetic data obtained from *in vitro* systems can be scaled and used in the prediction of *in vivo* clearance. (Parker and Houston 2008) Since the liver is the primary site of systemic metabolism, preparations from hepatic tissue are mostly used. *In vitro* hepatic metabolism can be measured in many different preparations that include the smallest and the least complex microsomes or cytosol, up to isolated hepatocytes, slices and perfused tissue. Subcellular fractions (i.e., cytosol, microsomes) have been used for decades in studies of xenobiotic metabolism. These preparations are cost-effective to prepare or to procure, are relatively stable over time, and offer a good means of isolating a group of enzymes. However, the nature of these preparations containing groups of enzymes often co-located to the same cellular compartment-produces a disadvantage compared to purified or recombinant systems

when attempting to study the metabolic contributions of a single enzyme. (Lipscomb *et al* 2008)

Microsomal protein is usually separated from the other constituents of the cell by differential ultracentrifugation (Guengerich 1994) and consists primarily of the enzymes associated with the endoplasmic reticulum. Microsomes contain CYPs, flavin-containing monooxygenases (FMO) and glutathione S-transferases. Metabolic activity is often initiated by the addition of substrate or cofactor, and the time of incubation is easily controlled. Because cofactors are exogenously added, they can be infinitely controlled and are typically added as native cofactor or in a cofactor regenerating system. (Lipscomb *et al* 2008)

2.6 Liver microsomes

Liver microsomes consist of vesicles of the hepatocyte endoplasmic reticulum and are prepared by differential centrifugation and thus contain almost only CYP and UGT enzymes. Liver preparations, other than from fresh human liver, can also be used (e.g., liver slices, liver cell lines, and primary hepatocytes) for preparation of microsomes. The CYP and UGT enzyme activity can be measured by various model substrates. In commercially available human liver microsomes (HLMs), the CYP activity is already characterized by the supplier. NADPH regenerating system or NADPH is required to supply the energy demand of the CYPs and UDPGA and alamethicin for UGT activity. The activity of HLMs can vary substantially between individuals. This problem, however, can be successfully solved by the application of pooled microsomes, which results in a representative enzyme activity. These pools can be purchased from different companies. Individual HLMs can also be used to screen for the inter-individual variability in the biotransformation of a drug. It is also possible to identify the critical CYP involved in the biotransformation of the drug using individual HLMs by correlating the enzyme activity of a particular CYP, using a bank of human donors, to the metabolism of the drug. (Brandon *et al* 2003) Therefore they are widely used as an *in vitro* model system in order to investigate the metabolic fate of xenobiotics. The most prominent group of drug metabolizing enzymes is the super family of CYPs. These haem-containing enzymes play a key role in the metabolism (mainly oxidation) of a variety of

chemically diverse compounds including food compounds, pharmaceutical agents, carcinogens, and environmental pollutants. (Pelkonen and Turpeinen 2007)

The preparation procedure includes the homogenization of a liver, followed by centrifugation at 9000 or 10000 g to remove nuclei, plasma membranes, and large organelles such as mitochondria. The resulting supernatant S9 is defined as the "supernatant fraction obtained from an organ (usually liver) homogenate by centrifuging at 9000 g for 20 minutes in a suitable medium; this fraction contains cytosol and microsomes." The microsome component of the S9 fraction contains cytochrome P450 isoforms (phase I metabolism) and other enzyme activities and S10 is the cytosolic fraction containing ribosomes and endoplasmic reticulum. (Duffus *et al* 2007)

Another centrifugation at 100000 g will pellet the endoplasmic reticulum which is the site of the CYP450s. This pellet is termed "microsomes". HLMS are used extensively for drug metabolism and DDIs. (Li 2004) The enzymatic activities are stable during the prolonged storage of the microsomes. In order to reflect the standard proportion of the enzymes in human or animal livers, liver microsomes are usually pooled. The major disadvantage of the model is the limited incubation time (the enzyme activities decrease after 2 hours of incubation). (Baranczewski *et al* 2006)

2.7 Cytochrome P450 Inhibition

Drug inhibition is usually regarded as potentially dangerous, or at least undesirable. A drug that inhibits a specific CYP450 enzyme can decrease the metabolic clearance of a co-administered drug that is a substrate of the inhibited enzyme. A consequence of decreased metabolic clearance is elevated blood concentrations of the co-administered drug, which may cause adverse effects or enhanced therapeutic effects. In addition, the inhibited metabolic pathway can lead to the decreased conversion of a pro-drug to its active form, resulting in reduced efficacy e.g. tamoxifen, codeine and clopidogrel. (Mannheimer and Eliasson 2010) Unwanted effects are most obvious and expected when they involve drugs with narrow therapeutic range, e.g. warfarin. (Lowery *et al* 2005) To determine whether a compound inhibits CYP activity, changes in the metabolism of a CYP-specific substrate by HLMS or hepatocytes at various

concentrations of the compound are monitored. Potency and rank order of the inhibition of CYP enzymes can be assessed by the determination of the IC_{50} and K_i .

The inhibition constant, K_i , denotes the equilibrium constant of the dissociation of the inhibitor-bound enzyme complex, while the IC_{50} value quantifies the concentration of inhibitor necessary to halve the reaction rate of an enzyme-catalyzed reaction observed under specified assay conditions. (Burlingham and Widlanski 2003)

K_i values are determined with HLMs and CYP isoenzyme selective substrates over a range of concentrations for both probe substrates and the compound. Unlike IC_{50} , K_i values are intrinsic values, which represent the extent to which a compound will affect a given CYP isoenzyme. Since the K_i values describe specific equilibrium-based interactions between the investigational drug and the tested CYP isoenzyme, these can be used to correlate *in vitro* inhibitory concentrations to predict *in vivo* plasma concentrations. (Bjornsson *et al* 2003)

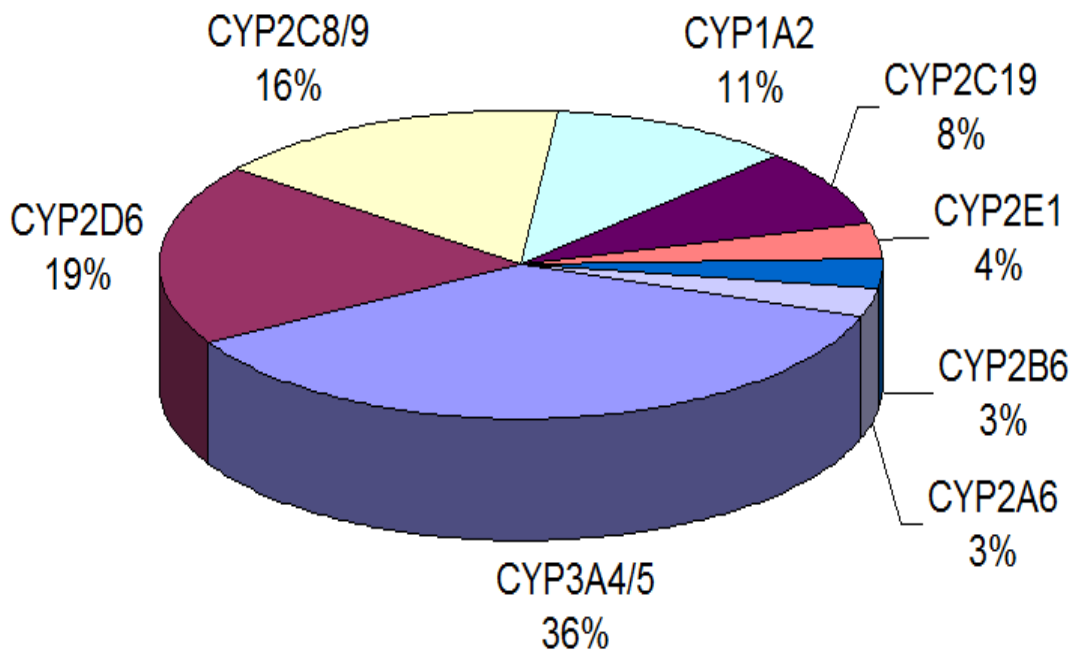


Figure 1: Proportion of drugs metabolized by different families of CYP450 enzymes (Wrighton and Steven 1992).

2.8 CYP450 enzymes

Drug metabolism in general consists of an oxygenation step, adding an oxygen moiety, usually a hydroxyl group, to the organic molecule. This is generally referred to as “phase I oxidation.” The most important phase I drug metabolizing enzymes belong to the cytochrome P450 (CYP) family, with the key human isoforms being CYP1A2, CYP2A6, CYP2B1, CYP2C9, CYP2C19, CYP2D6, CYP2E1 and CYP3A4, each with isoforms-specific substrates. CYP3A4 is believed to be the most abundant and important, being responsible for the metabolism of near 50% of the existing drugs. The CYP isoenzymes are responsible for the elimination of numerous xenobiotics. Inhibition or induction of CYP enzymes by other compounds may significantly alter plasma concentrations of drugs that are substrates for these enzymes. (Faber *et al* 2005)

The CYP superfamily of drug metabolizing enzymes is now established as being of primary importance for the metabolism and clearance of most drugs. For the CYP isoforms most relevant to human drug metabolism, each has its own distinct pattern of relative abundance, anatomic location, mechanism of regulation, substrate specificity, and susceptibility to inhibition and induction by other drugs or foreign chemicals. CYP comprises a superfamily of haemoproteins which functions as the terminal oxidase of the mixed function oxidase system. At least 481 CYP genes and 22 pseudogenes are known to exist across all species. Pseudogenes are dysfunctional relatives of genes that have lost their protein-coding ability or are otherwise no longer expressed in the cell. (Vanin 1985) These CYP genes are classified into families (designated by an arabic numeral) and subfamilies (designated by a letter) according to the amino acid identity of the encoded proteins. Of the thirty-five known human CYP genes, currently classified in families 1,2,3,4,5,7,8,11,17,19,21,24,27 and 51, only the eighteen forms comprising families 1 to 3 appear to substantially contribute to the metabolism of drugs and non-drug xenobiotics. The remainders are of importance in the metabolism and/or biosynthesis of endogenous compounds such as bile acids, biogenic amines, eicosanoids, fatty acids, phytoalexins, retinoids and steroids. (Miners and Birkett 1998)

In this thesis, only two CYP450 isoenzymes (CYP1A2 and CYP2C9) have been described, this was necessitated by the fact that this was only an initial exploratory

study which gave way to the ongoing more detailed *in vitro* study at the Division of Pharmacology. The selection of these two isoforms was based on practical considerations, such as the availability of standards and HPLC/MS/MS methods.

2.8.1 CYP1A subfamily

CYP1A subfamily enzymes are well known to play an important role in the metabolism of various carcinogens and drugs. For example, CYP1A1 and CYP1A2 catalyze the metabolic activations of the carcinogenic aryl hydrocarbons and aromatic amines, respectively. Furthermore, the activities of CYP1A1 and CYP1A2 in target tissues are one of the host factors that determine the susceptibility of experimental animals toward carcinogenic aryl hydrocarbons and aromatic amines respectively. (Kojima *et al* 2010)

HLMs contain relatively high constitutive levels of CYP1A2 (10-15% of the total P450 content of human liver). CYP1A2 metabolizes many clinically important drugs such as amitriptyline, imipramine, theophylline, clozapine, tacrine, and zileuton. Most of the investigators use phenacetin *O*-deethylation to form acetaminophen to represent CYP1A2 activity. However, industry investigators also use several substrates other than phenacetin to evaluate CYP1A2 activity. The frequent use of this substrate *in vitro* is due to the availability of a fast and simple high-performance liquid chromatography-ultraviolet detection assay with high sensitivity for the reaction. In HLMs, the *O*-deethylation of phenacetin displays biphasic kinetics. Studies with cDNA-expressed CYP1A2 chemical inhibitors and monoclonal antibodies show that the high-affinity component of phenacetin *O*-deethylation is CYP1A2. The *K_m* value of this pathway is reported at 10 to 50 μM , at least 10-fold lower than that of the low-affinity component. At a substrate concentration of 100 μM , the contribution of CYP1A2 is estimated to be 86%, but the contribution is reduced to 50% at a substrate concentration of 500 μM .

At concentrations $\geq 500 \mu\text{M}$, several enzymes, especially CYP2C9, contribute significantly to the *O*-deethylation of phenacetin in HLMs. Studies with organic solvents have indicated that at solvent concentrations $\leq 1\%$ (v/v), phenacetin *O*-deethylation is not significantly affected by dimethyl sulfoxide (DMSO) and methanol.

In summary, at substrate concentrations that reflect low K_m enzyme activity (i.e., at concentration lower than 100 μM), phenacetin *O*-deethylation is the preferred probe reaction for detecting CYP1A2-based DDIs potential *in vitro*. (Yuan *et al* 2002)

2.8.2 CYP2C subfamily

The CYP2C subfamily is the second most abundant CYP protein in the human liver, representing about 20% of the total CYP. (Shimada *et al* 1994) CYP2C9 is one of four known members of the human CYP2C subfamily, although genomic analysis suggests the possible existence of three additional CYP2C enzymes. Other known members of the human CYP2C subfamily include CYP2C8, CYP2C18 and CYP2C19. While CYP2C10 was originally also considered a discrete isoform, this enzyme is now thought to be a variant of CYP2C9. (Miners and Birkett 1998)

CYP2C9 is the principal CYP2C in human liver. It metabolizes many clinically important drugs including the diabetic agent tolbutamide, the anticonvulsant phenytoin, the *S*-enantiomer of the anticoagulant warfarin, Δ^1 -tetrahydrocannabinol and numerous anti-inflammatory drugs such as ibuprofen, diclofenac, piroxicam, tenoxicam, mefenamic acid, the antihypertensive losartan and several new drugs including the antidiabetic drug glipizide and the diuretic torasemide. (Goldstein 2002)

Diclofenac, tolbutamide, phenytoin, and celecoxib are structurally diverse CYP2C9 substrates. The first three compounds have been well established as the probes for CYP2C9 activity in human liver. (Tang *et al* 2000) Fluconazole, miconazole and sulfamethoxazole are potent inhibitors of CYP2C9. Co-administration of phenytoin, warfarin, sulfamethoxazole and losartan with fluconazole results in clinically significant drug interactions. (Venkatakrisnan *et al* 2000)

Although CYP2C9 activities are predominant in the liver, CYP2C8 and CYP2C9 activities overlap in that both enzymes can metabolize arachidonic acid, several NSAIDs, retinoic acid and others. CYP2C8 separately metabolizes various endogenous compounds including arachidonic acid, retinoic acid, and therapeutic drugs such as the widely used chemotherapeutic agent paclitaxel. (Speed *et al* 2009)

Table 1: Summary of xenobiotic-metabolizing human hepatic CYP450.

CYP	Relative amount in the liver (%)	Substrates (reaction)	Selective inhibitors	Other characteristics
1A2	~10	Ethoxyresorufin (<i>O</i> -deethylation) Phenacetin (<i>O</i> -deethylation)	Furafylline	Inducible
2A6	~10	Coumarin (7-hydroxylation)		Polymorphic
2B6	~1	S-Mephenytoin (<i>N</i> -demethylation)	Orphenadrine	
2C8	<1	Paclitaxel (6 α -hydroxylation)	Quercetin	
2C9	~20	Tolbutamide (methylhydroxylation) Diclofenac (hydroxylation) S-Warfarin (7-hydroxylation)	Sulfaphenazole	Polymorphic
2C19	~5	S-mephenytoin (4'-hydroxylation) Omeprazole (oxidation)		Polymorphic
2D6	~5	Dextromethorphan (<i>O</i> -demethylation) Debrisoquine (4-hydroxylation) Bufuralol (1'-hydroxylation)	Quinidine	Polymorphic
2E1	~10	Chlorzoxazone (6-hydroxylation) Aniline (4-hydroxylation)	Pyridine	Inducible
3A4	~30	Midazolam (1'- and 4-hydroxylation) Testosterone (6 β -hydroxylation) Nifedipine (dehydrogenation)	Azole antimycotics	Inducible

Data adapted from (Pelkonen and Breimer 1994) and (Pelkonen *et al* 1998; 2000).

2.9 Drug-drug interactions

Concomitant administration of several drugs is common and, indeed is often the situation in hospitalized patients. Whenever two or more drugs are administered over similar or overlapping time periods, the possibility for drug interactions exists. (Jiunn *et al* 1997)

DDIs were already recognized as a potential problem in the late 1960s. Over the last 4 decades, the problem has increased in magnitude with the introduction of several new classes of drugs. Prospective evaluations of DDIs indicate that the incidence of clinically significant DDIs probably lies between 1 and 10 in 1000 patients. Among these DDIs, a significant fraction is metabolic in nature, and is associated with inhibition of the enzymes responsible for drug clearance. (Yao *et al* 2001)

DDIs are of great interest to scientists involved in drug research, regulatory authorities who are responsible for public safety, physicians, and their patients. Since “polypharmacy”, or the practice of simultaneous prescription of more than one drug to treat one or more conditions in a single patient, has become a more common practice, drug interactions have been cited as one of the major reasons for hospitalization and even death. (Walsky *et al* 2004) A patient may be co-administered multiple drugs to allow effective treatment of a disease (e.g., TB, HIV infection) or for the treatment of multiple diseases or disease symptoms. It is now known that DDIs may have serious, sometimes fatal consequences. (Li 2007)

Interactions by mutual competitive inhibition between drugs is almost inevitable, because metabolism represents a major route of drug elimination and because many drugs can compete for the same enzyme system. The risk of clinical consequences from DDIs is higher with some drugs than with others. (Jiunn *et al* 1997)

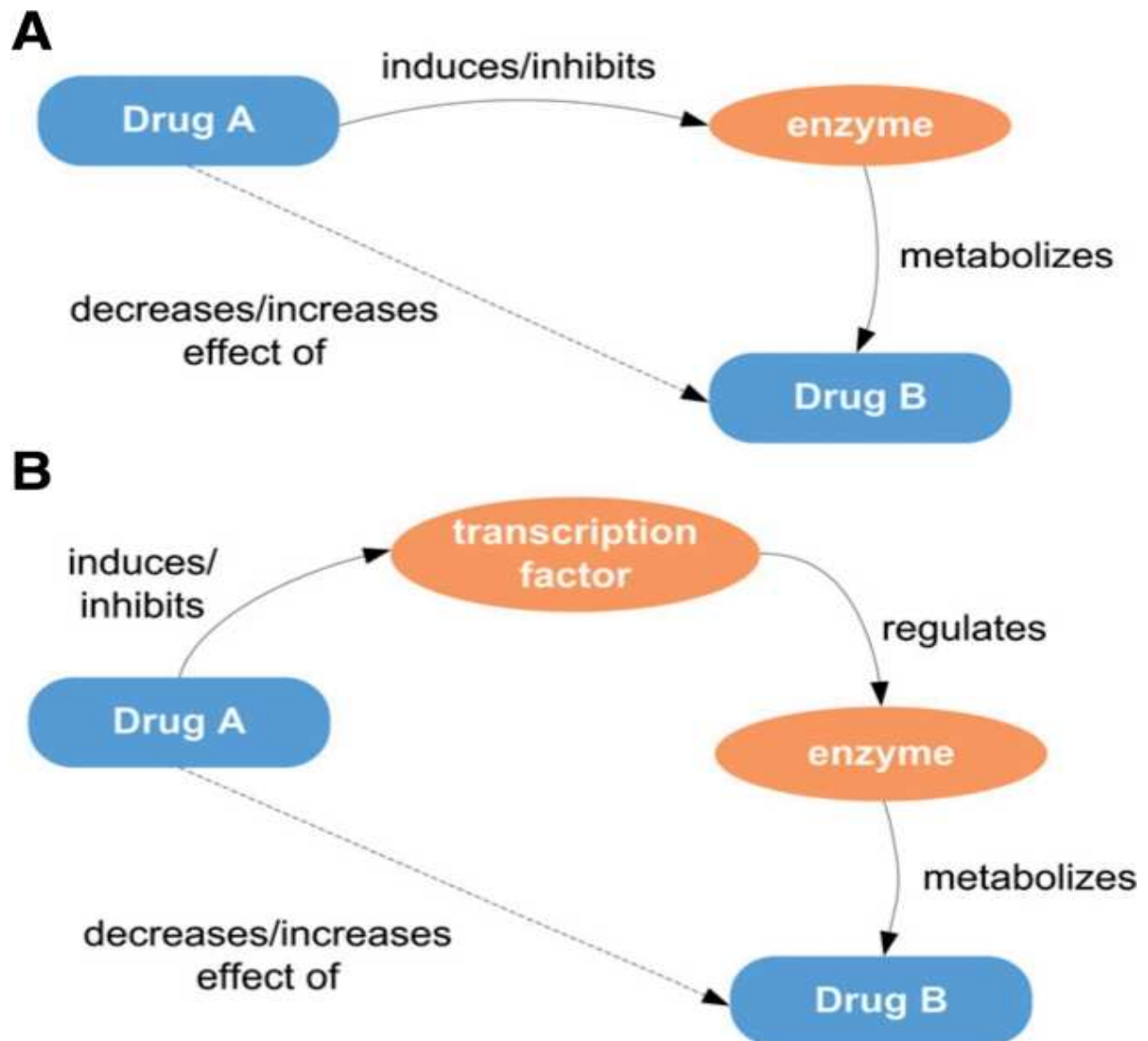


Figure 2: The effects of drug A on drug B through (A) direct induction/inhibition of enzymes; (B) indirect induction/inhibition of transcription factors that regulate the drug-metabolizing enzymes. (Tari *et al* 2010)

Many metabolic routes of elimination, including most of those occurring via the CYP family of enzymes, can be inhibited, activated, or induced by concomitant drug treatment. Observed changes arising from metabolic interactions can be substantial in order of magnitude or more decrease or increase in the blood concentration of a drug or metabolite and can include formation of toxic metabolite or increased exposure of a

parent compound. Depending on the extent and consequence of the interaction, the fact that drug metabolism can be significantly inhibited by other drugs and that the drug itself can inhibit the metabolism of other drugs can require important changes in either its dose or the dose of drugs with which it interacts, that is, on its labeled condition of use. Even drugs that are not substantially metabolized can have important effects on the metabolism of concomitant drugs. For this reason, metabolic DDIs should be explored, even for an investigational compound that is not eliminated significantly by metabolism. (Huang *et al* 2007)

Although modulation of other proteins such as p-glycoprotein and UDP-glucuronosyltransferases by co-administered drugs causes adverse side effects, inhibition of CYPs are currently recognized as the major mechanism for DDIs observed. As the most important drug metabolism enzymes in humans, CYP are responsible for metabolizing more than 95% of marketed drugs. Therefore, *in vitro* assessment of potential drug interactions has largely been focused on inhibition of CYPs. (Yan and Caldwell 2004) Several drugs in common use cause large increases in exposure to other drugs. Examples include ketoconazole, itraconazole, erythromycin, clarythromycin, diltiazem and nefazodone (CYP3A4); enoxacin (CYP1A2); and sulfaphenazole (CYP2C9); with some drugs possessing the potential to inhibit more than one P450 enzyme; fluconazole (CYP2C9 and CYP2C19) and fluvoxamine (CYP1A2 and CYP2C19). (Walsky *et al* 2004)

2.10 The rate of inhibition

The rate of inhibition depends on the affinity of the substrate for the enzyme being inhibited, the concentration of substrate required for inhibition, and the half-life of the inhibitor drug. The onset and offset of enzyme inhibition are dependent on the half-life and time to steady-state of the inhibitor drug. The time to maximum drug interaction (onset and termination) is also dependent on the time required for the inhibited drug to reach a new steady state. (Leucuta and Vlase 2006)

2.11 Enzyme induction

Enzyme induction is not as common as inhibition based drug interactions, but equally profound and clinically important. Enzyme induction occurs when hepatic blood flow is increased, or the synthesis of more CYP450 enzymes is stimulated. Like inhibitors, inducers tend to be lipophilic, and the time course of the interaction is dependent on the half-life of the inducer. The time course of induction is also dependent on the time required for enzyme degradation and new enzyme production. The half-life of CYP450 enzyme turnover ranges from 1 to 6 days. Enzyme induction is also influenced by age and liver disease. The ability to induce drug metabolism may decrease with age, and patients with cirrhosis or hepatitis may be less susceptible to enzyme induction. (Leucuta and Vlase 2006)

Table 2 lists some of the substrates metabolized by CYP isoenzymes and the agents which inhibit or induce these enzymes. This does not imply that any combination of inhibitor and substrate for a particular isoenzyme will result in an interaction of clinical significance.

Table 2: Examples of drugs with clinically important effects on CYP isoenzymes

Drug	Inhibition of:	Induction of:
Azole antifungals		
Ketoconazole	CYP3A	
Itraconazole	CYP3A	
Fluconazole	CYP3A, 2C9	
Terbinafine	CYP2D6	
Antidepressants		
Fluoxetine	CYP2D6	
Paroxetine	CYP2D6	
Fluvoxamine	CYP1A2, 2C19, 3A	
Nefazodone	CYP3A	
St. Johns wort		CYP3A
Antipsychotics		
Perphenazine	CYP2D6	
Anticonvulsants		
Carbamazepine		CYP3A
Antithrombotics		
Ticlopidine	CYP2D6, 2C19	
Antiinfectives		
Erythromycin	CYP3A	
Clarithromycin	CYP3A	
Ciprofloxacin	CYP1A2	
Rifampin		CYP3A
Viral protease inhibitors		
Ritonavir	CYP3A	CYP2C9, 2C19
Nonnucleoside reverse transcriptase inhibitors		
Delavirdine	CYP3A	
Nevirapine		CYP3A4, 2B6
Efavirenz		CYP2C9, 2C19, 3A4
Cardiovascular agents		
Quinidine	CYP2D6	
Diltiazem	CYP3A	
Verapamil	CYP3A	
Antiulcer agents		
Omeprazole	CYP2C19	

Adapted from (Greenblatt *et al* 2002)

2.12 Second-line anti-TB drugs

The backbone of regimens for the treatment of MDR-TB consists of an injectable drug (aminoglycoside or polypeptide) and a fluoroquinolone, supported by at least two additional SLDs in order to ensure that the regimen includes at least four drugs confirmed or expected to be effective. Aminoglycosides, polypeptides and fluoroquinolones are bactericidal, while thionamides, cycloserine/terizidone and PAS are bacteriostatic. Once the injectable drugs (i.e. aminoglycosides) and the fluoroquinolones are compromised by resistance, available treatment regimens become much weaker and the possibility for patient cure decreases significantly. Significantly more clinical data are needed to answer key questions relating to treatment outcomes in the presence of different combinations and permutations of drug resistance. (WHO 2008)

2.12.1 Para-aminosalicylic acid (PAS)

PAS was the second antibiotic found to be effective in the treatment of TB in the 1940s. (Lehmann 1946) It is at least as effective as other SLDs and probably more effective. (Peloquin *et al* 1994) PAS was widely used in combination chemotherapy against *MTB*. However, PAS caused gastrointestinal toxicity leading to poor patient compliance. As more easily tolerated antibiotics became available, PAS usage diminished considerably. The appearance of widespread epidemic of MDR-TB has necessitated the addition of PAS to the first line agents. The advancement of a new formulation of the drug with fewer gastrointestinal side effects has supported the use of this drug. Thus, PAS has become one of the principle SLDs for the treatment of MDR-TB. (Rengarajan *et al* 2004) An improved (granule) formulation of PAS has been used to treat patients with MDR-TB or patients who are intolerant of first-line anti-TB medications. This formulation, dosed as 4 grams (one packet of granules) in adults or 75 mg/kg body weight in children every 12 hours has several notable advantages over previous preparations. (Berning *et al* 1998)

Mechanism of action

The mechanism of action of PAS has yet to be elucidated, and it is believed that the mechanism is related to interference with bacterial folic acid synthesis and inhibition of iron uptake. The binding of PAS to pteridine synthetase is the first step in folic acid synthesis. PAS binds pteridine synthetase with greater affinity than para-aminobenzoic acid (PABA), effectively inhibiting the synthesis of folic acid. As bacteria are unable to use external sources of folic acid, cell growth and multiplication slows. PAS may inhibit the synthesis of the cell wall component, mycobactin, thus reducing iron uptake by *MTB*. (Arbex *et al* 2010)

Pharmacokinetics

The ingestion of 4 g of PAS granules leads to a maximum serum concentration of 20-60 µg/ml after 4-6 hrs. The serum levels of PAS peak within 90-120 min after the ingestion. The half life of PAS is 1 hr, and the plasma concentrations of the drug after 4-5 hrs are minimal, which justifies the need for doses of 10-12 g in order to maintain the bacteriostatic activity. PAS is metabolized in the intestines and liver, via acetylation, into N-acetyl-para-aminosalicylic acid. More than 80% of the drug is excreted by the kidney through glomerular filtration and tubular secretion. (Peloquin 2002)

Metabolism

There are two main products of the metabolism of PAS; acetylation by N-acetyltransferase-1 (NAT1) to form N-acetyl-*p*-aminosalicylate (APAS) and conjugation with glycine to form *p*-aminosalicyluric acid (PAA) accounting for approximately 70% and 25% of the absorbed dosage. (Wan *et al* 1974) A considerable proportion of metabolism occurs in the gut and liver and this first-pass effect can exercise a considerable effect on resulting blood concentrations. This first-pass effect also appears to be rate-limited and consequently higher PAS dosages lead to relatively higher blood concentrations of PAS and PAA, but this also has the consequence of more rapid excretion. (Lehman 1969) APAS appears to have little tuberculostatic effect, but PAA is reported to have approximately 75% of the inhibitory effect of PAS. (Lehman 1969)

Adverse effects

Gastrointestinal effects (anorexia, diarrhea, nausea, and vomiting) and hypothyroidism, the latter occurring especially when PAS is administered concomitantly with ethionamide, are common. Thyroid function returns to normal when the drug is discontinued. Hepatitis occurs in 0.3-0.5% of the cases, allergic reactions (fever, rash, and pruritus), hemolytic anemia, agranulocytosis, leukopenia, thrombocytopenia, malabsorption syndrome, and increased thyroid volume are rare, as are cardiovascular adverse effects (pericarditis), neurological adverse effects (encephalopathy), respiratory adverse effects (eosinophilic pneumonia), and ocular adverse effects (optic neuritis). PAS should be used with caution in patients with glucose-6-phosphate dehydrogenase (G6PD) deficiency and in those who are allergic to aspirin. (Arbex *et al* 2010)

Drug-drug interactions

Digoxin can reduce the absorption of PAS. Ethionamide can increase hepatotoxicity and hypothyroidism in patients treated with PAS. Isoniazid increases acetylation, which results in an increase in the serum levels of PAS. Concomitant use of angiotensin-converting enzyme inhibitors and PAS can reduce the antihypertensive effect of the latter. Concomitant use of PAS and carbonic anhydrase inhibitors potentiate the adverse effects of both drugs, and concomitant use of PAS and systemic corticosteroids can also increase the number and severity of adverse effects, especially gastrointestinal effects. PAS can reduce the effect of loop diuretics, and, conversely, loop diuretics can increase the serum levels of PAS. With the exception of diclofenac, nonselective NSAIDs can increase the adverse effects of PAS. PAS can increase the hypoglycemic effects of sulfonylurea, as well as increasing the risk of bleeding when administered in conjunction with oral anticoagulants, thrombolytics, or salicylates. (Arbex *et al* 2010)

The elimination of methotrexate, a widely used antifolate drug has been found to be prolonged in patients using salicylates, PAS being one of them, and sulphonamides. This can increase exposure to methotrexate, and may result in increased drug toxicity. (Joerger *et al* 2006) Other DDIs reported to occur between PAS and other drugs are listed in the table 3.

Table 3: Drug-drug interactions between PAS and other medications

Drug	Interaction
Azathioprine	PAS may increase the toxicity of azathioprine.
Mercaptopurine	PAS may increase the toxicity of mercaptopurine.
Sulindac	Risk of additive toxicity (e.g. bleed risk). PAS may decrease the serum concentration of sulindac.
Thioguanine	PAS may increase the toxicity of thioguanine.
Tiaprofenic acid	Increased risk of gastrointestinal bleeding.
Tolmetin	Additive effects increase the risk of GI bleeding
Trandolapril	PAS may reduce the efficacy of trandolapril
Treprostinil	The prostacyclin analogue, treprostinil, may increase the risk of bleeding when combined with PAS.
Warfarin	The antiplatelet effects of PAS may increase the bleeding risk associated with warfarin.

Adapted from <http://www.drugbank.ca/drugs/DB00233>.

2.12.2 Ethionamide

Thioamide drugs, ethionamide and prothionamide, have been widely used for many years in the treatment of mycobacterial infections caused by *MTB*. Both are bactericidal and are essentially interchangeable in a chemotherapy regimen. They are the most frequently used drugs for the treatment of drug-resistant TB and, therefore, are becoming increasingly relevant as the number of MDR and XDR cases is increasing worldwide. (Wang *et al* 2007)

Pharmacokinetics

Absorption: Approximately 80% of a gastrointestinal oral dose of ethionamide is rapidly absorbed from the gastrointestinal tract. Following a single 1 g oral dose in adults, peak plasma concentration of ethionamide averaging 20 µg/ml are attained within 3 hours and less than 1 µg/ml at 24 hrs. Following a single 250 mg oral dose in adults, the peak plasma concentrations of ethionamide average 1-4 µg/ml (McEvoy 1990).

Distribution: It is widely distributed throughout body tissues and fluids. It crosses the placenta and penetrates the meninges, appearing in the CSF in concentrations equivalent to those in the serum. (Reynolds 1989) The in vivo penetration of ethionamide into pulmonary macrophages and epithelial lining fluid (ELF) in humans has not been reported. (Conte *et al* 2000)

Metabolism: Ethionamide is extensively metabolized, to ethionamide sulfoxide, 2-ethylisonicotinic acid and 2-ethylisonicotinamide. The sulfoxide is the main active metabolite. The sulfoxide metabolite has been demonstrated to have antimicrobial activity against *MTB*. (DeBarber *et al* 2000)

Mechanism of action

Ethionamide may be bacteriostatic or bactericidal in action, depending on the concentration of the drug attained at the site of infection and the susceptibility of the infecting organism. Ethionamide, like prothionamide and pyrazinamide, is a nicotinic acid derivative related to isoniazid. It is thought that ethionamide undergoes intracellular modification and acts in a similar fashion to isoniazid. It inhibits the synthesis of mycolic acids, an essential component of the bacterial cell wall. (DeBarber *et al* 2000) Both the drug and the sulfoxide metabolite are active against *MTB*. 2-ethylisonicotinic acid and 2-ethylisonicotinamide are not active metabolites. (Reynolds 1989)

Adverse effects

Gastro intestinal disturbances are the most frequent. Adverse effects of the drug include nausea, vomiting, diarrhea, abdominal pain, excessive salivation, metallic taste,

stomatitis, anorexia, and weight loss. These adverse effects appear to be dose related, with approximately 50% of patients being unable to tolerate a single dose of 1 g. (Fox *et al* 1969) Transient increases in serum bilirubin, AST and ALT concentrations have been reported in patients receiving ethionamide. Hepatitis (with or without jaundice) has also been reported. (Fajardo *et al* 2006) Goiter, with and without hypothyroidism, has also been associated rarely with ethionamide therapy. (Drucker *et al* 1984)

2.12.3 Terizidone

The chemical structure of terizidone consists of two molecules of cycloserine and one molecule of terephthalaldehyde. (Zitkova and Tousek 1974) It is a broad spectrum antibiotic. Terizidone capsules of 250 mg each is recommended for TB both pulmonary and extra pulmonary by resistant strains of *MTB* or avium. Terizidone is being used in some countries like South Africa instead of cycloserine and is assumed to be as efficacious; however, there are no direct studies comparing the two drugs. (WHO Geneva 2008)

Mechanism of action

Terizidone acts by inhibiting cell wall synthesis by competitively inhibiting two enzymes, L-alanine racemase and D-alanine ligase, thereby impairing peptidoglycan formation necessary for bacterial cell wall synthesis. (Agam 2010)

Pharmacokinetics

Terizidone is completely and rapidly absorbed after oral administration. Maximum concentrations in blood are achieved in 2 to 4 hrs. Excretion in urine is faster in young than in older patients. It is well distributed in all body fluids and tissues. The half-life of terizidone is significantly greater than that of cycloserine with doses of 250 mg and 500 mg. Also, it is significantly higher in the elderly than in young patients. (Agam 2010)

Adverse effects

Dizziness, slurred speech, headache and convulsions are amongst the few reported side effects. Others include tremors, insomnia, confusion, depression. The most dangerous side effect is suicidal tendency. Nausea, vomiting, skin allergies and rashes are also reported. When used in higher doses that are more than 1 g per day liver function disorders, congestive cardiac failure, convulsions and coma are reported. (Agam 2010)

CHAPTER THREE

HYPOTHESES OF THE STUDY

The work contained in this thesis is based on the following hypotheses:

1. Because of developmental changes, the PK of PAS and resulting plasma levels may differ in children of different age groups.
2. Young children require a higher body weight adjusted dosage of the “second-line” anti-TB agent PAS than adults to achieve equivalent plasma concentrations.
3. The drugs under investigation are metabolically unstable and have a potential for drug-drug interactions.

CHAPTER FOUR

AIM OF THE STUDY

The primary aim of this thesis was to evaluate the PK of PAS in children, in comparison to PK data obtained in adults. In the second part of the study (exploratory *in vitro* study), stability and susceptibility to biotransformation of the second-line anti-TB drugs ETH and PAS were determined as well as the potential of PAS, ethionamide and terizidone towards the inhibition of CYP450 enzyme system. The specific aims were:

1. To document the descriptive PK of the second-line anti-TB agent PAS in children, in an attempt to provide an insight to the dosing rationale of this compound; these data were compared to the results obtained in adult patients as a reference group.
2. To compare the PK profile of PAS in children when administered once versus twice daily.
3. To generate baseline data and methodology for the future application of *in vitro* metabolism studies at the Division of Pharmacology.
4. To determine the potential DDIs of ethionamide, terizidone and PAS by inhibiting the CYP450 isoenzymes 1A2 and 2C9.

CHAPTER FIVE

MATERIALS AND METHODS

5.1 Study subjects

A prospective study was conducted in 22 patients who were on TB treatment at Brooklyn Chest Hospital (BCH), Cape Town, South Africa. BHC is a TB hospital, focusing on the long-term treatment of complicated and drug-resistant TB.

The diagnosis of TB was based on clinical symptoms, X-ray examinations, sputum and gastric washings microscopy, and *MTB* culture. Patients were included if they were on PAS on their treatment regimen. Patients who were below 3 months or above 60 years of age and those with severe anemia, vomiting and diarrhea were excluded.

The study was conducted according to the ethical guidelines and principles of the International Declaration of Helsinki, South African Guidelines for Good Clinical Practice and the Medical Research Council (MRC) Ethical guidelines for research and was approved by the Human Research Ethics Committee at Stellenbosch University (number N09/08/212).

Written informed consent was obtained from all subjects while in children one parent or a legal guardian was asked to provide written informed consent and children over 7 years of age provided assent. PAS was prescribed by the attending pediatrician or physician for all patients. Exclusion criteria were severe anemia, severe diarrhea, severe dehydration and/or severe illness.

5.2 Study design

1. A prospective, longitudinal, hospital based clinical pharmacokinetic study.
2. An *in-vitro* laboratory based analytical study.

5.3 Drug administration

All patients were prescribed oral doses of PAS (PASER granules, PAS Delayed-Release Granules-JACOBUS) produced by Jacobus Pharmaceutical Co, Inc., Princeton, NJ 08540 (USA). All children received a 75 mg/kg twice daily at least 2 weeks before enrolment into the study.

The study dosing regimens were adjusted on two occasions; at the first visit children patients received 75 mg/kg body weight twice daily and at the second visit children received 150 mg/kg body weight once daily, and this dose was received two days before the study day. All children received 75 mg/kg in between study days. However, they were given 150 mg/kg body weight dose for two days prior to the second visit study day.

Adult patients received the standardized dose of 4 g twice daily throughout the study period. Adherence to PAS was monitored by direct observation.

By necessity, concomitant drugs were allowed to vary across the patients according to the susceptibility pattern of each patient organism. None of the concurrent medications were known to have DDIs with PAS. Although breakfast typically was given to patients approximately 1 hr after dosing, the timing of food intake in relation to doses was not controlled. All doses were taken with an acidic beverage (orange juice) to prevent an early release of the drug in the stomach.

5.4 Sample collection and bioanalysis of PAS

Doses, dosing times, and blood sampling times were carefully recorded for each patient on a study flow sheet during each visit. For the adult patients blood was collected through a 20-gauge angiocatheter inserted into a forearm vein. For the pediatric patients a proportionate smaller angiocatheter was used.

A total of 6 ml of blood was scheduled to be collected immediately before and at 2, 4 and 6 hrs after drug ingestion in the first 3 patients (C1-C3) (children) and the same were repeated after 4 weeks of treatment. Following an interim analysis of the pharmacokinetic data, the protocol was amended for the subsequent patients. This amendment was approved by the Ethics Committee.

The blood samples were drawn prior to taking the dose and subsequently at 2, 4, 6, 8 and 12 hrs after ingestion of a drug on day one and the same procedure was repeated after 4 weeks. In the adult group, blood samples were drawn before and at 2, 3, 4, 5, 6, 8 and 12 hrs after drug ingestion on day one; this was then repeated after two to four weeks.

Samples with accurately recorded collection times between 0 hr and 12 hrs post-dose were acceptable for analysis. The blood samples were centrifuged at 4000 g for 10 min to obtain plasma. The plasma was promptly harvested and frozen at -80°C until assay. All samples were assayed in duplicate using a High Performance Liquid Chromatography/mass spectrometry method (LC-MS/MS) at the Division of Pharmacology, Department of Medicine, Faculty of Health Sciences, Stellenbosch University. The details of the assays are described in table 4.

5.5 PK analysis of PAS

PK parameters of PAS were calculated for each subject using a non compartmental model with first-order absorption and first order elimination, under the assumption that all subjects were at steady state. The maximum concentration of drug in plasma was defined as C_{max} and the time to this maximum concentration as T_{max} , C_{max} and T_{max} were determined directly from the plasma concentration time profiles. The area under the plasma concentration-time curve from time 0 to T (AUC_{0-T}), where T is the dosing interval, was calculated with the use of the linear trapezoidal method. Apparent oral clearance ($\text{CL}/\text{body weight}\cdot\text{F}$) was calculated as $\text{dose}/\text{AUC}_{0-T}\cdot\text{body weight}$.

5.6 Statistical analysis

The identified independent variables were age, sex, body weight, size of each dose and concomitant medications. The mean values, median, standard deviation (SD), Standard error (SE), coefficient of variation and range for each parameter were calculated and the results were presented in tables and graphs using Stata software, Version 10, 2009 (Stata Corp, Texas, USA) and Graph Pad Prism version 5.01. (GraphPad software, Inc, San Diego, California).

A repeated measures ANOVA was performed using the MIXED procedure in SAS version 9 (Local, XP_PRO). The comparison between groups (adults versus children, children receiving 75 mg/kg or 150 mg/kg and disease state) was treated as a between subject effect, whereas time after intake of study drug was regarded as a within subject effect.

5.7. Materials and methods for the *in vitro* study

5.7.1 Materials

5.7.1.1 Buffers

1. Potassium phosphate, monobasic (KH_2PO_4) (Sigma, St. Louis, MO).
2. Potassium phosphate, dibasic, trihydrate ($\text{K}_2\text{HPO}_4 \cdot 3\text{H}_2\text{O}$) (Sigma, St. Louis, MO).

5.7.1.2 Reagents for the NADPH-regenerating system

1. Glucose-6-phosphate (Sigma, St. Louis, MO).
2. N-Nicotinamide adenine dinucleotide phosphate, NADPH, reduced form (Sigma, St. Louis, MO).
3. Sodium citrate, tribasic (Sigma, St. Louis, MO).
4. Glucose-6-phosphate dehydrogenase (G6PDH) (Sigma, St. Louis, MO).
5. $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ (Sigma, St. Louis, MO).

5.7.1.3 CYP- specific substrates and metabolites

1. Phenacetin, a probe substrate for CYP1A2 (Sigma, St. Louis, MO).
2. Diclofenac, a probe substrate for CYP2C9 (Sigma, St. Louis, MO).

5.7.1.4 CYP selective inhibitors

1. Naphthoflavone, a CYP1A2 selective inhibitor (Sigma, St. Louis, MO).
2. Quercetin, a CYP2C9 inhibitor (Sigma, St. Louis, MO).

5.7.1.5 Human liver microsomes

1. Pooled HLMs were obtained from XENOTECH, LLC, Kansas City, MO. Each batch contained the pooled HLMs from 50 different donors. All the donors were dead (e.g. motor traffic accidents, cerebral vascular accidents and brain death). All samples were from the otherwise healthy donors and in all cases the cause of death did not result from any known biochemical deficiency in the liver.

5.7.1.6 Solvent, test compounds and internal standard

1. Deionized water.
2. Methanol.
3. Acetonitrile.

4. Test compounds; PAS, terizidone, ethionamide.
5. Thiacetazone as an internal standard (IS).

5.7.1.7 Other materials and instruments for the analysis

Agilent 1100 high-performance liquid chromatography (HPLC) system with an autosampler interfaced to an atmospheric turbulon ionisation chamber of an API 2000 tandem mass spectrometer (MS/MS).

5.7.2 Incubation conditions: General

Specific aspects of incubation condition for each assay (e.g., protein concentration, incubation time, reaction termination solvent) are described below. HLMS were thawed from -80°C . In general, graded concentrations of the substrates were incubated with the HLMS at protein concentration of $0.25\ \mu\text{g}/\text{ml}$ in potassium phosphate buffer (pH 7.4), and pre-warmed to 37°C in incubation eppendorf tubes immersed in shaking water bath (37°C). Metabolic reactions were initiated by the addition of NADPH-regenerating system made of 2.6mM NADP^+ , $6.7\ \text{mM}$ G6P, $6.6\ \text{mM}$ magnesium chloride and 0.8 units/ml G6PDH. Metabolic reactions were terminated by precipitation of microsomal proteins upon the addition of ice cold acetonitrile (-20°C) and centrifuged at $4042\ \text{g}$ for 10 minutes.

5.7.3 Determination of kinetic parameters in microsomal incubation

To determine the kinetic parameters for CYP-specific substrates, incubation conditions were first optimized. In the current method (Yan and Caldwell 2004) the final concentration of microsomal proteins was $0.25\ \text{mg}/\text{ml}$, and incubation time was 30 minutes. The concentration range of a particular substrate was estimated according to K_m values in the literature. Briefly, a CYP-specific substrate compound at different concentrations was mixed with microsomes. After a 5 minutes preincubation period, an NADPH-regenerating solution was added to initiate the reaction. The reaction was stopped by the addition of ice cold acetonitrile. The compound remained after a specific CYP-catalyzed oxidation were analyzed using LC-MS/MS.

5.6.3.1 Substrate working solutions

1. Microfuge tubes were labeled.

2. The substrate stock solution was diluted with deionized water to the highest desired concentration.
3. 400 ml of deionized water was added to each of tube.
4. 200 ml of substrate solution was transferred to the microfuge tubes and was mixed by vortexing.

5.7.3.2 Microsomal dilutions

1. To make a 13 ml microsomal solution, 2.6 ml 0.5 M potassium phosphate, (pH 7.4) was added to a 15 ml tube.
3. 325 ml HLMs was added (20 mg/ml proteins).
4. Deionized water was supplied to bring to the desired volume (13 ml).
5. The tube was inverted repeatedly to mix all components and was kept on ice.

5.7.3.3 NADPH- regenerating solution

In the current method, a NADPH-regenerating solution was prepared as described below, which contained 2.6 mM NADP⁺, 0.8 U/ml G6PDH, 6.7 mM glucose-6-phosphate, and 6.6 mM magnesium chloride.

1. 2.6 ml of 0.5 M of potassium phosphate, pH 7.4, was transferred to a 15-ml tube.
2. 8.84 ml deionized water was added to the tube.
3. 1.3 ml stock solution of cofactors was added.
4. 0.26 ml of G6PDH solution (40 U/ml) was added to complete the NADPH-regenerating system right before use.

5.7.3.4 Microsomal incubation

1. 10 ml of substrate was dispensed at different concentrations to corresponding incubation tubes.
2. 250 ml of diluted HLMs was dispensed to each tube using a pipette.
3. Preparation of NADPH-generating solution was completed by adding the G6PDH solution.
4. With a use of a pipette, 240 ml NADPH-generating solution was dispensed to each tube to initiate the reaction.
5. 1 ml ice-cold acetonitrile was used to stop the reaction.

6. The preparation was then centrifuged for 10 min at 4000 g to pellet down microsomal proteins.

7. The supernatant was transferred to HPLC vials for LC-MS/MS analysis.

5.7.3.5 Determination of IC₅₀ values in HLMs

After kinetic parameters of a CYP-specific substrate were determined, an experiment was performed to measure the IC₅₀ value of a test compound for this particular CYP enzyme. Basically, a test compound was serially diluted to desired concentrations and then mixed with microsomes containing a CYP-specific substrate at a concentration equal or close to the *K_m* value of the substrate determined under optimal conditions. After incubation with NADPH, the effect of the test compound on the CYP marker activity was then evaluated by measuring the remainder of the CYP substrate. For the quality control, CYP selective inhibitors were also included in the assay.

5.7.3.6 Preparation of known CYP inhibitors

Individual CYP-selective inhibitors were used to initially validate the inhibition assay when the assay was established and to verify the assay as positive controls.

1. Naphthoflavone, 0.1 mM dissolved in acetonitrile.
2. The CYP-specific inhibitors in tube were diluted to the desired concentration.

5.7.3.7 Test compound solutions

1. The test compounds were dissolved in an appropriate solvent to prepare a 10 mM solution.
2. Microfuge tubes were labeled accordingly
3. The compound stock solution was transferred to the appropriate tube.
4. Solvent (used to dissolve the compound) was added to each of tubes.
5. The compound stock solution was transferred to the tube and was mixed by vortexing.
6. Serial dilution (fourfold dilution) of compound in tubes was then performed.
7. Solvent alone was used as negative control.
8. A CYP-specific inhibitor was used as positive control

5.7.3.8 Inhibition assay

1. NADPH-regenerating solution and the test compound were prepared as described previously.
2. 5 µl of test compound was dispensed at different concentrations to corresponding tubes.
3. 245 µl of diluted HLMS solution was added to each tube.
6. 240 µl of NADPH-generating solution was added to each tube.
7. The mixture was incubated in a 37°C water bath for 5 min.
8. 10 µl of CYP-specific substrate was added to each tube, and incubation was continued for an additional 30 min.
9. Ice-cold acetonitrile was added to stop the reaction.
10. The mixture was centrifuged for 10 min at 4000 g.
11. The supernatant was transferred to HPLC vials for LC-MS/MS analysis, as described below.

5.8 Analytical methods

The developed analytical procedures (methods) were aimed to serve for a research project. All procedures were performed as accurately as possible, guidelines as laid down by the FDA and EMA were adhered to as far as possible. A full validation of the methods, as laid down by the FDA and/or EMA for clinical trials conducted by the pharmaceutical industry, was however not possible within the scope of the Division of Pharmacology.

Blood samples were collected in 1.0 ml sampling EDTA containing sampling vials and centrifuged at 3500 g for 5 minutes to separate plasma and cellular matter. Collected plasma samples were frozen at -80°C and stored until analysis. The stability of compounds under investigation in frozen samples was determined over a time period of 6 months; no degradation could be detected within this time period.

Patient samples were analyzed within this time window, usually within 3 weeks after collection. Freezing and thawing of samples did not significantly influence the

concentrations of high, medium and low calibrators of the compounds under investigation.

To 100 µl of plasma 300 µl of methanol, containing 1.0 µg/ml thiazetazone (IS) (Sigma, St Louis, USA), was added to precipitate plasma proteins. Samples were centrifuged at 11 000 g for 5 minutes. 250 µl of the supernatant was transferred into suitable autosampler vials for analysis. The temperature of the autosampler was set to be 6 °C. The respective injection volumes are indicated in table.

Specimens were analyzed using a binary HPLC (Agilent Series 1100 HPLC, Agilent Technologies, Waldbronn, Germany) equipped with an Agilent Zorbax analytical column (150 mm x 2.1 mm (ID), 3.5 µm particle size). The column temperature and flow rate are indicated for each compound in the table below. The mobile hydrophilic phase "A" was water containing 0.1% formic acid (FA) (Fluka Chemie GmbH, Buchs, Switzerland) whilst the lipophilic phase "B" was either methanol or acetonitrile (E. Merck, Darmstadt, Germany) both containing 0.1% FA. All solvents were of HPLC grade and were filtered through a 0.45 µm filter to remove possible particular matter. The respective used gradients are indicated below. Specifications of the pure compounds to create respective calibration curves are indicated in table 4. In all instances a quadratic equation was used for calibration including a 1/x weighting resulting in an R² value of better than 0.9900.

The concentration of the compounds was determined by means of an API 2000 tandem Mass Spectrometer (MS/MS) (Applied Biosystems, MDS Sciax, Foster City, Canada) equipped with an atmospheric turbulon ionization chamber. A single quantifier transition range for the IS (thiazetazone) with a precursor and product ion of m/z 237.12 / 119.96 (dwell 500 msec) was used whilst a transition of m/z 237.12 / 134.10 (dwell 500 msec) was used as qualifier. The retention time of thiazetazone was 7.0 min. A switching valve was used to only introduce the column effluent into the nebulizing chamber at specified time ranges including the time from 6.5 to 7.5 min for the IS to avoid unnecessary contamination of the nebulising chamber.

To each sample batch 8 calibrators of varying concentrations were included into the run as quality control (QC) samples.

For all compounds a variation of less than $\pm 5\%$ was found over the entire duration of the analysis. Duplicates of patient samples did not vary more than 2.5% from each other. Intraday variation was less than 3% whilst day to day variation was found to be less than 5% over the respective calibration range.

The quantifier m/z transition was used for quantification whilst the indicated qualifier m/z transition was used to ensure selectivity of the respective compound. The respective m/z transitions are listed in table 4.

Table 4: Summary of bioanalytical methods

Drug specific individual instrument settings	Phenacetin	Diclofenac	PAS
Pure compound specifications used for calibration and creation of respective standard curve	Aldrich, Cat # 77440 Lot S84263 - 289	Sigma, Cat # D-6899 Lot 100K1715	Aldrich, Cat # A7,960-4 Lot : S 44855 - 149
Solvents with 0.1% FA added	A: Water B: Acetonitrile	A: Water B: Methanol	A: Water B: Methanol
Gradient of Solvents A(hydrophilic):B(organic) Time range / (% A)	0.0-1.0 / 65% A 1.0-1.1 / 1.0% A 1.1-5.5 / 1% A 5.5-5.6 / 65% A 5.6-10.0 / 65% A	0.0-1.0 / 50% A 1.0-2.0 / 5% A 2.0-8.0 / 5% A 8.0-8.10 / 50% A 8.1-11.0 / 50% A	0.0-0.95 / 90% A 0.95-1.0 / 10% A 1.0-8.0 / 10% A 8.1-12.5 / 90% A
Injection volume	2.5 μ l	2 μ l	5 μ l
Flow speed	300 μ l / min	300 μ l / min	300 μ l / min
Column temperature	40 °C	40 °C	40 °C

Nebulizing temp.	375 °C	375 °C	375 °C
Mode	Positive mode	Negative mode	Positive mode
Quantifier transition (m/z)	180.2 / 110.1	293.88 / 249.00	154.2 / 136.2
Qualifier transition(s) (m/z)	180.2 / 138.1	No qualifier with a suitable intensity	154.2 / 119.2 and 154.2 / 108.2
Dwell time (msec)	400 msec	300 msec	500 msec
Retention time	3.35 min	6.65 min	5.42 min
Switching valve settings	2.5 to 4.5 min	6.0 to 8.0 min	4.5 to 6.0 min
Lower limit of detection	0.1 µg/ml	0.25 µg/ml	0.25 µg/ml
Calibration range	0.25 to 75.0 µg/ml	1.0 to 100 ug/ml	1.0 to 100.0 µg/ml

*FA : Formic acid

CHAPTER SIX

RESULTS

6.1 Pharmacokinetics of PAS in children

6.1.1 Patient baseline characteristics

Twenty two TB patients were included in this study. Demographic characteristics (age, body weight, sex, height and HIV status) are presented in table 5. Among these patients, ten were children, their mean age was 4.2 years (range: 1 to 12 years) and three (30%) were male. Mean body weight was 14.8 kg (range: 5.4 to 26.6 kg). Mean height was 93.4 cm (range: 60 to 130 cm). Four (40%) patients were HIV-positive, and epilepsy was found in one patient (10%). Twelve patients were adults, mean age was 31.3 years (range: 18 to 53 years). Four patients were male (33.3%). Mean body weight was 63.4 kg (range: 37 to 84 kg). Mean height was 169.8 cm (range: 163 to 183 cm). Four (33.3%) patients were HIV-positive and epilepsy was found in one patient (8.3%).

At least one first line anti-TB drugs was used by each of the patients, these included isoniazid, pyrazinamide and ethambutol. However, rifampicin was not among the drugs used by any of the patients. All patients received similar SLDs, these included the fluoroquinolone ofloxacin, the thionamide ethionamide, terizidone and the aminoglycoside capreomycin. One patient received rimactazide and one was on linezolid as part of the TB regimen.

All HIV positive patients were on an ART, which included stavudine, lamivudine and efavirenz, except for one adult patient who was on a zidovudine containing regimen. Two patients (one child and one adult) were epileptic; they received sodium valproate and haloperidol, respectively. The detailed information on concomitant medications for each individual patient is presented in tables 5 and 6.

All patients were studied on two occasions as planned. All PK assessments were completed without any events (e.g. vomiting) that may have affected the PK profiles that were recorded.

Table 5: Individual patient characteristics: Children (N=10)

Subject	Age (yrs/months)	Age	Sex	Weight (kg)	Z-score	Diagnosis	Concomitant Medications
C1	3/1	3	F	13	-1.04	XDR-TB	PZA, TZ, ETH, RIMACT, CAPRE.
C2	4/9	5	F	18	0.09	XDR-TB, HIV	INH, PZA, ETHA, ETH, TZ, OFLOX, D4T, 3TC, EFV.
C3	3/8	4	M	18	0.30	XDR-TB	INH, PZA, TZ, ETH, OFLOX.
C4	12/7	3	M	27	-2.32	XDR-TB, HIV, EPILEPSY	INH, PZA, TZ, ETH, D4T, 3TC, EFZ, SOD. VAL, LINEZ.
C5	9/1	9	F	25	-0.90	XDR-TB, HIV	INH, PZA, ETHA, ETH, OFLOX, D4T, 3TC, EFV.
C6	1/9	2	F	11	-1.00	XDR-TB	INH, PZA, ETH, TZ, OFLOX.
C7	3/8	4	F	15.5	-0.18	XDR-TB, HIV	INH, PZA, TZ, OFLOX, 3TC, EFV, D4T.
C8	0/10	1	M	8	-1.74	XDR-TB	INH, PZA, ETHA, TZ, ETH, OFLOX.
C9	1/0	1	F	9	-3.95	XDR-TB	INH, PZA, ETHA, TZ, OFLOX, CAPRE.
C10	1/8	1	F	13	-3.15	XDR-TB	INH, PZA, ETHA, TZ, ETH, OFLOX, CAPRE.

INH: Isoniazid, PZA: Pyrazinamide, ETHA: Ethambutol, ETH: Ethionamide, TZ: Teridizone, OFLOX: Ofloxacin, CAPRE: Capreomycin, RIMACT: Rimactazide, LINEZ: Linezolid, D4T: Stavudine, 3TC: Lamivudine, EFV: Efavirenz, AZT: Zidovudine, SOD.VAL: Sodium valproate.

Key:

Classification	Z-score value
Normal	$-2.0 < Z\text{-score}$
Moderately malnourished	$-3.0 < Z\text{-score} < -2.0$
Severely Malnourished	$Z\text{-score} < 3.0$ or oedema

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The Z-score is an indicator for how many standard deviations the child is above or below the mean. A z-score of less than -2 or more than +2 is usually seen as out of the normal range.

Table 6: Individual patient characteristics: Adult patients (N=12).

Subject	Age	Sex (yrs)	Weight (kg)	Height (cm)	BMI	Diagnosis	Concomitant Medications
A1	31	F	84	165	30.7	XDR-TB, HIV	INH, PZA, TZ, ETHA, ETH, OFLOX, D4T, 3TC, EFV.
A2	27	F	59	163	22.2	XDR-TB, HIV	INH, PZA, ETHA, ETH, TZ, OFLOX, CAPRE, D4T, 3TC, EFV.
A3	27	F	72	173	24.1	XDR-TB, HIV	INH, PZA, ETHA, TZ, ETH, OFLOX, CAPRE, D4T, 3TC, EFV.
A4	18	F	45	164	16.7	XDR-TB, HIV, EPILEPSY	INH, PZA, TZ, ETH, D4T, 3TC, EFZ, EPIL, LINEZ.
A5	26	F	38	161	14.5	XDR-TB, HIV	INH, PZA, ETHA, OFLOX, CAPRE, D4T, 3TC, EFV.
A6	53	M	74	183	21.9	XDR-TB, HIV	INH, PZA, ETH, TZ, OFLOX, CAPRE, D4T, 3TC, EFV.
A7	19	F	55	167	19.7	XDR-TB	PZA, ETHA, ETH, TZ OFLOX, CAPRE.
A8	34	F	58	169	20.1	XDR-TB	PZA, ETHA, TZ, ETH, OFLOX, CAPRE.
A9	41	M	76	175	24.8	XDR-TB	PZA, ETHA, TZ, OFLOX, CAPRE.
A10	48	M	70	170	24.8	XDR-TB	PZA, ETHA, TZ, ETH, OFLOX.
A11	30	M	71	178	24.2	XDR-TB, HIV	PZA, ETHA, ETH, OFLOX, CAPRE, D4T, 3TC, EFV, AZT, HAL.
A12	21	F	62	169	21.5	XDR-TB	INH, PZA, TZ, OFLOX.

INH: Isoniazid, PZA: Pyrazinamide, ETHA: Ethambutol, ETH: Ethionamide, TZ: Teridizone, OFLOX: Ofloxacin, CAPREO: Capreomycin, RIMACT: Rimactazide, LINEZ: Linezolid, D4T: Stavudine, 3TC: Lamivudine, EFV: Efavirenz, AZT: Zidovudine, HAL: Haloperidol, BMI: Body mass index.

BMI Categories:

Underweight: BMI < 18.5, Normal weight: BMI 18.5 - 24.9, Overweight: BMI 25 - 29.9, Obesity: BMI of 30 or greater.

Table 7: Summary of patient characteristics at the time of the PK assessment (average for both visits)

Characteristics	Children (N=10)	Adults (N=12)
Age - yr		
Mean	4.2	31.3
Median	4	28.5
Range	1-12	18-53
SD	2.5	11.1
Weight - kg		
Mean	14.8	63.4
Median	14.2	66
Range	5.4-26.6	37-8
SD	7.3	13.4
Height - cm		
Mean	93.4	169.8
Median	93	169
Range	60-130	163-183
SD	23.6	6.5
Male sex - No. (%)	3 (30)	4 (33.3)
HIV positive - No. (%)	4 (40)	4 (33.3)

SD: Standard deviation

6.1.2 PK of PAS in children and adults

Complete sets of plasma specimens were obtained during treatment with PAS, given as a twice versus once daily dosage. Plasma concentration-time profiles for PAS after treatment with 75 mg/kg twice daily, 150 mg/kg once daily and 4 g twice daily are depicted in figures 2 and 3. Because of the small sample size plasma profiles for each patient (each visit) are displayed. For the initial three patients (C1-C3), blood samples were taken at only three time points up to 6 hours after dosing. After availability of the data, it was decided to change the sampling schedule to six sampling time points up to twelve hours after dosing. The PK parameters of PAS for adults and children on both study days are presented in tables 6-9.

6.1.2.1 PK of PAS in children

Children were given PAS once a day (150 mg/kg) or twice a day (75 mg/kg). The results for the 75 mg/kg twice daily dosing regimen were: Geometric mean C_{max} was 45.4 $\mu\text{g/ml}$ (range: 3.8-68.1), geometric mean T_{max} was 4.8 hrs (range: 0-8), PAS exposure, as measured by the geometric mean AUC was 233.3 $\mu\text{g}\cdot\text{h/ml}$ (range: 22.1-46.1) and mean CL/F was 10.4 l/h/kg (range: 1.3-52).

Results for 150 mg/kg once daily dosing regimen were: Geometric mean C_{max} was 56.5 $\mu\text{g/ml}$ (range 3.7-115), geometric mean T_{max} was 4.8 hrs (range: 0-12), PAS exposure, as measured by the mean AUC was 277.9 $\mu\text{g}\cdot\text{h/ml}$ (range: 14.1-702) and mean CL/F was 47.1 l/h/kg (range: 2.2-283.1). The mean 12 hr concentration following the 75 mg/kg dose in the children was 6.8 $\mu\text{g/ml}$ (range: 0.7-19.4) compared to 21.3 $\mu\text{g/ml}$ (range: 0.6-76.7) following a dose of 150 mg/kg.

In this study 9/10 (90%) of children attained baseline plasma levels above MIC (1 $\mu\text{g/ml}$) after the dose of 75 mg/kg twice daily while only 6/7 children were above MIC at 12 hr plasma levels. In contrast 6/10 (60%) children reached the baseline plasma levels above 1 $\mu\text{g/ml}$ after the dose of 150 mg/kg once daily and only 5/7 (71.4%) children were above MIC at 12 hr mark. However, it is worthwhile to note that only 7 out of 10 children were subjected to the 12 hr measurements as described in the methodology section. Individual concentrations, mean values and standard deviations are presented in table 8.

Table 8: Pharmacokinetic parameters in 10 children after oral administration of twice daily dosage of 75 mg/kg of PAS

Subject	Dose (mg)	C _{max} (µg/ml)	12 h (µg/ml)	T _{max} (h)	AUC _{0-T} (µg•h/ml)	CL/F (l/h/kg)
1	1000	31.8	*	6	59.6	*
2	1340	53.4	*	4	227.1	*
3	1260	39.1	*	4	213.7	*
4	2000	82.7	10.9	0	395.3	5.1
5	1870	54.8	7.2	2	320.8	5.8
6	0780	36.3	1.0	6	197.9	3.9
7	1150	3.8	1.7	8	22.1	52.0
8	0590	26.1	6.5	8	191.1	3.1
9	0400	57.9	0.7	4	245.0	1.6
10	0580	68.1	19.4	6	461.1	1.3
Mean	1097	45.4	6.8	4.8	233.3	10.4
Range	400-2000	3.8-68.1	0.7-19.4	0-8	22.1-461.1	1.3-52
Median	1075	46.3	6.5	5.0	220.4	3.9
SD	539.7	22.7	6.7	2.5	135.1	18.4
SE	170.7	7.2	8.3	0.8	42.7	6.9
CV (%)	49.2	49.9	1.0	52.7	57.9	177.0

* The value was either not available or could not be calculated due to insufficient blood sampling time points, AUC_{0-T}: area under the concentration-time curve from 0 to time post dose, C_{max}: maximum concentration, T_{max}: time to maximum concentration, CL/F: oral total clearance SD: standard deviation; SE: standard error, CV: coefficient of variation.

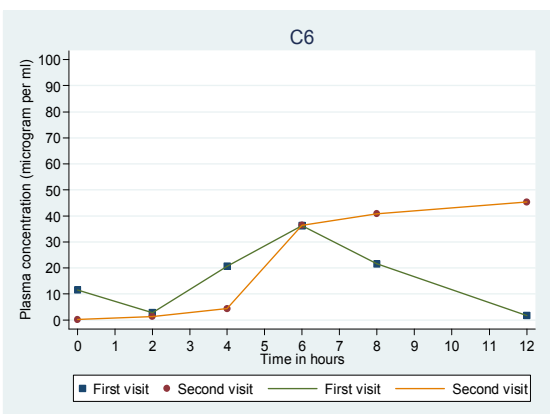
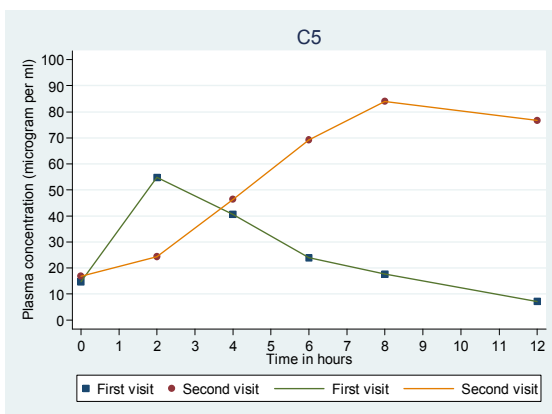
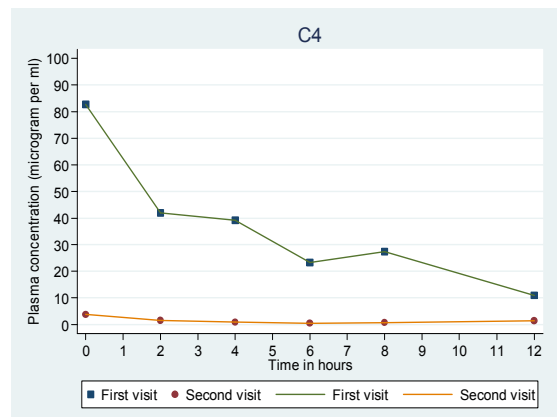
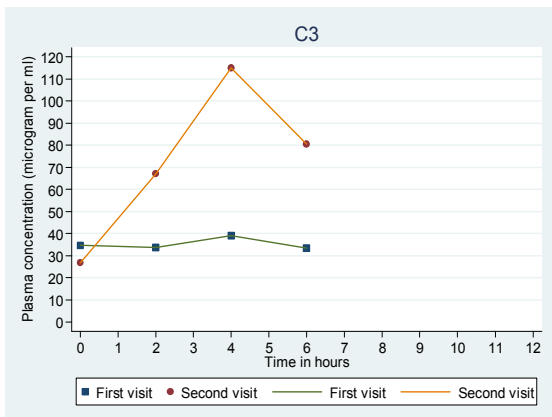
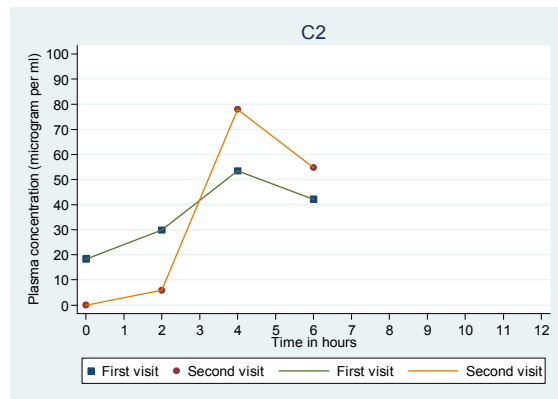
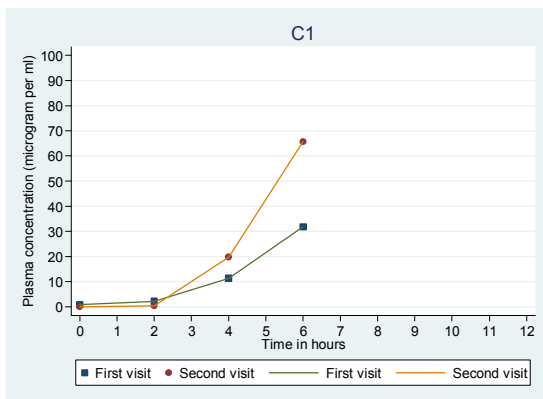
Table 9: Pharmacokinetic parameters in 10 children after oral administration of once daily dosage of 150 mg/kg of PAS

Subject	Dose (mg)	C _{max} (µg/ml)	12 h (µg/ml)	T _{max} (h)	AUC _{0-T} (µg•h/ml)	CL/F (l/h/kg)
1	2000	65.6	*	4	105.8	*
2	2800	78	*	4	222.5	*
3	2700	115	*	4	471.6	*
4	4000	3.7	1.4	0	14.1	283.1
5	3860	83.9	76.7	8	702	5.5
6	1690	45.3	45.3	12	298	5.7
7	2330	51.8	0.6	0	140.6	16.6
8	1200	20.3	2.1	6	94.3	12.7
9	0800	36.1	0.7	4	201.8	3.9
10	1170	65.3	21.9	6	527.9	2.2
Mean	2255	56.5	21.2	4.8	277.9	47.1
Range	800-4000	3.7-115	0.6-76.7	0-12	14.1-702	2.2-283.1
Median	2165	58.5	2.1	4	212.2	5.7
SD	1101	32.4	29.6	3.6	221.3	104.2
SE	348.2	10.3	16	1.1	69.9	39.4
CV	48.8	57.4	1.4	74	79.7	221

* The value was either not available or could not be calculated due to few time points, AUC_{0-T}: area under the concentration-time curve from 0 to time post dose, C_{max}: maximum concentration, T_{max}: time to maximum concentration, CL/F: oral total clearance, SD: standard deviation, SE: standard error, CV: coefficient of variation.

6.1.2.2 Individual plasma concentrations of PAS in children

Although PAS plasma concentrations from all subjects were pooled and analyzed together, the actual plasma concentrations in each individual patient were expressed to allow the better graphical presentations of common patterns of PAS variability in the study population (figures 3 and 4). The significant finding for both groups was that there was a considerable inter- and intra individual variations in the plasma profile of PAS.



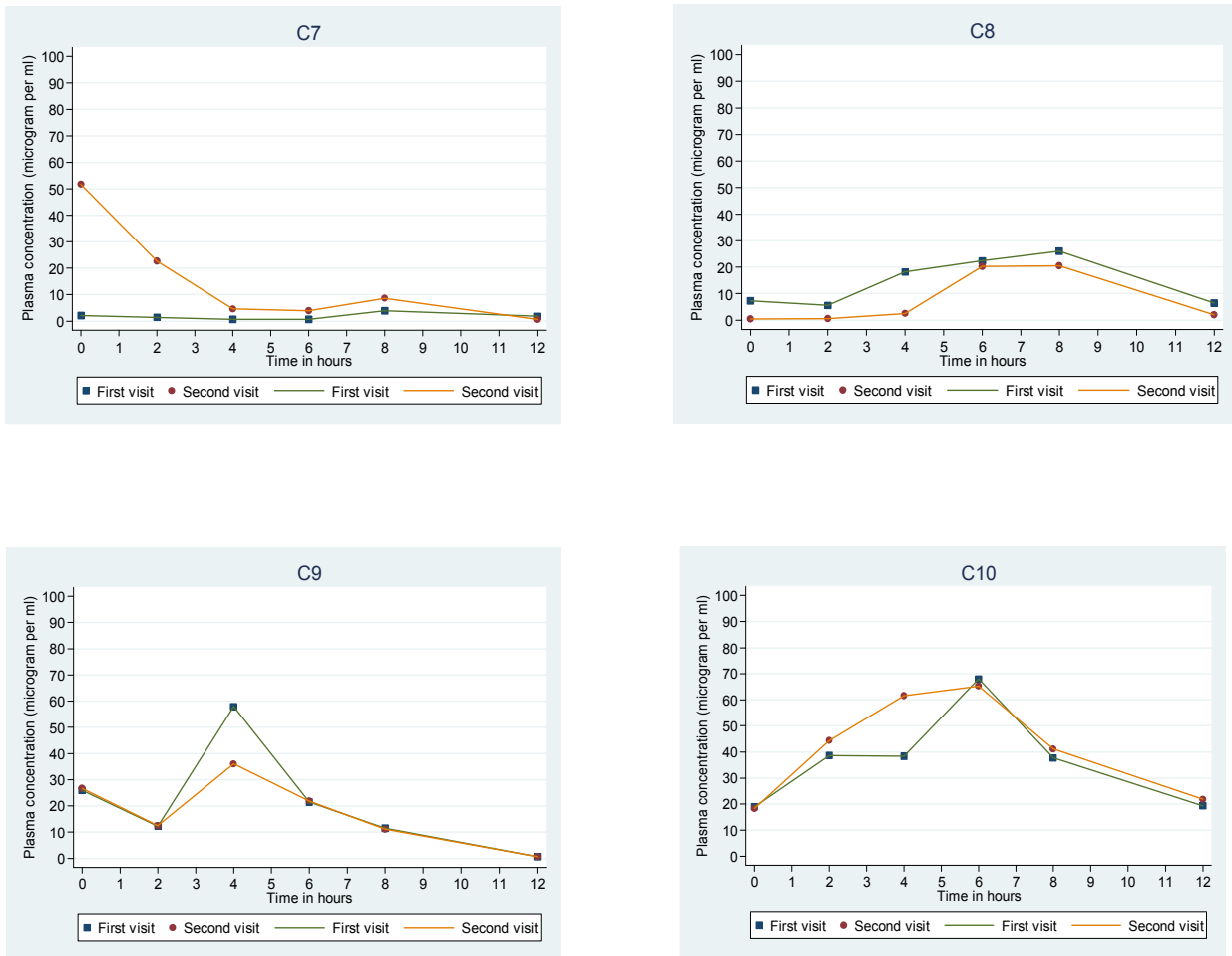


Figure 3: Plasma concentration-time profiles following administration of PAS in two different doses (75 mg/kg twice daily and 150 mg/kg once daily) in individual patients (children).

6.1.2.3 PK of PAS in adults

Adults were given a standard dose of 4 g PAS twice daily. Results for the first visit using a 4 g twice daily dosing regimen were: Geometric mean C_{max} was 51.3 $\mu\text{g/ml}$ (range: 25.8-93.1), geometric mean T_{max} was 5.2 hrs (range: 0-8), PAS exposure, as measured by the geometric mean AUC was 368 $\mu\text{g}\cdot\text{h/ml}$ (range: 146.5-853.3) and mean CL/F was 13.2 l/h/kg (range: 7-27.3).

On the second visit, using the same 4 g twice daily dosing regimen, the results were: Geometric mean for C_{max} was 37.6 $\mu\text{g/ml}$ (range: 15.7-69.5), geometric mean T_{max} was 5.2 hrs (mean: 2-8), PAS exposure, as measured by the geometric mean AUC was 230.6 $\mu\text{g}\cdot\text{h/ml}$ (range: 64.9-539.5) and mean CL/F were 23.9 l/h/kg (range: 7.4-61.6). The mean 12 hrs concentration following the 4 g dose in the adults was 19.7 $\mu\text{g/ml}$ (range: 1.8-91.5) on the first visit compared to 13.8 $\mu\text{g/ml}$ (range: 2.3-34.3) on the second visit.

Although the baseline plasma levels were all above MIC (1 $\mu\text{g/ml}$) after the dose of 4 g twice daily in the first visit, 2 (16.7%) patients were below MIC on the second visit. All 12 patients attained plasma levels above MIC at 12 hr mark in both first and second visits. Individual concentrations mean values, medians, standard error, coefficient of variation and standard deviations have been given in table 10.

Table 10: Pharmacokinetic parameters obtained from 12 adults after oral administration of 4 g of PAS (first visit)

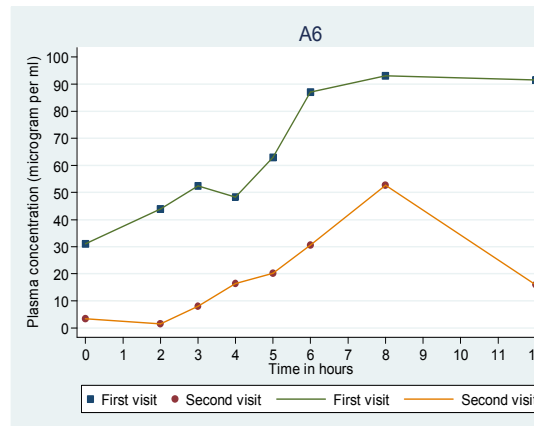
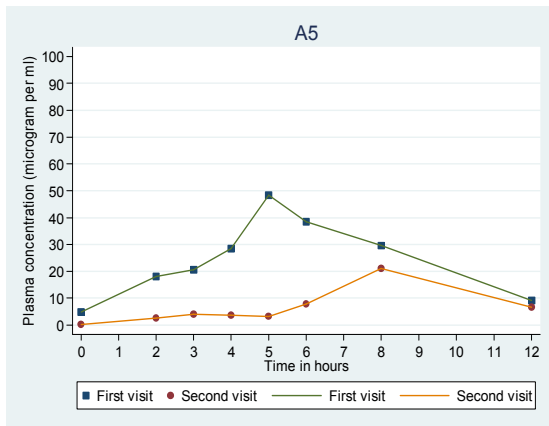
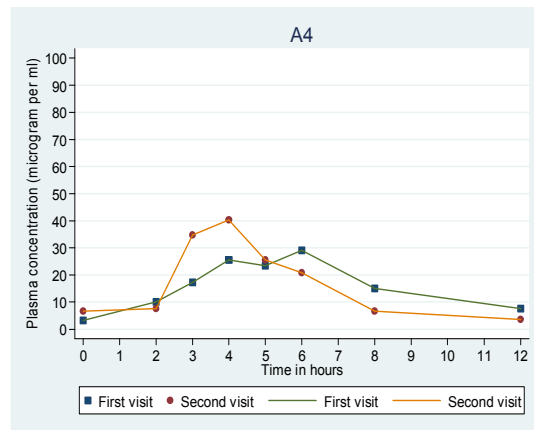
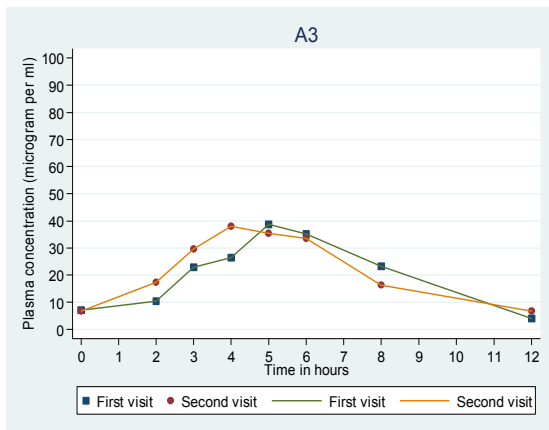
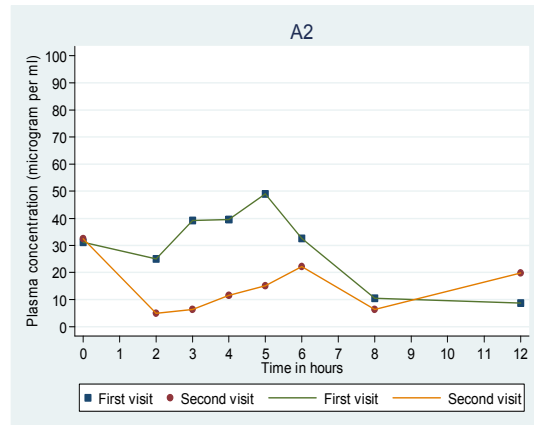
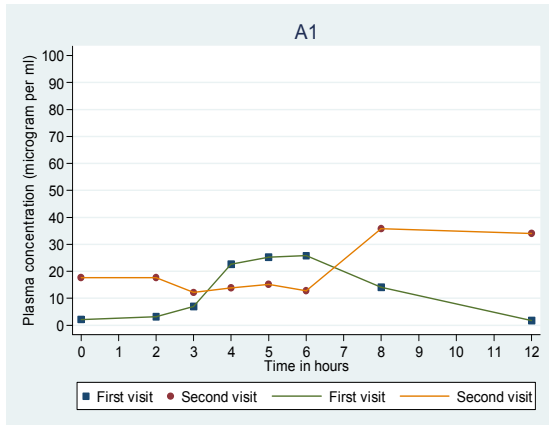
Subject	Dose (mg/kg)	C_{max} (µg/ml)	12 h (µg/ml)	T_{max} (h)	AUC_{0-T} (µg·h/ml)	CL/F (l/h/kg)
1	47.6	25.8	1.8	6	146.5	27.3
2	68	48.9	8.8	5	294.3	13.6
3	55.9	38.8	4.1	5	241	16.6
4	88.9	38.8	7.6	5	241	16.6
5	105.3	48.4	9.1	5	294.2	13.6
6	54.8	93.1	91.5	8	853.3	4.7
7	72.7	48.9	8.8	5	294.2	13.6
8	70.2	37.3	12.3	6	310.8	12.9
9	52.6	87.1	37.2	5	570.2	7
10	57.1	44.7	15.8	8	324.4	12.3
11	56.3	61.1	16.8	4	543	7.4
12	65.6	42.8	29.4	0	303.1	13.2
Mean	66.3	51.3	19.7	5.2	368	13.2
Range	47.6-105.3	25.8-93.1	1.8-91.5	0-8	146.5-853.3	7-27.3
Median	61.4	46.5	9.0	5	298.7	13.4
SD	16.7	20.0	27.1	2	194	5.8
SE	4.8	5.8	8.8	0.6	56	1.7
CV (%)	25.2	39.1	1.4	39.4	52.7	43.7

AUC_{0-T}: area under the concentration-time curve from 0 to time post dose, C_{max}: maximum concentration, T_{max}: time to maximum concentration, CL/F: oral total clearance, SD: standard deviation, SE: standard error, CV: coefficient of variation.

Table 11: Pharmacokinetic parameters obtained from 12 adults after oral administration of 4 g of PAS (second visit)

Subject	Dose (mg/kg)	C _{max} (µg/ml)	12 h (µg/ml)	T _{max} (h)	AUC _{0-T} (µg•h/ml)	CL/F (l/h/kg)
1	48.2	35.8	34	8	268.0	14.9
2	66.7	32.5	19.8	0	164.7	24.3
3	55.6	38.0	6.8	4	248.6	16.1
4	88.9	40.3	3.6	4	176.0	22.7
5	108.1	21.1	6.6	8	103.1	38.8
6	54.1	52.7	16	8	285.0	14.0
7	72.7	29.0	4.8	4	172.4	23.2
8	68.9	16.4	5.4	8	109.2	36.6
9	52.6	69.5	29	5	539.5	7.4
10	57.1	50.3	2.5	5	230.1	17.4
11	57.1	50.3	34.3	6	406.0	9.9
12	64.5	15.7	2.3	2	64.9	61.6
Mean	66.2	37.6	13.8	5.2	230.6	23.9
Range	48.2-108.1	15.7-69.5	2.3-34.3	2-8	64.9-539.5	7.4-61.6
Median	60.8	36.9	6.7	5	203.1	20.1
SD	17.2	16.2	12.5	2.6	134.8	15.2
SE	4.9	4.7	34	0.7	38.9	4.4
CV (%)	25.9	43.0	0.9	50.1	58.5	63.7

AUC_{0-T}: area under the concentration-time curve from 0 to time post dose, C_{max}: maximum concentration, T_{max}: time to maximum concentration, CL/F: oral total clearance, SD: standard deviation, SE: standard error, CV: coefficient of variation.



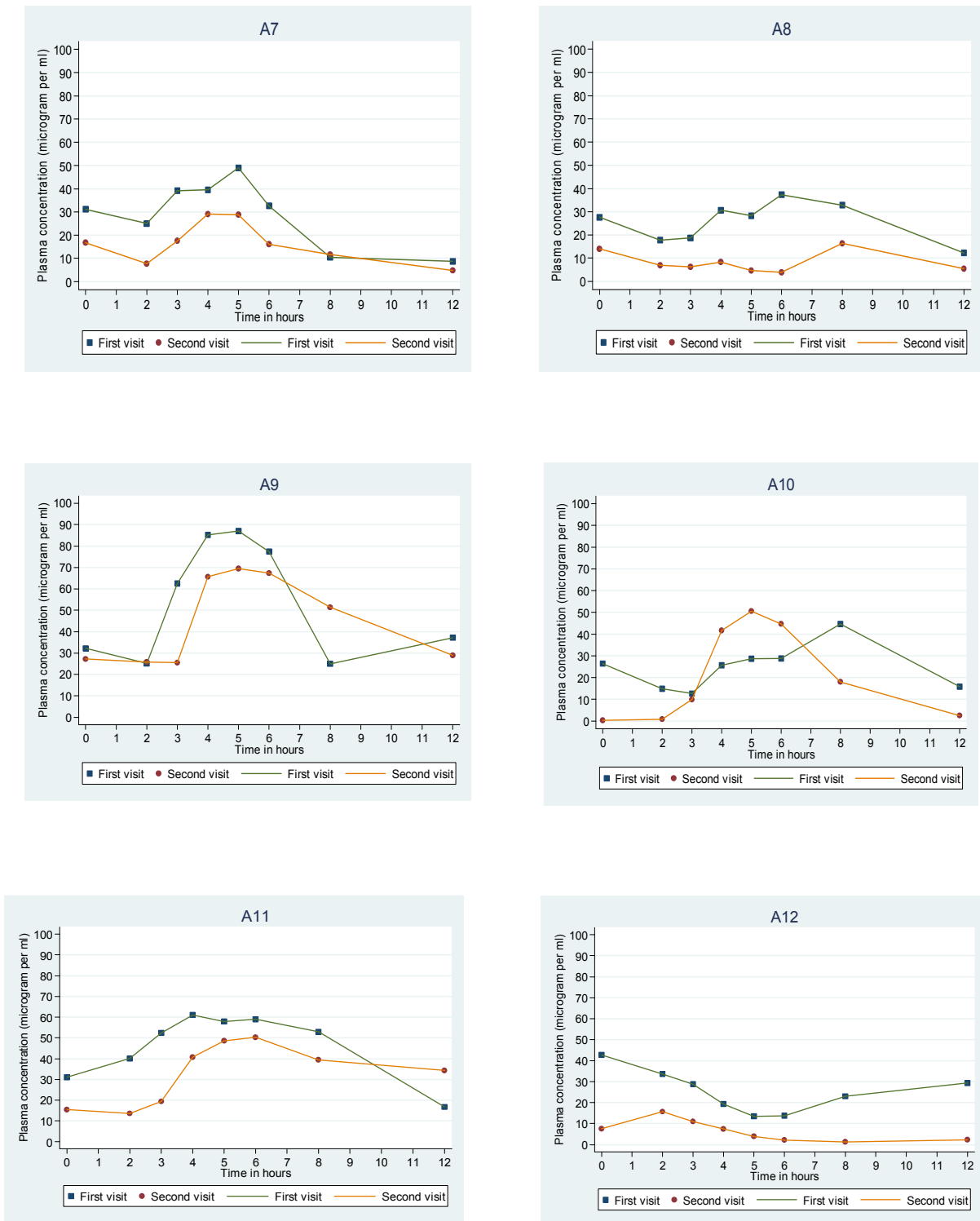


Figure 4: Plasma concentration-time profiles following administration of PAS (4 g twice daily) given at steady state at two different visits in adult patients.

6.1.2.4 Comparison analysis

Comparison between the first and second visits

The mean values as well as individual patient plasma concentrations indicated a considerable discrepancy between the two visits. In the group of children the mean plasma concentrations of PAS differed between two visits but were not statistically significant with the *P*-value of 0.33. Although adults received a similar dose for each visit a higher plasma concentration was noted during the first visit. The difference between both visits was not statistically significant with the *P*-value of 0.82.

Comparison between children and adults

The results indicated that adult patients attained higher plasma concentrations when compared to children on the first visit when children received a 75 mg/kg dose. The same was noted when comparing the plasma concentrations between adults and children at the second visit when children received a 150 mg/kg dose. These differences were not statistically significant with the *P*-value of 0.74.

Comparison between HIV positive and HIV negative patients

Because of the presence of the HIV positive patients in the study population who were on the ART, the comparison was made between HIV positive and HIV negative patients from both groups. The results revealed that there were no significant PK differences between HIV positive and HIV negative patients.

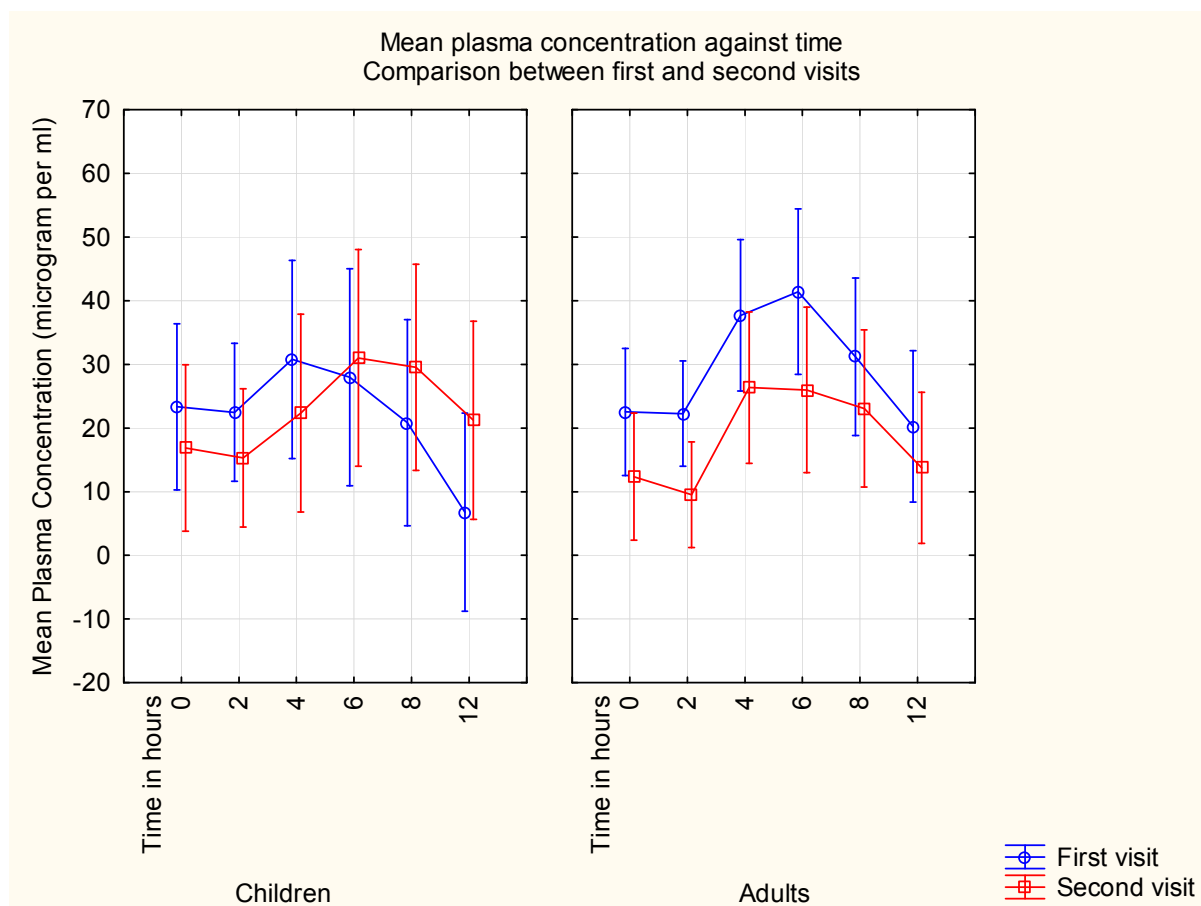


Figure 5: Mean steady-state plasma concentration-time profiles of PAS in children and adults. Children (N=10) received a dose of 75 mg/kg twice daily on the first visit and 150 mg/kg once daily on the second visit. Adults (N=12) received a dose of 4 g PAS on both visits. Vertical bars denote 95 % confidence intervals.

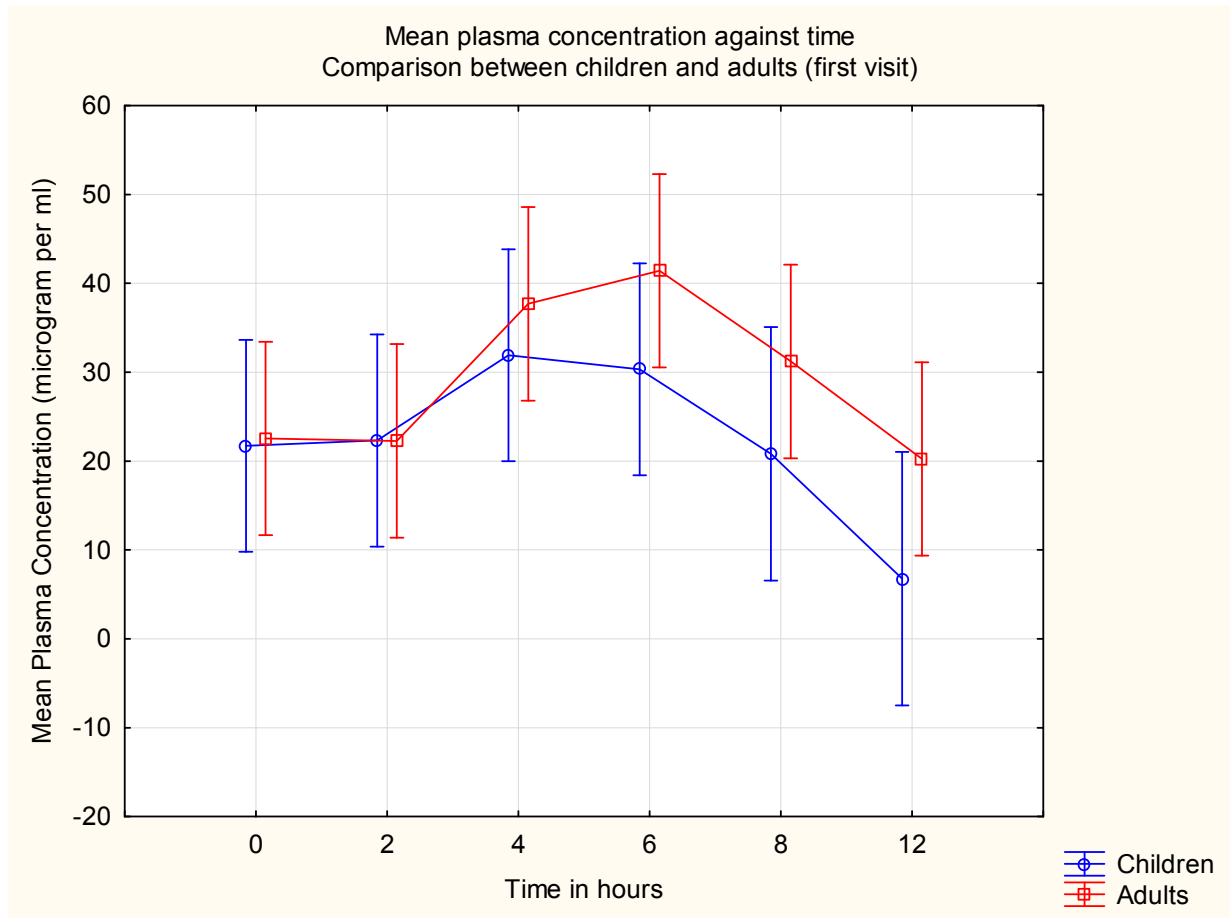


Figure 6: Mean steady-state plasma concentration-time profiles of PAS in children (75 mg/kg twice daily) and adults (4 g twice daily, first visit). Vertical bars denote 95% confidence intervals.

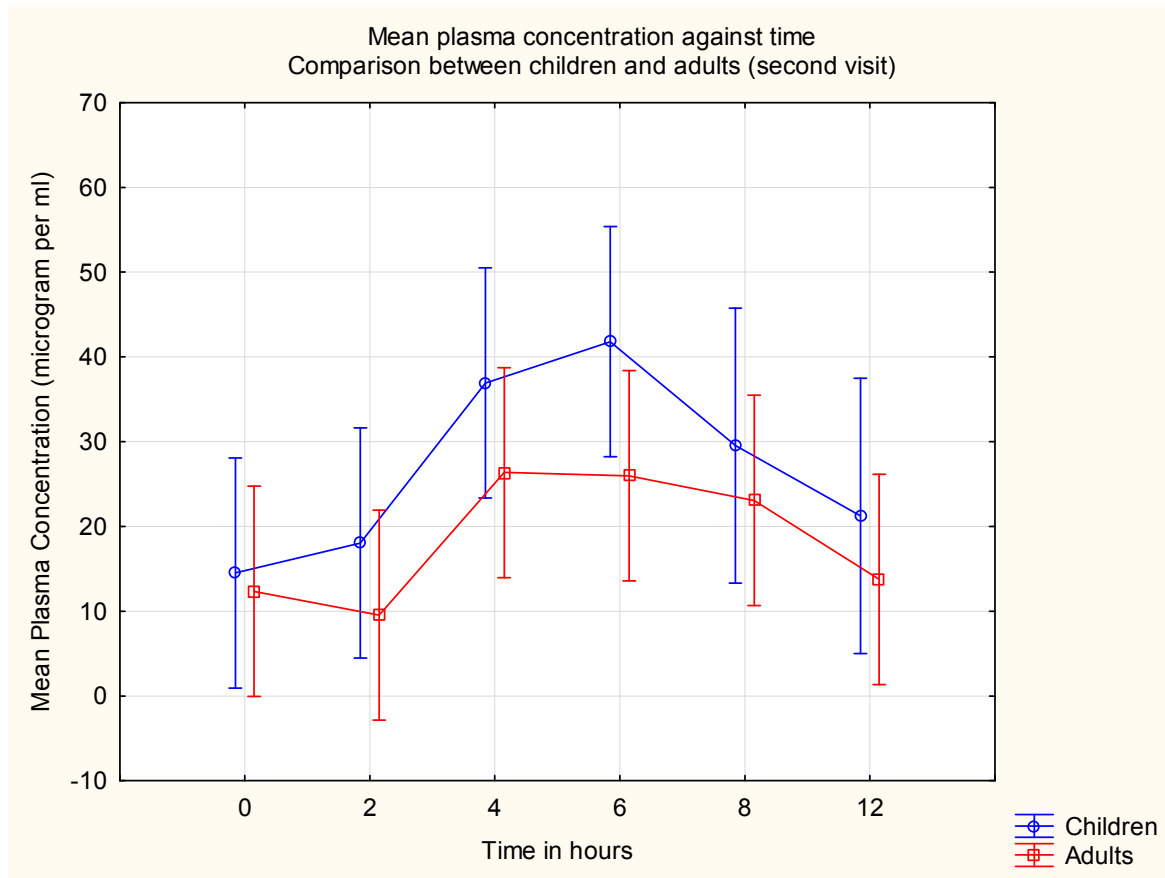


Figure 7: Mean steady-state plasma concentration-time profiles of PAS in children (150 mg/kg once daily) and adults (4 g twice daily, second visit). Vertical bars denote 95% confidence intervals.

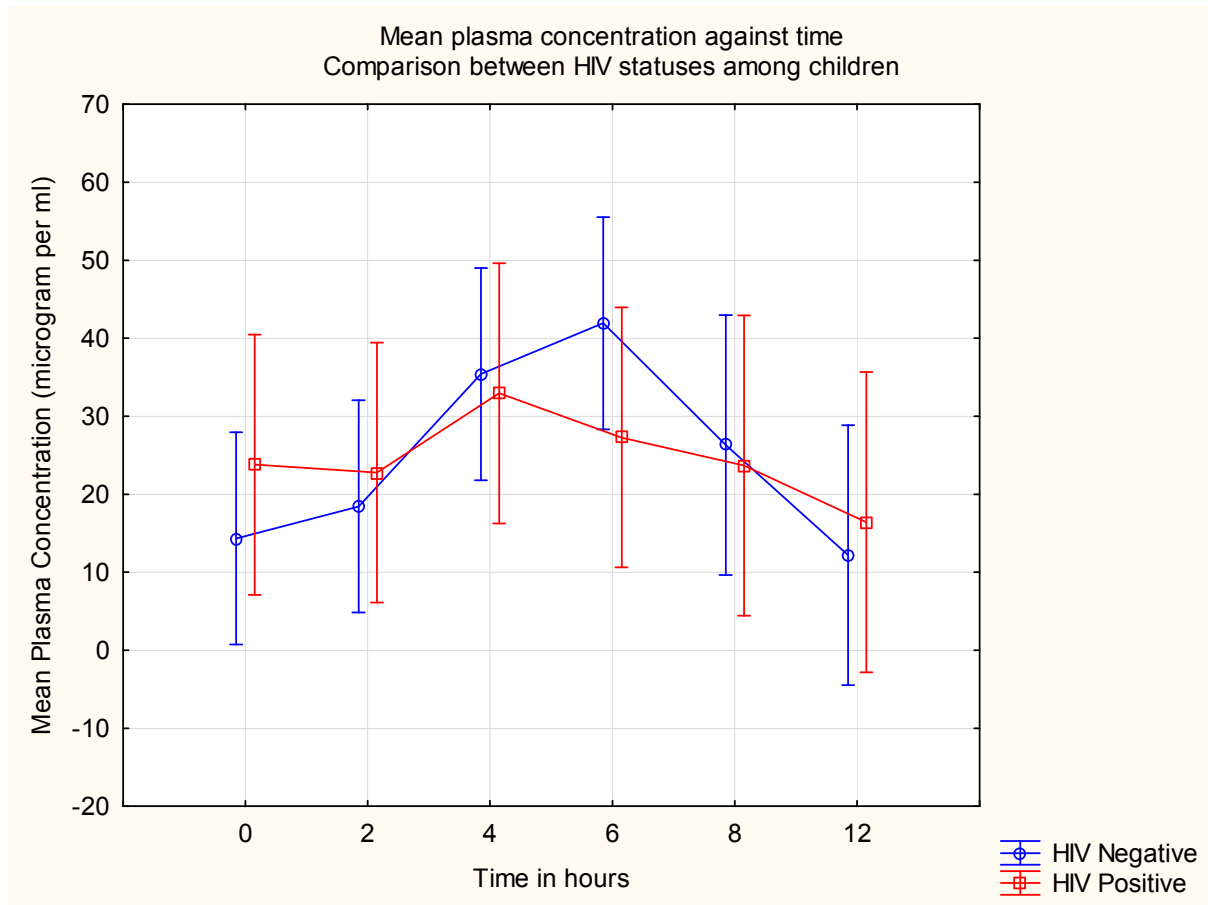


Figure 8: Mean steady-state plasma concentration-time profiles of PAS in HIV negative and HIV positive children. Vertical bars denote 95% confidence intervals.

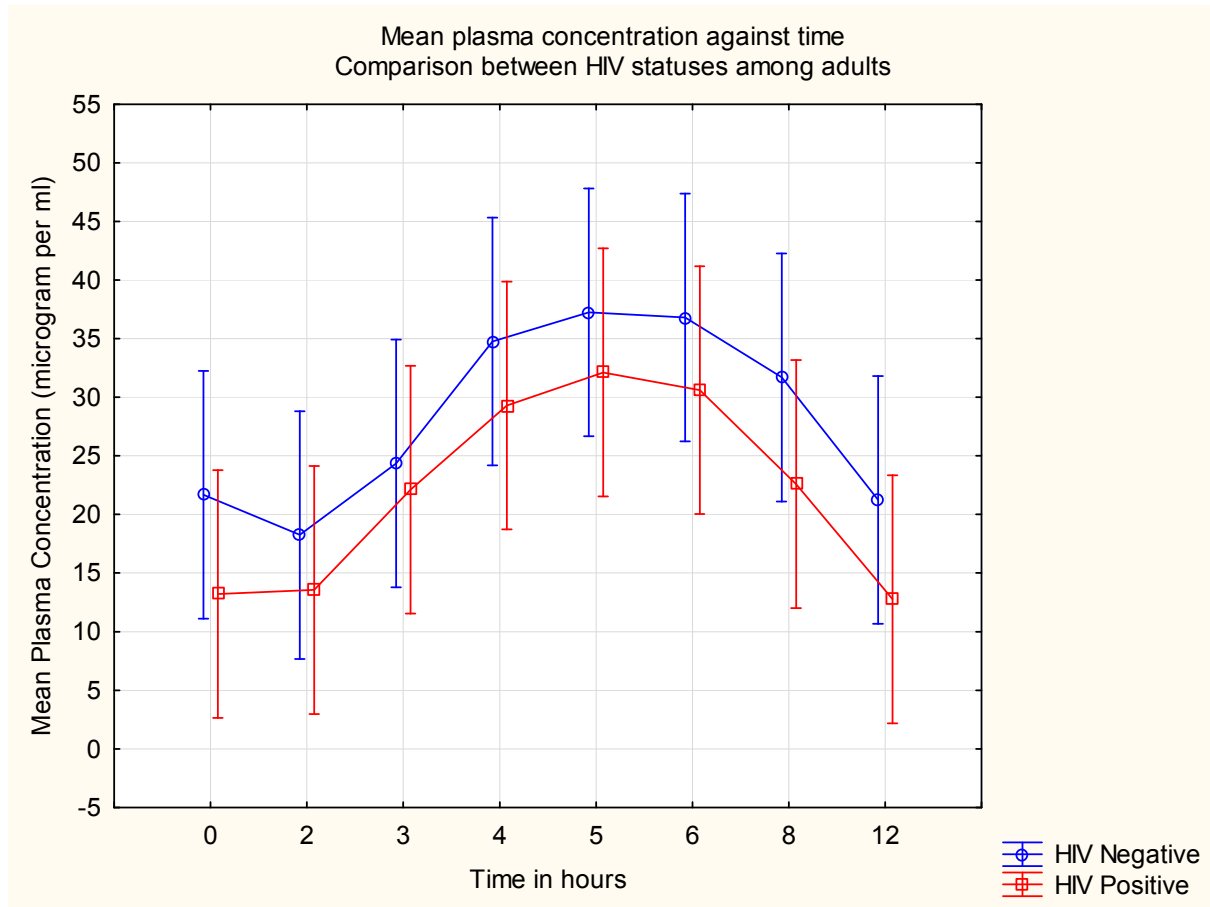


Figure 9: Mean steady-state plasma concentration-time profiles of PAS in HIV negative and HIV positive adult patients. Vertical bars denote 95% confidence intervals.

6.2 *In vitro* metabolism study

In this part of the work, the CYP interaction potential of PAS, terizidone and ethionamide towards the CYP isoforms (CYP1A2 and CYP2C9) has been assessed. The source of variability and inaccuracy resides with the fact that the assays were not performed in duplicates because of the exploratory nature of the study.

The Michaelis-Menten equation

The Michaelis-Menten equation was plotted to describe the relationship between the rates of substrate conversion by an enzyme to the concentration of the substrate. In this equation, V is the rate of conversion, V_{\max} is the maximum rate of conversion, $[S]$ is the substrate concentration, and K_m is the Michaelis-Menten constant. The Michaelis-Menten constant is equivalent to the substrate concentration at which the rate of conversion is half of V_{\max} . K_m approximates the affinity of enzyme for the substrate.

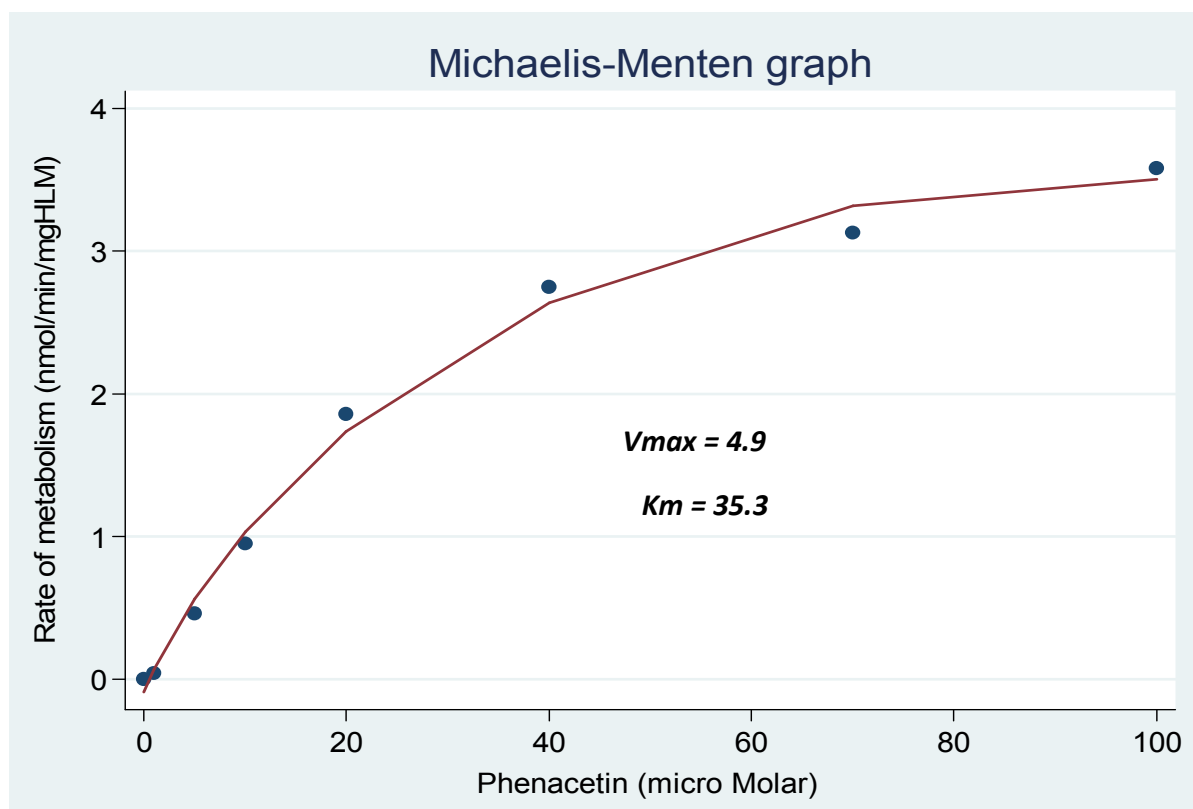


Figure 10: Michaelis-Menten plot depicting the K_m of phenacetin (substrate for CYP1A2).

6.2.1 *In vitro* CYP1A2 inhibition by PAS, ethionamide and terizidone

The effects of specific inhibitor on phenacetin metabolism, a marker substrate for CYP1A2 metabolism (substrate loss) were investigated in parallel with the test compounds (PAS, terizidone and ethionamide).

The metabolism of phenacetin was shown to be inhibited by quercetin by 100% (positive control). For the test compounds, PAS, terizidone and ethionamide, the inhibition was investigated at concentrations up to 200 μM . Over this concentration range there was no significant inhibition by any of the test compounds as shown in figure 11.

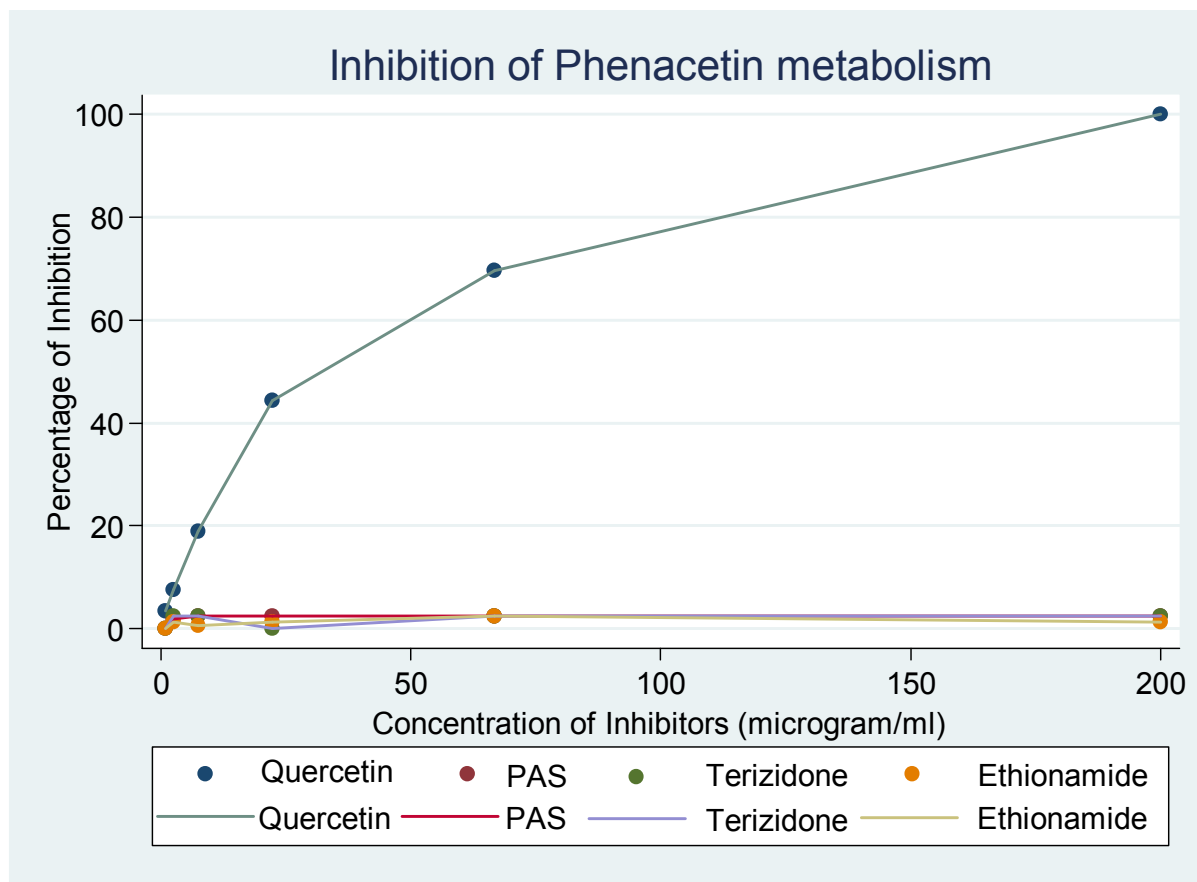


Figure 11: Percentage inhibition of CYP1A2 dependent phenacetin metabolism by quercetin and the test compounds, PAS, terizidone and ethionamide.

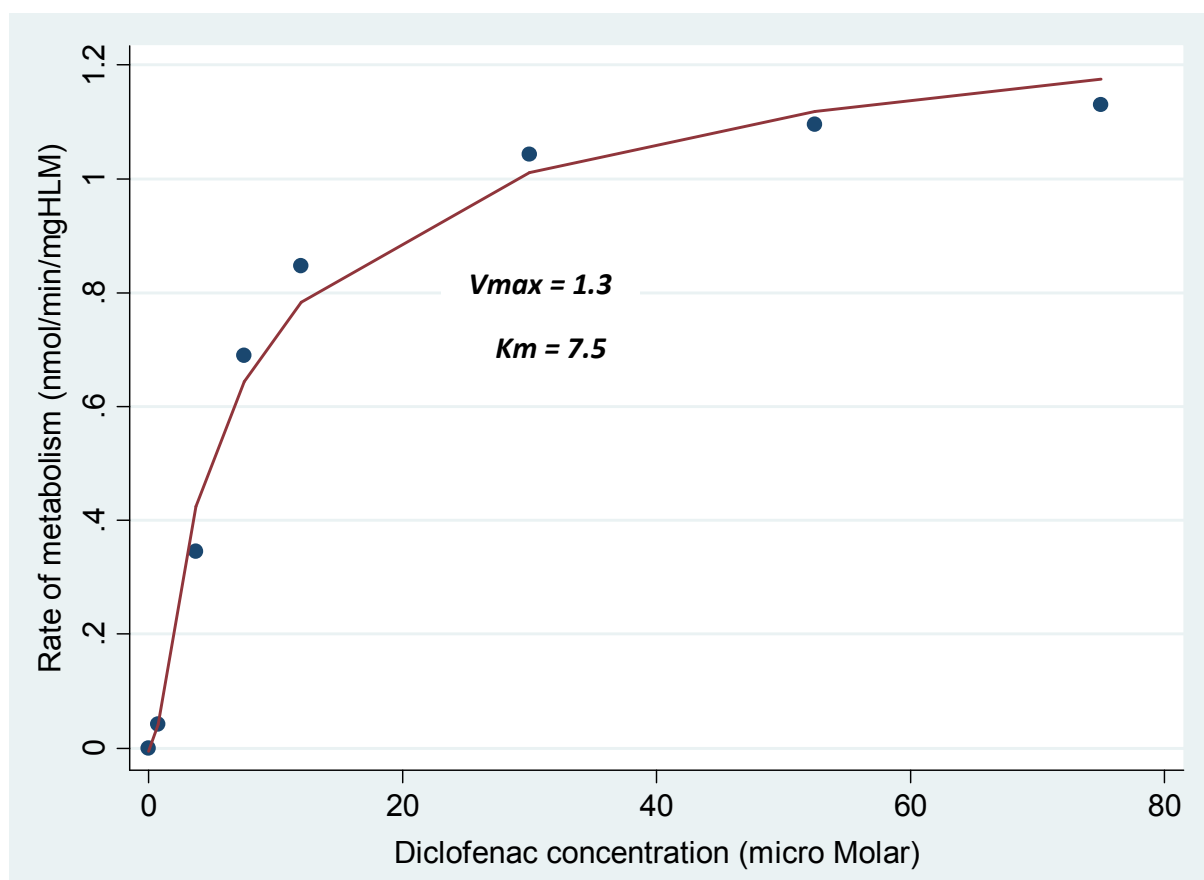


Figure 12: Michaelis-Menten plot depicting the K_m of diclofenac, a substrate for CYP2C9.

6.2.3 *In vitro* CYP2C9 inhibition by PAS, ethionamide and terizidone

The effects of specific inhibitor (quercetin) on diclofenac, a known substrate for CYP2C9 metabolism (substrate depletion) were investigated in parallel with the test compounds (PAS, terizidone and ethionamide).

The metabolism of diclofenac was clearly inhibited by quercetin by 100%. The inhibition was investigated at concentrations up to 200 μM of the test compounds. Over this concentration there was no significant inhibition by the any of the test compounds as shown in figure 13.

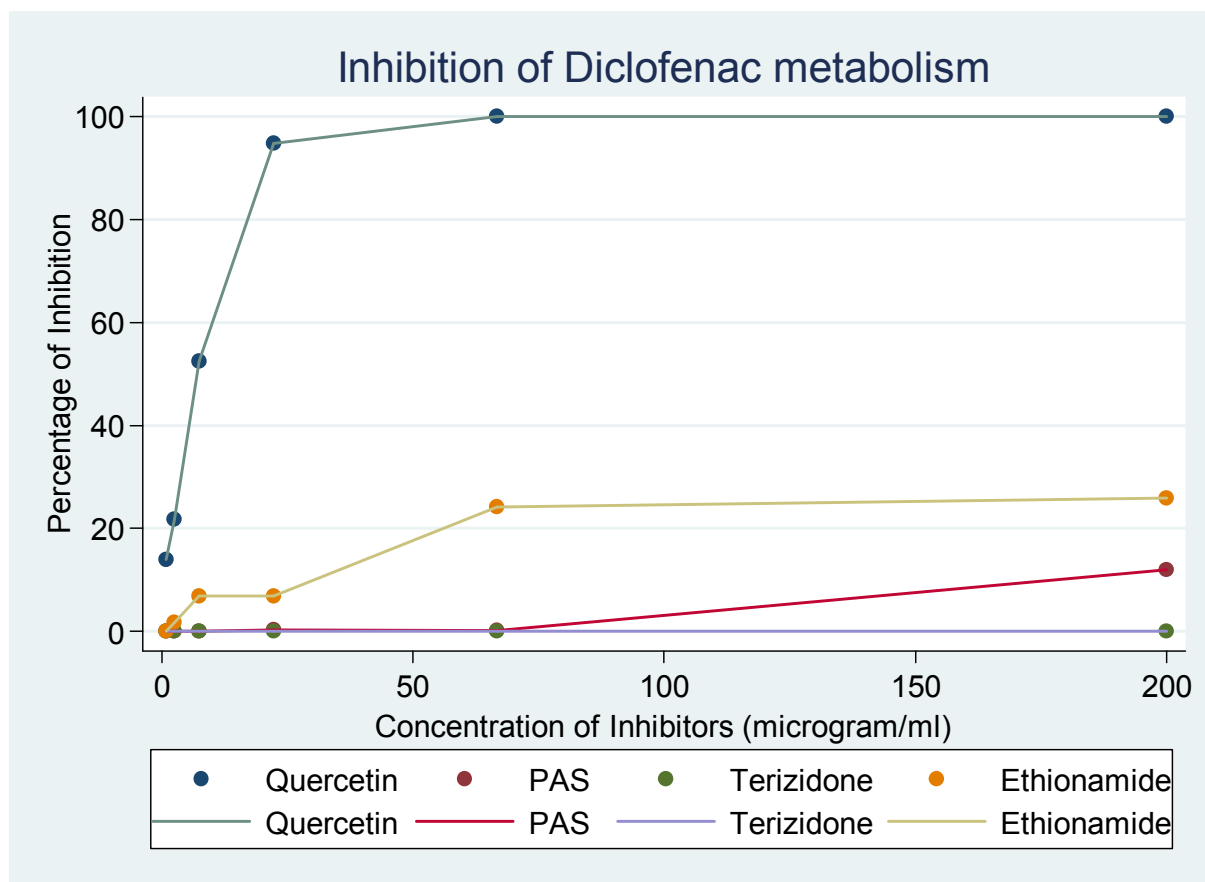


Figure 13: Percentage of inhibition of the known inhibitor quercetin versus the three test compounds (PAS, terizidone and ethionamide) on CYP2C9 dependent diclofenac metabolism.

CHAPTER SEVEN

DISCUSSION

7.1 PK of PAS in children

PAS was the first effective anti-TB agent to be administered to a child (Lehman 1964) and, after the introduction of streptomycin and isoniazid, PAS became an integral part of a standard regimen to treat TB until around 1970 when rifampicin and pyrazinamide were introduced. PAS was particularly valued for its role in preventing resistance to companion drugs. PAS became unpopular due its adverse effects and the advancement of newer drugs which were more effective with fewer side effects.

With the current trends of MDR and XDR-TB, PAS has become an important component for treatment of resistant TB particularly XDR-TB. The introduction of PAS has been inevitable despite the fact that there is a paucity of information on the PAS usage in children.

Preparations available: PAS preparation used in this study (Paser granules) is a delayed release preparation specifically designed to provide sustained, relatively low concentrations of PAS on the supposition that PAS is bacteriostatic in action and prolonged concentrations above MIC are of more importance the peak blood concentrations. An enteric coating protects the preparation in the acid environment of the stomach, but allows the rapid dissolution of the capsule within the neutral conditions of the small intestine. Previously PAS was available as sodium, potassium or calcium salts or as aminosalicic acid; absorption from the salts is rapid and complete, but from the free acid is incomplete resulting in only 77% of the dose being absorbed. (Wan *et al* 1974)

Single dose versus several daily doses: Current practice is to aim for sustained concentrations of PAS above the MIC for *MTB* of approximately 1 µg/ml throughout the dosing interval. This is also thought to reduce the intolerance to PAS, which is a significant deferent to consistent compliance. (Peloquin 1999) In an early British Medical Research Council study, INH was accompanied by two possible dosages of INH, either 10 mg/kg daily in two 5 mg/kg dosages or 20 mg/kg daily in four 5 mg/kg dosages; on

evaluation at 3 months 75% of 73 patients and 73% of 67 patients respectively were sputum culture negative indicating that increasing the total daily dosage given intermittently did not improve efficacy. (Medical Research Council 1955) In a later study a PAS dosage of 15 g daily given daily in either one or two doses to retreatment pulmonary TB patients together with streptomycin. After 6 months treatment the proportion of patients who were culture negative was similar in the two groups. (East African/British Medical Research Council 1968) Nevertheless it should be noted that several studies found that once daily dosing of PAS resulted in less intolerance than dosing several times daily. (Bridge and Carr 1958, Riska and Tennberg 1962, Lehman 1969)

From the perspective of efficacy an experiment in guinea pigs found that a single daily dose of PAS was as effective therapeutically as the same dosage given in several daily doses; furthermore a single daily dosage of 200 mg was as effective as two 100 mg dosages daily. (Karlson and Carr 1958) PAS in a single dosage of 15 g in a small group of 4 patients had the second highest early bactericidal activity during the first two days of monotherapy and this was exceeded only by that of isoniazid. (Jindani *et al* 1980) When a dosage of 10-12 mg/kg PAS in divided doses accompanied by isoniazid was compared to the effects of a dosage of 5-6 mg/kg also in divided doses and accompanied by isoniazid in 231 and 233 adult patients respectively culture negativity after 16 weeks was 85% and 68% respectively, implying a better result with higher PAS concentrations. (Bowerman 1957)

Despite this long experience with PAS, there is very little published information regarding its PK in children. In one of the few reports in English, Söderjelm (1949) described PAS serum concentrations in four children with TBM and spinal TB. PAS was given five times daily at four hourly intervals to a total dosage of 300 mg/kg daily. The results are presented as figures and the maximum concentrations following dosages of 50-60 mg/kg are between 5-10 µg/ml. The levels rose rapidly after oral dosing reaching a maximum after about 60 minutes; C_{max} was followed by a sharp decline. The author comments "The total dosage of 300 mg/kg body weight per day given in 5 doses or every 4 hours during the daytime appears to establish adequate bacteriostatic serum

levels except during the early morning hours because of the omission of the night dose.” (Söderjelm 1949) In an Italian publication the authors Maggioni and Assensio studied the concentrations of PAS, both free and combined, in blood urine and cerebrospinal fluid (CSF), after oral, intravenous and rectal administration. Their aim was to sustain a PAS concentration above 1 mg/100 ml. In the English summary they state “In order to achieve constantly a blood level above 1 mg, a total daily dose of 0.3-0.4 g for every kg administered every 4 hrs is considered necessary.” They also determined CSF concentrations that were 0.5-20% of the blood concentrations, but if the blood/brain barrier was normal the entry of PAS into the CSF was poor. Rectal administration of PAS was not advised. (Maggioni and Assensio 1950)

The clinical PK assessment study

This initial clinical part of this project was designed to study the PK of terizidone and PAS in children. However, due to various analytical problems it was not feasible to perform the terizidone part of the study. Terizidone basically consists of two molecules of cycloserine, which is known for its instability in aqueous solution. During the analytical method development (HPLC methods), chemical instability was experienced, possibly due to hydrolysis of isoxazolidine ring. After several attempts of method development, including the use of different buffers and freezing of the samples, the instability of the drug was confirmed in this matrix. It was therefore decided to focus on the clinical PK of PAS in children alone.

The objectives of this study were to evaluate the PK of PAS in children; the group of adult patients was included as reference population to allow a direct comparison between both groups. The primary questions posed were; (i) do children experience a similar PK profile in comparison to adults; (ii) will the PK profile differ when the dose is administered in two different regimens; (iii) will the PK profile differ in HIV positive and negative patients?

There were three issues responsible for promoting these questions. First, drug dosing regimen in children ideally should be based on pediatric PK data. Unfortunately, a well defined PK profile in children is often not available for many drugs, and therefore their

relative safety and efficacy have been questioned. The second issue is that currently PAS is given twice daily without sufficient PK evidence for the appropriateness of this dosing regimen in the pediatric population. It was therefore considered necessary to assess if dosing frequency can be reduced. Administration of a single 150 mg/kg dose would simplify drug administration and may therefore be desired for children. Third, it is necessary to determine if concomitant medications and disease states i.e. HIV and ARV may have an impact on the disposition of PAS.

At the time of its inception, PAS was employed as a first-line anti-TB drug. It was introduced into anti-TB chemotherapy during an era when the benefits of combination drug therapy had recently been acknowledged but the number of drugs available was quite limited. As additional anti-TB drugs with increased activity and better tolerance became available, the usage of PAS decreased markedly. Much of our knowledge of PAS activity and toxicity today is consequently derived from published experience accrued during its use in the 1950s. Today PAS is utilized as an alternative drug for the management of drug-resistant TB or for patients with intolerance to other drugs (Wilson *et al* 2003).

Because PAS is bacteriostatic and because it lacks a post-antibiotic effect, it may be desirable to maintain plasma concentrations above the typical MIC for *MTB* of 1 µg/ml throughout the dosing interval. This may be true particularly when the companion drugs are weak, such as ethionamide and cycloserine. It was demonstrated that twice-daily dosing with this PAS formulation at standard 4 g doses in adults maintained steady plasma concentrations above the typical MIC for *MTB* of 1 µg/ml throughout the dosing interval. The success of PAS and other drugs in the management of MDR-TB, defined as resistance to at least isoniazid and rifampicin depends upon therapeutic, non-toxic drug plasma concentrations reaching the tubercle bacilli. (Peloquin *et al* 1999)

In this study, all patients had either MDR-TB or XDR-TB and were receiving a PAS containing regimen. Initially blood sampling for up to six hours after drug administration was planned. However, since plasma concentrations were still rather high after six hours, this sampling schedule was later changed and extended to twelve hours. This unique population allowed characterizing and comparing the PK profile between

children and adults, two different dose regimens, concomitant medications and the effect of HIV infection. No such direct comparison of PK profiles in these divergent populations has been performed previously. The approach taken in this study was to provide this critical PK information for the second-line anti-TB drug PAS. The results of this study provide the descriptive PK and several answers to the basic questions posed.

Individual PK profile

The PK profiles for the individual patients i.e. C_{max} , T_{max} , AUC and CI/F indicated that there was a considerable intra- and inter-patient variability as shown in figures 3 and 4 above. These discrepancies could not be readily been accounted for but could have been attributed to the genetic polymorphism and/or since multiple medications were administered to these patients, it is difficult to incriminate PAS or the researcher as the causal agent for the baseline variability observed.

These patients were on anti-TB regimens that consist of not less than 4 drugs, some of the patients were HIV positive as well they were on ARV regimens. Moreover, two patients (one child and one adult) had epilepsy and were also taking sodium valproate and haloperidol respectively.

Comparisons between groups

A comparison between pediatric patients population with the consecutive visits indicated that the mean plasma concentration for PAS was reasonably higher in the second visit where the patients (children) received 150 mg/kg dose once daily as expected compared to the first visit where patient (children) received a dose of 75 mg/kg twice daily. The comparisons between the visits in adult group showed the discrepancy despite the same dosage of 4 g for each visit. This could explain the inter- and intra-individual variability.

The comparison between children and adults indicated that adults achieved higher plasma concentration than children when children received 75 mg/kg dose. It should be noted that adults had lower plasma concentrations when compared to children on the second visit.

The comparison between patients who were HIV positive and those who were HIV negative showed no significant difference in the PK parameters between HIV positive and HIV negative patients (both adults and children).

Influence of dosage on plasma profiles

A review of the dosages revealed that that children were receiving much precise body weight adjusted doses as their dosages were calculated on basis of their respective exact body weights (75 mg/kg twice daily on the first visit versus 150 mg/kg once daily), while adults received a standard dose of 4 g twice daily. Since the individual body weight in adults varied considerably, patients with 50 kg, 60 kg and 70 kg body weight will actually receive a PAS dose of 80 mg/kg, 66.67 mg/kg and 57 mg/kg respectively. This may explain partially plasma concentrations in adults were sometimes lower than in children.

Plasma profiles before and after dosing

The plasma levels before and after dosing (trough levels) was recorded for each patient. Trough levels were recorded approximately after 12 hours (75 mg/kg) and after 24 hours (150 mg/kg). The mean trough plasma concentrations were all above the MIC of 1 µg/ml. Since the sampling time was up to 12 hours after dosing it was only possible to record the values just before dosing for the previous dose and after 12 hours post dosing. It was not possible to document the values of 24 hrs post dose on the sampling day. Therefore is difficult to conclude whether the once daily dosage (150 mg/kg) will maintain the plasma levels above the MIC of 1 µg/ml after a 24 hrs period in children.

Baseline Values

As shown in the individual plasma profiles there was a huge inter-individual variation on the baseline plasma profiles of both groups of patients e.g. the range for the children after a 75 mg/kg dose was 0.90 to 82.7 µg/ml with mean of 28.2 µg/ml. The reasons for this variation and a high baseline values can not readily be explained, especially when you compare with the variation after a 150 mg/kg dose which was 0 to 51.8 µg/ml. However, this may be due to the acetylation status of the patients (slow versus fast) or

may be due to the concomitant medications which the patient received, i.e. some patients received up to 10 different drugs during the time of this research, the example being patient number C4 who was HIV positive and epileptic at the same time. This trend was also true in the group of adult patients although in a much lesser extent.

7.2 Discussion: *In vitro* metabolism study

Simultaneous co-administration of multiple drugs to a patient is highly probable. A patient may be co-administered multiple drugs to allow effective treatment of a disease (e.g., TB, HIV infection) or for the treatment of multiple diseases or disease symptoms. It is now known that drug-drug interactions may have serious, sometimes fatal consequences. (Li 2007)

DDIs remain an important issue in clinical practice and the discovery and development of new drugs. Frequently, HLMs, a rich source of human drug-metabolizing enzymes such as the cytochrome P450 family, are used as an *in vitro* assay system. Of the human P450 isoenzymes, five have been described to contribute to the metabolism of the vast majority of drugs. These are CYP1A2, CYP2C9, CYP2C19, CYP2D6, and CYP3A4, albeit some other human P450 enzymes, such as CYP3A5, CYP2B6, and CYP2C8, have been gaining increased attention as potentially important drug-metabolizing enzymes for some drugs. (Obach *et al* 2006)

Inhibition of CYP450 enzymes is frequently the underlying mechanism for DDIs. (Walsky *et al* 2004) A significant proportion of drugs undergo metabolism; therefore, the issue DDIs mediated by drug metabolizing enzymes is of considerable clinical interest. (Kneip *et al* 2008)

In vitro systems are utilized to provide answers to fundamental clinical questions regarding drug metabolism and drug interactions and to guide the planning and interpretation of clinical PK studies. HLMs generally are an important component of currently utilized *in vitro* systems. These preparations contain the various human CYPs in proportion to their abundance in human liver *in vivo*. *In vitro* models have the advantage of lower cost, more rapid implementation, no risk of human drug exposure, no need for animal studies, and the availability of greater number of potential chemical inhibitors for experimental purposes. In preclinical *in vitro* studies, tests of the affinity of an NCE to CYPs are usually among the first series of assays performed. Inhibition of CYP enzymes is frequently the underlying mechanism for DDIs. The understanding of this area has led to increased confidence in the use of *in vitro* data for qualitative projection of DDIs *in vivo*.

Although quantitative projections of interactions cannot reliably be made from *in vitro* data, *in vitro* inhibition data can be used in planning drug interaction studies in humans. Positive inhibition findings for a given compound can lead to the conduct of *in vivo* studies to determine the effect of the inhibitory drug on the PK of drugs that are cleared by the specific P450 enzyme affected. (Walsky *et al* 2004)

In the present study an exploratory *in vitro* method was utilized which aimed at generating baseline data and methodology for the future application of *in vitro* metabolism studies. However, due to the exploratory pilot nature of this part of the project, only two CYP isoforms were used to determine the potential for second line anti-TB drugs (PAS, ethionamide and terizidone) to inhibit selected important CYP enzymes. PAS, ethionamide and terizidone were incubated at various concentrations with the marker substrates and known inhibitors. The disappearance of the marker substrates was then determined and compared between the inhibitors and test compounds. In the present study it has been demonstrated that SLDs (PAS, ethionamide and terizidone) did not inhibit the selected isoenzymes *in vitro*, signifying that these compounds are not likely to significantly inhibit drug metabolism mediated by CYP2C9 or CYP1A2 isoenzymes.

CHAPTER EIGHT

CONCLUSIONS

8.1 PK of PAS in children

Due to the variety of physiologic stages that children pass through during the early years of life, it is clear that extrapolation of adult dosimetry will not suffice to describe the PK in children. Rather it is important to characterize the PK profiles in children to optimize dosing and thereby to improve cure rates and reduce both toxicity and emergency of resistance. Optimizing current treatment regimens could provide major health benefits and is a necessary prerequisite for appropriate TB treatment.

Due to the nature of this study and paucity of published PK data for PAS in children, it is difficult to compare the current results to the previous data. However, the results revealed that a single daily dose of 150 mg/kg PAS administered as the slow release PASER formulation can hold certain advantages. With this dosing regimen, single daily dosage maximum serum concentrations and AUC_{0-12} were higher than those after the 75 mg/kg dosage and to judge by the 12 hr concentrations might be maintained for several more hours. Thus it is likely that greater bactericidal activity might be engendered, but concentrations above the MIC maintained still for much longer than 12 hours.

These data support further clinical study to assess the PK of PAS involving a larger patient population to confirm and provide a more robust evidence for the proposed optimal PAS dosage regimen in children.

8.2 *In vitro* metabolism study

An *in vitro* assay for assessment of the enzyme activity of two clinically relevant CYP isoenzymes (CYP2C9 or CYP1A2) and inhibition assays were successfully developed using an *in vitro* method in HLMs. The results have shown that PAS, ethionamide and terizidone do not inhibit any of these two isoenzymes.

The results were shown to be relevant, sensitive and reliable, and it is proposed that these methods are suitable for this kind of work.

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APPENDIX I

Participant information leaflet and consent form for use by parents/legal guardians

Title; Pharmacokinetics and dosing rationale of terizidone and para-aminosalicylic acid (PAS) in children and the evaluation of the in vitro metabolism of ethionamide, terizidone and PAS.

Principal Investigator: Prof Bernd Rosenkranz

Address: Department of Medicine, Division of Pharmacology, 7th Floor Clinical Building, Faculty of Health Sciences, Stellenbosch University, Francie van Zijl Drive, Tygerberg, 7505

Contact Numbers: Office phone; +27 21 938 9331

Mobile phone; +27 82 9550017

Your child is being invited to take part in a research project. Please take some time to read the information presented here, which will explain the details of this project. Please ask the study staff or doctor any questions about any part of this project that you do not fully understand. It is very important that you are fully satisfied that you clearly understand what this research entails and how your child could be involved. Also, your child's participation is **entirely voluntary** and you are free to decline to participate. If you say no, this will not affect you or your child negatively in any way whatsoever. You are also free to withdraw him/her from the study at any point, even if you do initially agree to let him/her take part.

This study has been approved by the **Committee for Human Research at Stellenbosch University** and will be conducted according to the ethical guidelines and principles of the international Declaration of Helsinki, South African Guidelines for Good Clinical Practice and the Medical Research Council (MRC) Ethical Guidelines for Research.

Introduction

Tuberculosis (TB) is a very important health problem in South Africa and is often complicated by resistance to the commonly used medicines. When resistance is present other medicines (second-line medicines) must be used. These are not as effective as the medicines usually given for tuberculosis and they often have complications. The medicines used to treat drug resistant tuberculosis include para-ethionamide (ETH), terizidone /cycloserine (CS) and para-aminosalicylic acid (PAS). All of the second-line drugs are not very effective and it is important that they be given in the best possible dosage. There is no information regarding the amount of these medicines that get into the blood of children. Such information would make it possible for doctors to give much more accurate dosages of these medicines than is possible at present. This study aims to determine the amount of terizidone and PAS that gets into the blood of children of different ages following different doses of terizidone and PAS.

Before you decide if you want your child to be a part of this study, we want you to know about the study. You may choose not to be part of this study. No health care will be withheld for your child should you choose not to be part of the study. Your child will still receive the standard treatment for drug-resistant tuberculosis used at this hospital.

This is a consent form. It gives you information about this study. The study staff will talk with you about this information. You are free to ask questions about this study at any time. If you agree to allow your child to take part in this study, you will be asked to sign this consent form. You will get a copy to keep.

Why is this study being done?

The purpose of this study is to evaluate the absorption of the antituberculosis medicines, terizidone and PAS when given in different doses in children of different ages with drug-resistant tuberculosis. To date, the dosage used is based on studies done in adults and we do not have reliable data for children.

What will happen to my child and what will I have to do if my child is in this study?

If you decide to allow your child to take part in this study a number of investigations will be done. The absorption of the medicines will be studied by placing a needle in a vein. This needle will then be removed and a soft plastic tube will be left in your child's arm for 8 hours. A small amount of blood will be taken from this tube a total of 5 times in eight hours. No further pricking is required during this period. After 8 hours, the plastic tube will be removed. The next morning, another single blood sample will be taken. The amount of the medicine in the blood will then be measured. All of the above investigations (all 6 blood draws in total) will mean taking less than 2 teaspoonfuls (less than 10ml) of blood from your child.

How many children will take part in the study?

About 30 children will be enrolled in the study.

How long will your child be in the study?

The investigations on your child will be completed as soon after being admitted to Brooklyn Chest Hospital or Tygerberg Children's Hospital as is possible and the examination of your child by the doctor and the blood investigations should be completed within 24 hours.

Why might the doctor not admit my child / baby to this study?

The study doctor may decide that your child is too sick to take part in the study. The study might also be cancelled by the site's Ethics Committee (the committee that watches over the safety and rights of research subjects).

What are the risks of the study?

Adverse reactions to second-line medicines needed to treat drug-resistant TB medicine can occur and your child will be carefully observed for these. Your child/baby's participation in this study will not increase the risk that your child might be affected by

any of the tuberculosis medicines, but will increase the chance that such reactions will be detected early.

There is a risk of pain and bleeding at the site of insertion of the IV cannula (blood drawing needle), but this will soon heal. A gentle application of pressure for a few minutes may be required for bleeding after the cannula has been removed.

Are there benefits to taking part in this study?

It is likely that your child/baby may derive no benefit from being in this study. Information from this study may, however, help experts decide what dosage of terizidone and PAS should be recommended for use in other children in future. The study will therefore only show benefit at a later stage.

What other choices does my child/baby have besides this study?

Should you decide that you do not want your child/baby to take part in this study your child's/baby's treatment will be unaffected and will continue as usual. Please talk to your doctor about the study if you should be uncertain as to whether your child should take part in the study.

What about confidentiality?

Every effort will be made to keep your child's/baby's personal information confidential, however we cannot guarantee absolute confidentiality. Your child's/baby's personal information may be disclosed if required by law. On our study record we will use codes instead of your child's/baby's name. Only the study staff will know these codes. The study workers will not give out any information about your child/baby without written consent from you. Your family's privacy will be respected. The public health authorities will be notified as usual if your child/baby has TB. Any publication of this study will not use your child's/baby's name or identify your child/baby personally.

Your child's/baby's study records may be inspected by the South African Department of Health, study staff or study monitors. The study records will be kept separate from the normal medical records.

What are the costs to me?

There is no extra cost to you resulting from your child's/baby's participation in the study.

Will I receive any payment?

We will re-imburse you transportation money to enable you to attend the hospital should you wish to be with your child on the day that the study is undertaken.

What happens if my child/baby is injured?

If your child/baby is injured as a result of being in this study, your child/baby will be given immediate treatment for his/her injuries at no cost to you.

What are my rights and my child's rights as a research subject?

Participation in this study is completely voluntary. You may choose not to allow your child/baby to take part in this study or take your child/baby out of the study at any time. Your child/baby will be treated the same no matter what you decide, there will be no penalty.

We will tell you about new information from this or other studies that may affect your child's/baby's health, welfare or willingness to stay in this study.

What do I do if I have questions or problems?

For questions about this study or a research-related injury, contact: **Prof. Bernd Rosenkranz** with above address or the person who gave you this form.

You can contact the committee for Human Research at 021-938 9207 if you have any concerns or complaints that have not been adequately addressed by your child's study doctor.

You will receive a copy of this information and consent form for your own records.

Declaration by parent/legal guardian

By signing below, I (*name of parent/legal guardian*)
agree to allow my child (name of child) who is
years old, to take part in a research study entitled: Pharmacokinetics and dosing
rationale of terizidone and/or PAS in Children.

I declare that:

- I have read or had read to me this information and consent form and that it is written in a language with which I am fluent and comfortable.
- If my child is older than 7 years, he/she must agree to take part in the study and his/her ASSENT must be recorded on this form.
- I have had a chance to ask questions and all my questions have been adequately answered.
- I understand that taking part in this study is **voluntary** and I have not been pressurised to let my child take part.
- I may choose to withdraw my child from the study at any time and my child will not be penalised or prejudiced in any way.
- My child may be asked to leave the study before it has finished if the study doctor or researcher feels it is in my child's best interests, or if my child does not follow the study plan as agreed to.

Signed at (*place*) on (*date*)

.....
Signature of parent/legal guardian

.....
Signature of witness

Declaration by an investigator

I (*name*) declare that:

- I explained the information in this document to
- I encouraged him/her to ask questions and took adequate time to answer them.
- I am satisfied that he/she adequately understand all aspects of the research, as discussed above
- I did/did not use an interpreter (*if an interpreter is used, then the interpreter must sign the declaration below*).

Signed at (*place*) On (*date*)

.....

Signature of investigator

.....

Signature of witness

Declaration by interpreter

I (*name*) declare that:

- I assisted the investigator (*name*) to explain the information in this document to (*name of parent/legal guardian*) using the language medium of Afrikaans/Xhosa.
- We encouraged him/her to ask questions and took adequate time to answer them.
- I conveyed a factually correct version of what was related to me.
- I am satisfied that the parent/legal guardian fully understands the content of this informed consent document and has had all his/her questions satisfactorily answered.

Signed at (*place*) On (*date*)

.....

Signature of interpreter

.....

Signature of witness

Title; Pharmacokinetics and dosing rationale of terizidone and para-aminosalicylic acid (PAS) in children and the evaluation of the in vitro metabolism of ethionamide, terizidone and PAS.

We want to ask you to take part in a study about one of the tablets (terizidone or PAS) that you are taking as part of your TB treatment. In the study we measure the amount of this tablet that goes into your blood and in this way we can see whether we are giving too much, too little or just enough of this specific medicine. To do this, we have to draw a small amount of blood from you. We will apply some ointment to your arm so that the needle prick will be less painful.

A few minutes later we will use a needle to put a soft plastic tube in your arm. The needle will then be taken out and the plastic tube will stay in.

From this plastic tube we will then take small amounts of blood 5 times in a few hours. We only have to prick you once in this time because we can take blood from the soft tube without pricking you again. We will then measure the amount of the medicine in your blood. The soft plastic tube will be taken out. The next morning we will take one more little bit of blood. You can decide if you want to be part the study or not. You do not have to take part if you don't want to. You can decide at any time to stop being a part of the study if you are unhappy.

Assent of minor

I (*Name of Child/Minor*)..... have been invited to take part in the above research project.

- The study doctor/nurse and my parents have explained the details of the study to me and I understand what they have said to me.
- They have also explained that this study will involve.
- I also know that I am free to withdraw from the study at any time if I am unhappy.
- By writing my name below, I voluntary agree to take part in this research project. I confirm that I have not been forced either by my parents or doctor to take part.

.....
Name of child

.....
Independent witness

(To be written by the child if possible)

Participant information and informed consent form

Title of research project: Pharmacokinetics and dosing rationale of terizidone and para-aminosalicylic acid (PAS) in children and the evaluation of the in vitro metabolism of ethionamide, terizidone and PAS.

Principal Investigator: Prof Bernd Rosenkranz

Address: Faculty of Health Sciences, Stellenbosch University, Francie van Zijl Drive, Tygerberg, 7505

Contact Numbers: Tel +27-21-938 9331 (Office); Cellular phone: +27-82-9550017

You are invited to take part in a research project. Please take some time to read the information presented here, which will explain the details of this project. Please ask the study staff or doctor any questions about any part of this project that you do not fully understand. It is very important that you are fully satisfied that you clearly understand what this research entails and how your child could be involved. Also, your participation is **entirely voluntary** and you are free to decline to participate. If you say no, this will not affect you negatively in any way whatsoever. You are also free to withdraw from the study at any point, even if you do initially agree to take part.

This study has been approved by the **Committee for Human Research at Stellenbosch University** and will be conducted according to the ethical guidelines and principles of the international Declaration of Helsinki, South African Guidelines for Good Clinical Practice and the Medical Research Council (MRC) Ethical Guidelines for Research.

Introduction

Tuberculosis (TB) is a very important health problem in South Africa and is often complicated by disseminated forms of TB such as meningitis or widespread lesions (miliary) TB, and resistance to the commonly used medicines. PAS, a second-line medicine is mostly used in combination with the commonly used drugs for the treatment of extensively resistant TB. When resistance is present other medicines (second-line medicines) must be used. These are not as effective as the medicines usually given for TB and they have more complications than the commonly used (first-line) medicines. The medicines used to treat extensively resistant TB almost always include PAS. The second-line drugs such as PAS are not as effective as first-line TB drugs and it is therefore important that PAS be given in the best possible dosage. The information regarding the amount of PAS that gets into the blood is very little. Such information would make it possible for doctors to give much more accurate dosages of PAS than is

possible at present. This study aims to determine the amount of PAS that gets into the blood of TB patients following the currently recommended and used dose of PAS.

Before you decide if you want to be a part of this study, we want you to know about the study. You may choose not to be part of this study. No health care will be withheld for you should you choose not to be part of the study. You will still receive the standard treatment for drug-resistant tuberculosis at this hospital.

This is a consent form. It gives you information about this study. The study staff will talk with you about this information. You are free to ask questions about this study at any time. If you agree to take part in this study, you will be asked to sign this consent form. You will get a copy to keep.

Why is this study being done?

The purpose of this study is to evaluate the absorption of the antituberculosis medicine PAS when given in the currently recommended dose in patients with drug-resistant TB.

What will happen to me and what will I have to do if I participate in this study?

If you decide to take part in this study, a number of investigations will be done. The absorption of the medicines will be studied by placing a needle in a vein and taking a small amount of blood 6 times soon after the medicines have been given. The amount of the medicine in the blood will then be measured. All of the above investigations will mean taking less than 2 teaspoonfuls of blood from you at two occasions approximately a month apart.

How many patients will take part in the study?

About 30 patients will be enrolled in the study.

How long will you be in the study?

The investigations will be completed as soon after being admitted to Brooklyn Chest Hospital as possible and the examination by the doctor and the blood investigations should be completed within a day and will be repeated after a further 1 month.

Why might the doctor not admit me to this study?

The study doctor may decide that you are too sick to take part in the study. The study might also be cancelled by the site's Ethics Committee (the committee that watches over the safety and rights of research subjects).

What are the risks of the study?

Your participation in this study will not increase the risk that you might be affected by PAS, but will increase the chance that such reactions will be detected early. A small amount of bleeding may occur at the site where the blood is taken from and this may leave a bruise which should soon heal.

Are there benefits to taking part in this study?

It is likely that you may derive no benefit from being in this study. Information from this study may, however, will help experts decide what dosage of PAS should be recommended for use.

What other choices do I have besides this study?

Should you decide that you do not want to take part in this study your treatment will be unaffected and will continue as usual. Please talk to your doctor about the study if you should be uncertain as to whether you should take part in the study.

What about confidentiality?

Every effort will be made to keep your personal information confidential. However we cannot guarantee absolute confidentiality. Your personal information may be disclosed if required by law. On our study record we will use codes instead of your name. Only the study staff will know these codes. The study workers will not give out any information about you without written consent from you. Your family's privacy will be respected. The public health authorities will be notified as usual if you have TB. Any publication of this study will not use your name or identify you personally.

Your study records may be inspected by the South African Department of Health, study staff or study monitors. The study records will be kept separate from the normal medical records.

What are the costs to me?

There is no cost to you resulting from participation in the study. All medical care will be provided by the study doctor.

Will I receive any payment?

We will re-imburse your transportation money to enable you to attend the hospital on the day that the study.

What happens if I get injured?

If you get injured as a result of being in this study, you will be given immediate treatment for your injuries at no cost to you.

What are my rights as a research subject?

Participation in this study is completely voluntary. You may choose not to take part in this study or withdraw from the study at any time. You will be treated the same no matter what you decide, there will be no penalty.

We will tell you about new information from this or other studies that may affect your health, welfare or willingness to stay in this study. If you want the results of the study inform the study staff.

What do I do if I have questions or problems?

For questions about this study or a research-related injury, contact either:

You can contact the Committee for Human Research at 021-938 9207 if you have any concerns or complaints that have not been adequately addressed by your child's study doctor.

You will receive a copy of this information and consent form for your own records.

Declaration by patient

By signing below, I agree to take part in a research study.

I declare that:

- I have read or had read to me this information and consent form and that it is written in a language with which I am fluent and comfortable.
- I have had a chance to ask questions and all my questions have been adequately answered.
- I understand that taking part in this study is **voluntary** and I have not been pressurised to take part.

- I may choose to withdraw from the study at any time and will not be penalised or prejudiced in any way.
- I may be asked to leave the study before it has finished if the study doctor or researcher feels it is in my best interests, or if I do not follow the study plan as agreed to.

Signed at (*place*) On (*date*)

.....

Signature of a Participant

.....

Signature of witness

Declaration by investigator

I (*name*) declare that:

- I explained the information in this document to
- I encouraged him/her to ask questions and took adequate time to answer them.
- I am satisfied that he/she adequately understand all aspects of the research, as discussed above
- I did/did not use an interpreter (*if an interpreter is used, then the interpreter must sign the declaration below*).

Signed at (*place*) On (*date*)

.....

Signature of investigator

.....

Signature of witness

Declaration by interpreter

I (*name*) declare that:

- I assisted the investigator (*name*) to explain the information in this document to (*name of parent/legal guardian*) using the language medium of Afrikaans/Xhosa.
- We encouraged him/her to ask questions and took adequate time to answer them.
- I conveyed a factually correct version of what was related to me.
- I am satisfied that the parent/legal guardian fully understands the content of this informed consent document and has had all his/her questions satisfactorily answered.

Signed at (*place*) On (*date*)

.....

Signature of interpreter

.....

Signature of witness

APPENDIX II

Research Protocol



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**Clinical Pharmacology of Second-line
Antituberculosis Drugs.**

RESEARCH PROTOCOL

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Preface

In the current protocol we propose to study:

1. The *in vivo* pharmacokinetics (PK) of the second-line antituberculosis drugs terizidone and para aminosalicylic acid (PAS) in children
2. The pharmacokinetics (PK) of the second-line antituberculosis drug para-aminosalicylic acid (PAS) in adults
3. The pharmacogenetics of genes relevant for drug elimination such as N-acetyltransferase among patients treated with PAS for treatment of drug resistant tuberculosis.
4. The *in vitro* metabolism of the second-line anti tuberculosis drugs determination using liver microsomes.

The primary objective of the protocol is to evaluate the PK profile of terizidone and PAS in children by single dose study under strictly managed conditions following a defined protocol at set time points after dosing in children and adults. In parallel, we plan to assess the pharmacogenetics of PAS, the *in vitro* metabolic stability and the effect of ethionamide, terizidone and PAS on different metabolizing enzymes by using standardized methodology.

Research protocol

Title: Pharmacokinetics and dosing rationale of terizidone and para-aminosalicylic acid (PAS) in children and the evaluation of the in vitro metabolism of ethionamide, terizidone and PAS.

Protocol synopsis

Tuberculosis continues to cause an unacceptably high toll of disease and death among children worldwide. Available data has suggested an exponential rise in the proportion of the tuberculosis case load caused by children as the incidence of tuberculosis rises so that children may constitute nearly 40 per cent of the case load in certain high incidence communities. Until now no trials have been done in children to establish optimum antituberculosis treatment regimens and current treatment guidelines are largely inferred from adult data. This is despite the fact that pharmacokinetics in children and especially very young children are likely to be different from that in adults. Furthermore resistance to frontline therapeutics has resulted in treatment of patients with “second line” agents that are considered more toxic, and the experience of these drugs with children is even more limited. Tuberculosis management in children therefore presents particularly difficult challenges, and advances in paediatric tuberculosis research could provide insights and opportunities for tuberculosis control.

In the proposed protocol we aim to conduct the pharmacokinetic study of second line anti tuberculosis drugs terizidone and PAS in childhood TB across the range of dosages and ages in order to determine the validity of the currently suggested dose based on adult data.

We will also conduct the in vitro study of the susceptibility and stability of the second line anti TB drugs (ETH, terizidone and PAS) to biotransformation when subjected to metabolic enzymes. These drugs will also be incubated with the substrates of major enzymes/isoenzymes i.e. cytochrome P450 enzymes

(CYPs), flavin containing monooxygenases (FMO) and uridine glucuronosyl transferases (UGT) to determine whether they have any effects on these enzymes.

Research hypothesis

1. Because of developmental changes, the pharmacokinetics of terizidone and PAS and resulting serum levels may differ in children of different age groups.
2. Young children require a higher body weight adjusted dosage of the “second-line” antituberculosis agents, terizidone and PAS than adults to achieve equivalent serum concentrations.
3. Because of the genetic variations of the metabolizing enzymes (*N*-acetyltransferase) the serum concentrations may differ in different individuals.
4. The drugs under investigation are metabolically unstable and we therefore will investigate the *in vitro* metabolism and potential for drug-drug interactions.

Aim of this study

1. To document the descriptive pharmacokinetics of the second-line anti-TB agents terizidone and PAS in children, in an attempt to provide an insight to the dosing rationale of these compounds; these data will be compared to the results obtained in adult patients as a reference group.
2. To investigate the genetic variation of NAT1 and NAT2 in patients with TB treated by PAS.
3. To evaluate the stability and susceptibility to biotransformation of the second-line anti-TB drugs (ETH, terizidone and PAS) when subjected to metabolic enzymes

4. To generate baseline data and methodology for the future *in vitro* metabolic analysis of new compounds at the Division of Pharmacology.

Study methodology

The proposed study is a prospective longitudinal clinical as well as laboratory study; We plan to recruit children 13 years or younger and a group of adults 18 years or older with probable or confirmed drug resistant tuberculosis admitted to Brooklyn Hospital for Chest Diseases (BHCD) and In this explorative study, up to 40 children (for both drugs) and up to 40 adults will be enrolled.

We plan to measure the serum concentrations at different set points after dosing in different age groups. The methodology requires 1 ml of blood for each assay and blood will be drawn during each assessment via an indwelling catheter that will be inserted on the morning of the evaluation. The indwelling catheter will be used to minimize the number of venipunctures required throughout the investigation. The serum concentrations will be determined by an established high performance liquid chromatography/mass spectrometry method (HPLC-MS).

Methodology for the *in-vitro* study will involve the incubation of the test compound at concentrations with the enzyme source (Human liver microsomes (HLM)), and S9 fractions) with appropriate positive and negative controls. The time spacing and replicate number will be according to standardized procedures. The amount of parent drug remaining in the incubation media will be analyzed by HPLC/MS detection. The current procedure will also involve the incubation of substrates for specific isoenzymes with the test compounds using HLM to determine the effects of these drugs on different metabolizing enzymes.

Microsoft excel will be used to capture the data and STATISTICA version 8 (StatSoft Inc. (2008) STATISTICA (data analysis software system) will be used to analyse the data. Summary statistics will be used to describe the variables. Distribution of variables will be presented as histograms and or as frequency

tables. Medians or means will be used as the measures of central location for ordinal and continuous responses and standard deviations and quartiles as indicators of spread.

Time plan

We have planned to submit the protocol between August-September 2009 and have full Ethics approval for the study to start shortly thereafter. The aim is to collect data according to the protocol within a year. Data analysis and writing of the manuscript could be completed in 2 months. It would be possible to aim at the final manuscript at the end of year 2010 for the final master's thesis presentation and graduation. At the time of the submission of this amendment, the clinical project is ongoing (paediatric population).

The in vitro study will have a specific timeline, its implementation will commence after the approval from the ethical committee and the arrival of the required study materials. At the time of submission of this amendment, the laboratory work is being initiated.

Conclusion

The data generated in this study will be used as guidance for future studies involving much larger populations. We plan to present the data at congresses and to write this up for publications.

Funding and budget

Partial funding from the departmental funds is readily available. In addition, funding is available from NRF grants provided to Prof. P. Donald.

Introduction

Tuberculosis continues to cause an unacceptably high toll of disease and death among children worldwide. **(Newton et al., 2008)** Approximately 8.3million new TB cases were diagnosed globally in 2000. It is estimated that 884,019 (11%) cases occurred among children, with the highest burden noted in areas of endemicity. A survey conducted in Cape Town, South Africa, indicated that 13% of cases entered into the register were found among children < 13 years of age. **(Marais et al., 2004)** Children are particularly vulnerable to severe disease and death following infection. Few trials have been done in children to establish optimum antituberculosis treatment regimens and current treatment guidelines are largely inferred from adult data. **(Newton et al., 2008)** Resistance to frontline therapeutics, most notably, isoniazid (INH) and rifampicin, results in treatment of patients with “second-line” agents that are less effective and/or more toxic. Among the second tier drugs for the treatment of multidrug-resistant tuberculosis, ethionamide (ETH), **(Vanneli et al., 2002)** and terizidone or cycloserine (CS) are generally used first because of efficacy, side effect profile, and historical use in tuberculosis **(Murkherjee et al., 2004)**. Dosages for children are based on weight and extrapolated from data from studies with adults, yet pharmacokinetics for children, especially very young children, is likely to be different than for adults. Studies of ethambutol and pyrazinamide have found lower plasma drug levels and shorter half-lives in children than in adults using the same dosages. **(Graham et al., 2006)** Similar conclusions were drawn from a study of isoniazid pharmacokinetics in South African children. **(Schaaf et al., 2005)**

In the light of the above remarks we conclude that tuberculosis management in children presents particularly difficult challenges, and that advances in paediatric tuberculosis research could provide wider insights and opportunities for tuberculosis control.

In the proposed protocol we aim to study the pharmacokinetics of second-line anti tuberculosis drugs terizidone and PAS in childhood TB across the range of dosages and ages in order to determine the validity of the currently suggested

doses based on adult target concentrations as well as pharmacokinetic data obtained in a reference group of adult patients in the present study.

We also aim to conduct the in vitro evaluation of susceptibility and stability of the second line anti TB drugs (ETH, terizidone and PAS) to biotransformation when subjected to metabolic enzymes. These drugs will also be incubated with the substrates of major enzymes/isoenzymes i.e. cytochrome P450 enzymes (CYPs), flavin containing monooxygenases (FMO) and uridine glucuronosyl transferases to determine whether they have any inhibitory effects on these enzymes.

Background information and literature review

Burden of TB

Tuberculosis (TB) is a highly contagious chronic granulomatous bacterial infection, and is still a leading killer worldwide. TB has returned with a new face and the global scourge of multi-drug resistant TB (MDR TB) is reaching epidemic proportions. Rates of HIV infection among TB patients are correspondingly high, exceeding 60% in South Africa, Botswana, Zambia, and Zimbabwe. **(Du Toit et al., 2006)** It is estimated that one third of the world's population is infected with *Mycobacterium tuberculosis*, and that each year, about 9 million people develop TB, of whom about 2 million die. Of the 9 million annual TB cases, about 1 million (11%) occur in children. Of these childhood cases, 75% occur annually in 22 high burden countries. **(WHO Guidelines., 2006)** TB in the Western Cape Province was 520/100,000 in 1998 and 678/100,000 in 2003. **(Cape Town TB Control Progress report 1997-2003)**

Tuberculosis in children

Children are mainly infected by adult pulmonary TB source cases and childhood TB therefore reflects the intensity of ongoing transmission of *Mycobacterium tuberculosis* within a community. **(Schaaf et al., 2005)** Since most children acquire the organisms from adults in their surroundings, the epidemiology of childhood tuberculosis follows that in adults. Tuberculosis case load represented

by children suggest an exponential rise in the proportion of the tuberculosis case load caused by children as the tuberculosis rises, nearly 40 per cent of the case load in certain high incidence communities. **(Donald PR., 2002)** In Cape Town, South Africa, children suffer considerable morbidity: 207/439 (47.1%) had disease manifestations other than uncomplicated lymph node disease, while 26/439 (5.9%) were diagnosed with disseminated (miliary) disease and/or tuberculous meningitis (TBM). **(Marais et al., 2004)**

Pediatric pharmacology: Consequences of immaturity

Developmental changes are responsible for differences in drug disposition seen throughout childhood, therefore the weight-adjusted drug dose may not be the same for different age groups. During a period of latent or rapid growth, some drugs that may cause severe or protracted toxicity can alter the final mature expression of a system.. These concepts of drug effects on host effect on drug need to be appreciated for appropriate use of drugs in children. **(Pradhan et al., 1986)** Age-related differences in PK, in addition to those of body size, can be used to guide calculations. Altered absorption, distribution, and elimination are most marked in the newborn, but for many drugs, disposition process may equal or exceed the adult capacity by late infancy and /or childhood. **(Pradhan et al., 1986)**

Drug-resistant Tuberculosis

The treatment and control of TB is more complex when the organism is resistant to the action of antituberculosis drugs. MDR-TB are associated with mortality as high as 43–93% in adults, despite limited information in children, resistance patterns in children have generally been found to be similar to those of adults from the same areas and similar backgrounds. **(Nelson et al., 2004)** Traditionally, patients with drug-resistant tuberculosis are classified as having acquired or primary drug resistance on the basis of a history of previous treatment. In the survey in 35 countries, the median prevalence of primary resistance to any antituberculous drug was 9.9% (range 2-41) and that of acquired resistance was 36%. **(Van Rie et al., 2000)**. In a study conducted at

Tygerberg Children Hospital (TCH). Among 313 children (range 2 weeks - 12.9 years) in whom drug susceptibility test (DST) results were available, 40 (12.8%) and 17 (5.4%) were infected with strains resistant to isoniazid and rifampicin, respectively. All who had rifampicin-resistant strains had co-existent resistance to isoniazid (MDR TB). Only 1/40 (2.5%) children with resistance to isoniazid and/or rifampicin was also resistant to ethambutol. **(Schaaf et al., 2007)**

Extensively drug resistant tuberculosis has now been reported from 45 countries, though this almost certainly underestimates its true extent as many countries lack laboratory facilities to detect resistance to second line drugs. The outbreak in South Africa was particularly alarming because, most patients with extensively drug resistant tuberculosis had no history of tuberculosis treatment, implying person to person transmission of extensively drug resistant tuberculosis, and because of evidence of transmission in healthcare settings. **(Grant et al., 2008)**

Second-line anti-TB drugs

Terizidone

Terizidone (a derivative of cycloserine) It has a bacteriostatic effect at the usual dose, is used in combination with other second-line agents in the treatment of MDR-TB, as it does not share cross resistance with other anti TB drugs. It is believed to have lower incidence of side effects than cycloserine. **(WHO, Geneva., 2002. SAMF 8th Ed, 2008)**

Para-aminosalicylic acid (PAS)

PAS is bacteriostatic drug, *in-vitro*, most strains of the mycobacterium tuberculosis are sensitive to a concentration of 1µg/ml. It is administered orally in a daily dose of 10 to 12g. **(Goodman and Gilman., 2006)** PAS is used primarily as a second-line drug to treat MDR TB. **(Mathys et al., 2009)**

Hypothesis

4. Because of developmental changes, the pharmacokinetics of terizidone and PAS and resulting serum levels may differ in children of different age groups.
5. Young children require a higher body weight adjusted dosage of the “second-line” antituberculosis agents, terizidone and PAS than adults to achieve equivalent serum concentrations.
6. Because of the genetic variations of the metabolizing enzyme (*N*-acetyltransferase) the serum concentrations may differ in different individuals.
7. The drugs under investigation are metabolically unstable and have a potential for drug-drug interactions.

Rationale

Children in endemic settings are highly susceptible to TB infection and disease. Because of the rising rates of MDR-TB and because children are at a higher risk of developing disseminated forms of tuberculosis, information on drug dosing for second line drugs in this patient population is urgently needed.

Aims of study

1. To document the descriptive pharmacokinetics of the second-line antituberculosis agents terizidone and PAS in children, in an attempt to provide an insight to the dosing rationale of these compounds; these data will be compared to the results obtained in adult patients as a reference group.
2. Investigate the genetic variation of NAT1 and NAT2 in patients with tuberculosis treated by PAS.

3. To evaluate the susceptibility and stability of the second line anti TB drugs (ETH, terizidone and PAS) to biotransformation when subjected to metabolic enzymes.
4. To generate baseline data and methodology for the future application if *in-vitro* metabolism studies at the Division of Pharmacology.

Study methodology

Study design

3. A prospective, longitudinal, hospital based clinical pharmacokinetic study.
4. An *in-vitro* laboratory based analytical study

Study setting and study population

The study recruitment sites will be Tygerberg Children's Hospital (TCH) and Brooklyn Chest Hospital (BCH) both associated with the Stellenbosch University and situated in the district of Cape Town. **TCH** is a tertiary level hospital and serves as referral hospital for complicated TB cases from the surrounding high-burden communities.

BHC is a specialized TB hospital, focusing on the long-term treatment of complicated and drug-resistant TB.

Subjects and sites

Children with probable or confirmed drug-resistant tuberculosis admitted to the above mentioned hospitals, which are on treatment with **terizidone** and/or **PAS**.

Sample size considerations

In this explorative study a valid estimation of number of cases needed for statistical significance cannot be made as there is neither data on terizidone, PAS serum levels nor on standard deviations in children of different age groups.

For reasons of practicability and according to the incidence of a therapy regimen containing terizidone and PAS in childhood tuberculosis, we plan to enrol up to 30 children for terizidone, up to 10 children for PAS and up to 40 adults for PAS, however this relatively small sample size may have less statistical power.

Inclusion criteria

Children between 2 month and 13 years of age and adults older than 18 years who require treatment with second line anti-TB drugs terizidone and PAS and are admitted to one of the participating hospitals, who are medically stable and accompanied by a parent or legal guardian who has given written, informed consent for the participation of the child in the study. Patients included in another study (**Schaaf et al**) involving PK of ethionamide may also be included in the present protocol

Exclusion criteria

Children who are medically unstable such as those with severe anaemia, severe malnutrition, diarrhea and dehydration or other medical condition such as to make the drawing of blood inadvisable, or whose parents or legal guardian are unwilling to give permission for their children to participate in the study.

Procedure for *in-vivo* study

The serum concentration of terizidone will be determined at time **0, -1, -2, -3, -4-, -6, - and 24 hours hours** after dosing in groups of up to 10 children from each age group, in children 3 months -<2 years, 2-<6 years, and 6-12 years of age receiving the currently recommended dosage. This methodology requires **1 ml of blood** for each assay and **a total of 7 ml of blood** will thus be drawn during each assessment.

The serum concentrations of PAS will be determined at time **0, - 2, - 4, - 6, - 8, - and 12 or 24** hours in one group of up to 10 children aged between 3 months and 13 years after receiving a single dose per day. In adults, blood sampling

times will be **0, -2, -3, -4, -5, -6, -8 and 12 or 24** hours. Blood sampling will be repeated after 2 to 4 weeks after receiving a multiple dose per day (twice daily). The dose will be kept refrigerated and will be given with an orange juice under observation from the researcher. This methodology requires **1 ml of blood** for each assay and **a total of 12 ml of blood** will thus be drawn for both assessments in children and **a total of 20 ml** in adults.

These amounts does not exceed the allowable sample volume of 9.6, 36, 96 ml for the full term neonates, children (3 years) and children (12 years), respectively (**Abdel-Rahman et al., 2007**). However, considerations will be given to patients with diarrhea, severe anaemia, severe malnutrition or any other medical conditions that restrict blood sampling.

In the paediatric population, an anesthetic patch will be applied for 45 minutes before inserting an indwelling catheter that will be used so that venipunctures are reduced throughout the investigation.

Serum concentrations will be determined by an established high performance liquid chromatography with mass spectrometry (HPLC-MS) methodology.

Procedure for *in-vitro* metabolism

The test compound will be incubated at concentrations with the enzyme source (Human liver microsomes (HLMs), and/or S9 fractions) with appropriate positive and negative controls. At varying time intervals, the reactions will be terminated. The time spacing and replicate number will be according to the standardized procedures. The amount of parent drug remaining in the incubation media will be analyzed by HPLC-MS.

The current procedure will involve the incubation of substrates for specific isoenzymes with the test compounds and the enzyme source (Human liver microsomes) to determine the effects of these drugs to different metabolizing enzymes.

Procedure for pharmacogenetics assays

The blood cell pellet obtained from blood samples taken for pharmacokinetic assays will be stored frozen at -40°C or below. In an initial stage it is planned to determine the genotype for both types of N-acetyl-transferase (NAT-1 and NAT-2). Samples may also be analysed for other proteins involved in drug elimination (such as P-glycoprotein) at a later stage.

Data management and statistical analysis

Microsoft Excel will be used to capture the data and STATISTICA version 8 (StatSoft Inc. (2008) STATISTICA (data analysis software systems. will be used to analyse the data. Data will be collected on case report forms (CRF) and study flowcharts and then entered into an Access or SPSS database.

Summary statistics will be used to describe the variables. Distribution of variables will be presented as histograms and or as frequency tables. Medians or means will be used as the measures of central location for ordinal and continuous responses and standard deviations and quartiles as indicators of spread. The relationship between continuous response variables and nominal input variables will be analysed using appropriate analysis of variance (ANOVA). When ordinal response variables are compared versus a nominal input variable, non-parametric ANOVA methods will be used. For completely randomized designs the Mann-Whitney test or the Kruskal-Wallis test will be used and for repeated measures the Wilcoxon- or Friedman tests will be used.

A population pharmacokinetic analysis will also be conducted to determine the pharmacokinetic effects of different covariates and to quantify the covariates contributing to the studied drugs' clearance and volume of distribution population parameters including variability in order to establish an initial estimate of the major covariates required for dosing predictions.

Ethical consideration

The study will be conducted according to the principles of Good Clinical Practice, South African and internationally accepted ethical standards taking into account the Declaration of Helsinki and International Conference on Harmonisation (ICH) guidelines.

All patients referred to the hospital for treatment might participate if they are eligible and a written informed consent (for children: by parents or legal guardians) is given. Before enrolment, children older than 7 years will be asked to assent after being fully informed about the rationale, procedure, data management as well as potential benefits and risks of the study.

The patients participating in the study will not directly benefit from the study; however, the study will provide important information for the optimal treatment and the use of terizidone and PAS in childhood tuberculosis.

This study is in the interest of improving medical care for children with tuberculosis in need of the second line drugs, terizidone and PAS. No alteration in the prescribed treatment of the children will be made other than that prescribed by the attending physicians.

The consent form will be available in English, Afrikaans and isiXhosa, the parents will be informed of the possibility to revoke informed consent even though it was made in writing. There will be no financial incentive offered for study participation.

Blood samples for measurements of serum levels and additional parameters will be collected through a permanent angiocatheter that will be inserted at the beginning of each study day to minimize the number of necessary venipunctures.

Obtained data will be collected in a database using a unique anonymous subject code for each patient. Identified information linked to the study code will be recorded separately. The confidentiality of all information of a personal nature will

be maintained and ensured at all times. Only the researcher and authorized officials affiliated to control bodies will be allowed access to personal information.

It is the intention of the researcher to present and publish the findings of the study in an appropriate medical journal without, as indicated, divulging any information of a personal nature.

Risks associated with participation in the study

The participants will be treated as usual by their attending physician. The investigators will not in any way alter their drug therapy. The risks are therefore those associated with venipuncture. These include pain, bruising and bleeding at the site of IV cannula insertion.

To minimize these risks, an indwelling IV cannula will be inserted so that repeat venipuncture is minimized and a topical anaesthetic gel will be used. These symptoms usually resolve spontaneously or may require the application of gentle pressure to the area for a few minutes after cannula removal.

A further risk is the drawing of blood in children who are anaemic or dehydrated. To prevent this, children who are medically unstable such as those with severe anaemia, severe malnutrition, diarrhea and dehydration or other medical condition such as to make the drawing of blood inadvisable will be excluded from the study.

Furthermore, the amount of blood drawn will be limited to 8ml in total, which is less than the upper limit recommended in guidelines (**Abdel-Rahman et al., 2007**).

Feasibility of the study

The participating hospitals admit good number of children per year (approximately 120-130) of whom great majority have either drug-resistant tuberculosis and require second line anti tuberculosis agents. It should therefore be possible to enroll the proposed number of patients at these facilities within a

time a year. Furthermore, the Division of Pharmacology has a long standing and extensive experience in conducting pharmacokinetic studies in childhood tuberculosis. It has previously been involved in studies which characterized the pharmacokinetic disposition of isoniazid in children and has produced refined the methodology for determining the serum concentrations of several antituberculosis agents.

For the in vitro study we are collaborating with experts from Synexa group, South Africa, who have considerable experience in such kind of laboratory work; the reagents for the in vitro study are readily available commercially.

Conclusion

The data generated in this study will be used as guidance for future studies involving much larger populations. We plan to present the data at congresses and to write this up for publications.

Time plan

We have planned to submit the protocol between August-September 2009 and have full Ethics approval for the study to start shortly thereafter. The aim is to collect data according to the protocol within a year. Data entry analysis and writing of the manuscript could be completed in 2 months. It would be possible to aim at the final manuscript at the end of year 2010 for the final master's thesis presentation. At the time of the submission of this amendment, the clinical project is ongoing (paediatric population).

The in vitro study will have a specific timeline, its implementation will commence after the approval from the ethical committee and the arrival of the required study materials.

Funding and budget:

Partial funding from the departmental funds is readily available. In addition, funding requests/ applications have been submitted to Medical Research Council and Harry Crossley Foundation and we await for feedback and possible approval. The budget summary is as indicated in the table below:

Table 1: Budget				
		Unit cost (R)	No. of units	Sub-total (R)
Consumables				
	Blood collection and specimens	75	180	13 500
	Micro centrifuge polypyrrolene tubes	950	1	950
Reagents				
	Liver microsomes	1660	25	41 500
	S9 fractions	650	20	13 000
	Co-factors (<i>NADPH, UDPGA</i>)			9 000
	Other reagents (<i>DMSO, Phosphate buffer, Methanol, Acetonitrile</i>)			4 000
	Substrates			20 000
Laboratory analyses				
	Development and Validation of assays			10 000
	Analysis of specimens	800	180	144 000
Travel				
	Parents/ caregivers to and from the hospital	100	30	3 000
	Cost for courier services	-	-	8 000
Total				266 950

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Appendix

Terms and definitions relating to tuberculosis and drug resistance

- **Drug resistant tuberculosis** -Tuberculosis that is resistant to any first line antituberculosis drug
- **Multidrug resistant tuberculosis (MDR-TB)** -Tuberculosis that is resistant to at least isoniazid and rifampicin
- **Extensively drug resistant tuberculosis (XDR-TB)**-Tuberculosis that is resistant to at least isoniazid and rifampicin and also to a fluoroquinolone and a second line injectable agent (amikacin, capreomycin, or kanamycin)
- **Drug resistance in new tuberculosis cases (primary drug resistance)**- Drug resistant tuberculosis in a person with no history of tuberculosis treatment, implying they were infected with a resistant organism. This reflects person to person transmission of drug resistant tuberculosis
- **Drug resistance among previously treated cases ("acquired" drug resistance)**-Drug resistant tuberculosis in a person with a history of tuberculosis treatment. This reflects drug resistance acquired during tuberculosis treatment but may also reflect infection or reinfection with a resistant organism (**Grant et al., 2008**)

The standardized regimen for treatment of multidrug-resistant TB in South Africa is as shown in the table below:

Standardized Regimen for Treatment of Multidrug-Resistant TB (MDR-TB) in SA						
Treatment should be started in a referral centre and be adjusted according to a patient weight						
Pre-treatment body weight	Intensive phase: 4 months					
	Doses given daily					
	Kanamycin	Ethionamide	PZA	Ofloxacin	Ethambutol or terizidone	
< 50 kg	750 mg	500 mg	1000 mg	600 mg	800mg	750 mg
50-65	1000 mg	750 mg	1500 mg	600 mg	1200mg	750 mg
≥ 65	1000 mg	750 mg	2000 mg	800 mg	1200 mg	750 mg
Pre-treatment body weight	Continuation phase 12-18 months depending on culture conversion					
	Doses give daily					
	Ethionamide		Ofloxacin		Ethambutol or terizidone	
< 50 kg	500 mg		60 mg		800 mg	500 mg
50-65 kg	750 mg		600 mg		1200 mg	750 mg
≥ 65 kg	750 mg		800 mg		1200 mg	750 mg

Ethambutol is used if strain still susceptible. Terizidone is used if strain resistant to ethambutol. Reduce terizidone dose to 500 mg if weight ≤ 35 kg. Pyridoxine (Vit B6) 150 mg to be given to patients on terizidone. Some provinces may be using cycloserine instead of terizidone. **(Begley, 2007)**

STELLENBOSCH UNIVERSITY

**Participant information leaflet and consent form for use by
parents/legal guardians**

Title; Pharmacokinetics and dosing rationale of terizidone and para-aminosalicylic acid (PAS) in children and the evaluation of the in vitro metabolism of ethionamide, terizidone and PAS.

Reference number:

Principal Investigator: Prof Bernd Rosenkranz

Address: Department of Medicine, Division of Pharmacology, 7th Floor Clinical Building, Faculty of Health Sciences, Stellenbosch University, Francie van Zijl Drive, Tygerberg, 7505

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Your child is being invited to take part in a research project. Please take some time to read the information presented here, which will explain the details of this project. Please ask the study staff or doctor any questions about any part of this project that you do not fully understand. It is very important that you are fully satisfied that you clearly understand what this research entails and how your child could be involved. Also, your child's participation is **entirely voluntary** and you are free to decline to participate. If you say no, this will not affect you or your child negatively in any way whatsoever. You are also free to withdraw him/her from the study at any point, even if you do initially agree to let him/her take part.

This study has been approved by the **Committee for Human Research at Stellenbosch University** and will be conducted according to the ethical guidelines and principles of the international Declaration of Helsinki, South

African Guidelines for Good Clinical Practice and the Medical Research Council (MRC) Ethical Guidelines for Research.

Introduction

Tuberculosis (TB) is a very important health problem in South Africa and is often complicated by resistance to the commonly used medicines. When resistance is present other medicines (second-line medicines) must be used. These are not as effective as the medicines usually given for tuberculosis and they often have complications. The medicines used to treat drug resistant tuberculosis include para-ethionamide (ETH), terizidone /cycloserine (CS) and para-aminosalicylic acid (PAS). All of the second-line drugs are not very effective and it is important that they be given in the best possible dosage. There is no information regarding the amount of these medicines that get into the blood of children. Such information would make it possible for doctors to give much more accurate dosages of these medicines than is possible at present. This study aims to determine the amount of terizidone and PAS that gets into the blood of children of different ages following different doses of terizidone and PAS.

Before you decide if you want your child to be a part of this study, we want you to know about the study. You may choose not to be part of this study. No health care will be withheld for your child should you choose not to be part of the study. Your child will still receive the standard treatment for drug-resistant tuberculosis used at this hospital.

This is a consent form. It gives you information about this study. The study staff will talk with you about this information. You are free to ask questions about this study at any time. If you agree to allow your child to take part in this study, you will be asked to sign this consent form. You will get a copy to keep.

Why is this study being done?

The purpose of this study is to evaluate the absorption of the antituberculosis medicines, terizidone and PAS when given in different doses in children of different ages with drug-resistant tuberculosis. To date, the dosage used is based on studies done in adults and we do not have reliable data for children.

What will happen to my child and what will I have to do if my child is in this study?

If you decide to allow your child to take part in this study a number of investigations will be done. The absorption of the medicines will be studied by placing a needle in a vein. This needle will then be removed and a soft plastic tube will be left in your child's arm for 8 hours. A small amount of blood will be taken from this tube a total of 5 times in eight hours. No further pricking is required during this period. After 8 hours, the plastic tube will be removed. The next morning, another single blood sample will be taken. The amount of the medicine in the blood will then be measured. All of the above investigations (all 6 blood draws in total) will mean taking less than 2 teaspoonfuls (less than 10ml) of blood from your child.

How many children will take part in the study?

About 30 children will be enrolled in the study.

How long will your child be in the study?

The investigations on your child will be completed as soon after being admitted to Brooklyn Hospital or Tygerberg Children's Hospital as is possible and the examination of your child by the doctor and the blood investigations should be completed within 24 hours.

Why might the doctor not admit my child / baby to this study?

The study doctor may decide that your child is too sick to take part in the study. The study might also be cancelled by the site's Ethics Committee (the committee that watches over the safety and rights of research subjects).

What are the risks of the study?

Adverse reactions to second-line medicines needed to treat drug-resistant TB medicine can occur and your child will be carefully observed for these. Your child/baby's participation in this study will not increase the risk that your child might be affected by any of the tuberculosis medicines, but will increase the chance that such reactions will be detected early.

There is a risk of pain and bleeding at the site of insertion of the IV cannula (blood drawing needle), but this will soon heal. A gentle application of pressure for a few minutes may be required for bleeding after the cannula has been removed.

Are there benefits to taking part in this study?

It is likely that your child/baby may derive no benefit from being in this study. Information from this study may, however, help experts decide what dosage of Terizidone and PAS should be recommended for use in other children in future. The study will therefore only show benefit at a later stage.

What other choices does my child/baby have besides this study?

Should you decide that you do not want your child/baby to take part in this study your child's/baby's treatment will be unaffected and will continue as usual. Please talk to your doctor about the study if you should be uncertain as to whether your child should take part in the study.

What about confidentiality?

Every effort will be made to keep your child's/baby's personal information confidential, however we cannot guarantee absolute confidentiality. Your

child's/baby's personal information may be disclosed if required by law. On our study record we will use codes instead of your child's/baby's name. Only the study staff will know these codes. The study workers will not give out any information about your child/baby without written consent from you. Your family's privacy will be respected. The public health authorities will be notified as usual if your child/baby has TB. Any publication of this study will not use your child's/baby's name or identify your child/baby personally.

Your child's/baby's study records may be inspected by the South African Department of Health, study staff or study monitors. The study records will be kept separate from the normal medical records.

What are the costs to me?

There is no extra cost to you resulting from your child's/baby's participation in the study.

Will I receive any payment?

We will re-imburse you transportation money to enable you to attend the hospital should you wish to be with your child on the day that the study is undertaken.

What happens if my child/baby is injured?

If your child/baby is injured as a result of being in this study, your child/baby will be given immediate treatment for his/her injuries at no cost to you.

What are my rights and my child's rights as a research subject?

Participation in this study is completely voluntary. You may choose not to allow your child/baby to take part in this study or take your child/baby out of the study at any time. Your child/baby will be treated the same no matter what you decide, there will be no penalty.

We will tell you about new information from this or other studies that may affect your child's/baby's health, welfare or willingness to stay in this study.

What do I do if I have questions or problems?

For questions about this study or a research-related injury, contact: **Prof Bernd Rosenkranz** with above address or the person who gave you this form.

You can contact the committee for Human Research at 021-938 9207 if you have any concerns or complaints that have not been adequately addressed by your child's study doctor.

You will receive a copy of this information and consent form for your own records.

Declaration by parent/legal guardian

By signing below, I (*name of parent/legal guardian*)
..... agree to allow my child (*name of child*)
..... who is years old, to take part in a
research study entitled: Pharmacokinetics and dosing rationale of terizidone
and/or PAS in Children.

I declare that:

- I have read or had read to me this information and consent form and that it is written in a language with which I am fluent and comfortable.
 - If my child is older than 7 years, he/she must agree to take part in the study and his/her ASSENT must be recorded on this form.
 - I have had a chance to ask questions and all my questions have been adequately answered.
- I understand that taking part in this study is **voluntary** and I have not been pressurised to let my child take part.
- I may choose to withdraw my child from the study at any time and my child will not be penalised or prejudiced in any way.
- My child may be asked to leave the study before it has finished if the study doctor or researcher feels it is in my child's best interests, or if my child does not follow the study plan as agreed to.

Signed at (*place*) on (*date*)

.....
Signature of parent/legal guardian

.....
Signature of witness

Declaration by investigator

I (*name*) declare that:

- I explained the information in this document to
- I encouraged him/her to ask questions and took adequate time to answer them.
- I am satisfied that he/she adequately understand all aspects of the research, as discussed above
- I did/did not use an interpreter (*if a interpreter is used, then the interpreter must sign the declaration below*).

Signed at (*place*) On (*date*)

.....
Signature of investigator

.....
Signature of witness

Declaration by interpreter

I (*name*) declare that:

- I assisted the investigator (*name*) to explain the information in this document to (*name of parent/legal guardian*) using the language medium of Afrikaans/Xhosa.
- We encouraged him/her to ask questions and took adequate time to answer them.
- I conveyed a factually correct version of what was related to me.
- I am satisfied that the parent/legal guardian fully understands the content of this informed consent document and has had all his/her questions satisfactorily answered.

Signed at (*place*) On (*date*).....

.....
Signature of interpreter

.....
Signature of witness

Title; Pharmacokinetics and dosing rationale of terizidone and para-aminosalicylic acid (PAS) in children and the evaluation of the in vitro metabolism of ethionamide, terizidone and PAS.

We want to ask you to take part in a study about one of the tablets (terizidone or PAS) that you are taking as part of your TB treatment. In the study we measure the amount of this tablet that goes into your blood and in this way we can see whether we are giving too much, too little or just enough of this specific medicine. To do this, we have to draw a small amount of blood from you. We will apply some ointment to your arm so that the needle prick will be less painful.

A few minutes later we will use a needle to put a soft plastic tube in your arm. The needle will then be taken out and the plastic tube will stay in.

From this plastic tube we will then take small amounts of blood 5 times in a few hours. We only have to prick you once in this time because we can take blood from the soft tube without pricking you again. We will then measure the amount of the medicine in your blood. The soft plastic tube will be taken out. The next morning we will take one more little bit of blood.

You can decide if you want to be part the study or not. You do not have to take part if you don't want to. You can decide at any time to stop being a part of the study if you are unhappy.

Assent of minor

I (*Name of Child/Minor*)..... have been invited to take part in the above research project.

- The study doctor/nurse and my parents have explained the details of the study to me and I understand what they have said to me.
- They have also explained that this study will involve.
- I also know that I am free to withdraw from the study at any time if I am unhappy.

- By writing my name below, I voluntary agree to take part in this research project. I confirm that I have not been forced either by my parents or doctor to take part.

.....

Name of child

.....

Independent witness

(To be written by the child if possible)

**PARTICIPANT INFORMATION AND INFORMED CONSENT FORM FOR
RESEARCH INVOLVING GENETIC STUDIES**

TITLE OF RESEARCH PROJECT: “Pharmacokinetics and dosing rationale of terizidone and para-aminosalicylic acid (PAS) in children and in-vitro metabolism of ethionamide, terizidone and PAS.”

REFERENCE NUMBER: N09/08/212

PRINCIPAL INVESTIGATOR: Prof Bernd Rosenkranz

ADDRESS: Department of Pharmacology

7th Floor, Clinical Building

Stellenbosch University

Tygerberg Campus

7505

CONTACT NUMBER: 021 938 9331/ 0829550017

We would like to invite your child to participate in a research study that involves genetic analysis and possible long-term storage of blood or tissue specimens. Please take some time to read the information presented here which will explain the details of this project. Please ask the study staff or doctor any questions about any part of this project that you do not fully understand. It is very important that you are fully satisfied that you clearly understand what this research entails and how your child could be involved. Also, your participation is **entirely voluntary** and you are free to decline to participate. If you say no, this will not affect your child negatively in any way whatsoever. You are also free to withdraw your child from the study at any point, even if you do agree to take part initially.

If you do not wish your child to participate in this genetic analysis part of the study, they can still be part of the main study of antituberculosis drug levels in the blood. The genetic analysis will then not be performed on your child's blood.

This research study has been approved by the ethics **Committee for Human Research at Stellenbosch University** and it will be conducted according to international and locally accepted ethical guidelines for research, namely the Declaration of Helsinki, and the SA Department of Health's 2004 Guidelines: *Ethics in Health Research: Principles, Structures and Processes*.

What is Genetic research?

Genetic material, also called DNA or RNA, is usually obtained from a small blood sample. Genes are found in every cell in the human body. Our genes determine what we look like and sometimes what kind of diseases we may be susceptible to. Worldwide, researchers in the field of genetics are continuously discovering new information that may be of great benefit to future generations and also that may benefit people today, who suffer from particular diseases or conditions.

What does this particular research study involve?

In the main part of the study in which your child is involved, the amount of antituberculosis drug in the blood will be measured at various time points. A number of factors can affect the amount of drug in a patient's blood. One of these is the ability of the liver to produce enzymes (substances which speed up chemical reactions). These enzymes are responsible for the metabolism (breakdown) of drugs which are then passed out of the body.

Some people produce more powerful enzymes than others and they are therefore able to break down drugs more quickly than other people. This is because different people have differences in the genes which control how these enzymes are made. These variations in the genetic material of individuals are known as polymorphisms.

This could possibly help us in the future to predict which children are likely to develop very high (which may be dangerous) or very low (the drug doesn't work) concentrations, based on their genetic makeup. If we know their genetic makeup, we could perhaps take precautions or use a lower or higher dose of these drugs.

Why has your child been invited to participate?

- Your child has been invited to participate because you have shown interest in the main part of our study and so we are going to measure blood concentrations of antituberculous drugs in your child. Performing genetic testing on your child will therefore help us determine if genetic differences (polymorphisms) can lead to high or low blood concentrations as explained above.

What procedures will be involved in this research?

Your child will not be required to give any additional blood or participate in additional procedures. The blood obtained from the main study will be used to perform genetic testing.

Are there any risks involved in genetic research?

In this study, we will be focusing on those genes which are responsible for the production of enzymes which break down or metabolize the drugs your child is receiving or drugs which broken down by the same kind of enzymes.

This study therefore will not identify any diseases your child may develop or genetic problems which may lead to diseases later in life.

Are there any benefits to your taking part in this study and will you get told your results?

Your child will not benefit directly from taking part in this genetic research study. You will not be informed of the results. Blood will be stored and only tested at a later date once more is known about the enzymes responsible for drug breakdown and the genetic polymorphisms possibly involved.

It is possible that in the future we can use the study results to predict which children are likely to develop very high (which may be dangerous) or very low (the drug doesn't work) concentrations. If we know their genetic composition, we could perhaps take precautions or use a lower or higher dose of these drugs.

How long will your blood be stored and where will it be stored?

The blood will be stored in the Division of Clinical Pharmacology and analyzed by the University of Stellenbosch.

If your blood is to be stored is there a chance that it will be used for other research?

Your blood will only be used for genetic research that is directly related to the metabolism of anti-TB drugs. Also if the researchers wish to use your stored blood for additional research in this field they will be required to apply for permission to do so from the Human Research Ethics Committee at Stellenbosch University.

If you do not wish your blood specimen to be stored after this research study is completed you will have an opportunity to request that it be discarded when you sign the consent form.

How will your confidentiality be protected?

The blood for DNA analysis will be labelled with your child's participant number and not name, prior to storage. It will be labelled because we wish to compare your child's blood concentration to their genetic results and so these must be linked by a participant number.

All study records and samples will be kept confidentially in a sealed and locked area only accessible to the investigators. No one other than the investigators or the ethics committee of Stellenbosch University will have access to your child's personal information. In the publication of results, your child's name or identifying information will not be used

Will you or the researchers benefit financially from this research?

You will not be paid to take part in this study.

<p>Important information: In the unlikely event that this research leads to the development of a commercial application or patent, you or your family will not receive any profits or royalties</p>
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Declaration by participant

By signing below, I agree to take part in a genetic research study.

I declare that:

- I have read or had read to me this information and consent form and it is written in a language with which I am fluent and comfortable.
- I have had a chance to ask questions and all my questions have been adequately answered.
- I understand that taking part in this study is voluntary and I have not been pressurised to take part.
- I have received a signed duplicate copy of this consent form for my records.

Tick the option you choose:

- I agree that my blood or tissue sample can be stored **indefinitely/ stored for,.....years**, but I can choose to request at any time that my stored sample be destroyed. My sample will be identified with a special study code that will remain linked to my name and contact details. I have the right to receive confirmation that my request has been carried out. *(NB This option can be excluded completely if the genetic research has no clinical relevance to the patient and you plan to completely and permanently anonymise all samples)*

OR

- Please destroy my blood sample as soon as the current research project has been completed.

Signed at (*place*) on (*date*)

.....
Signature of participant

.....
Signature of witness

Declaration by investigator

I (*name*) declare that:

- I explained the information in this document to
- I encouraged him/her to ask questions and took adequate time to answer them.
- I am satisfied that he/she adequately understands all aspects of the research as discussed above.
- I did/did not use an interpreter. (*If an interpreter is used then the interpreter must sign the declaration below.*)

Signed at (*place*) on (*date*)
2005.

.....

Signature of investigator

.....

Signature of witness

Declaration by Interpreter

I (*name*) declare that:

- I assisted the investigator (*name*) to explain the information in this document to (*name of participant*) using the language medium of Afrikaans/Xhosa.
- We encouraged him/her to ask questions and took adequate time to answer them.
- I conveyed a factually correct version of what was related to me.
- I am satisfied that the participant fully understands the content of this informed consent document and has had all his/her question satisfactorily answered.

Signed at (*place*) on (*date*)
2005.

.....

Signature of interpreter

.....

Signature of witness

PARTICIPANT INFORMATION AND INFORMED CONSENT FORM FOR RESEARCH

Title of research project: Pharmacokinetics and dosing rationale of terizidone and para-aminosalicylic acid (PAS) in children and the evaluation of the in vitro metabolism of ethionamide, terizidone and PAS.

Principal Investigator: Prof Bernd Rosenkranz

Address: Faculty of Health Sciences, Stellenbosch University, Francie van Zijl Drive, Tygerberg, 7505

Contact Numbers: Tel +27-21-938 9331 (Office); Cellular phone: +27-82-9550017

You are invited to take part in a research project. Please take some time to read the information presented here, which will explain the details of this project. Please ask the study staff or doctor any questions about any part of this project that you do not fully understand. It is very important that you are fully satisfied that you clearly understand what this research entails and how your child could be involved. Also, your participation is **entirely voluntary** and you are free to decline to participate. If you say no, this will not affect you negatively in any way whatsoever. You are also free to withdraw from the study at any point, even if you do initially agree to take part.

This study has been approved by the **Committee for Human Research at Stellenbosch University** and will be conducted according to the ethical guidelines and principles of the international Declaration of Helsinki, South African Guidelines for Good Clinical Practice and the Medical Research Council (MRC) Ethical Guidelines for Research.

Introduction

Tuberculosis (TB) is a very important health problem in South Africa and is often complicated by disseminated forms of TB such as meningitis or widespread lesions (miliary) TB, and resistance to the commonly used medicines. PAS, a second-line medicine is mostly used in combination with the commonly used drugs for the treatment of extensively resistant TB. When resistance is present other medicines (second-line medicines) must be used. These are not as effective as the medicines usually given for TB and they have more complications than the commonly used (first-line) medicines. The medicines

used to treat extensively resistant TB almost always include PAS. The second-line drugs such as PAS are not as effective as first-line TB drugs and it is therefore important that PAS be given in the best possible dosage. There is only very little information available regarding the amount of PAS that gets into the blood. Such information would make it possible for doctors to give much more accurate dosages of PAS than is possible at present. This study aims to determine the amount of PAS that gets into the blood of TB patients following the currently recommended and used dose of PAS.

Before you decide if you want to be a part of this study, we want you to know about the study. You may choose not to be part of this study. No health care will be withheld for you should you choose not to be part of the study. You will still receive the standard treatment for drug-resistant tuberculosis at this hospital.

This is a consent form. It gives you information about this study. The study staff will talk with you about this information. You are free to ask questions about this study at any time. If you agree to take part in this study, you will be asked to sign this consent form. You will get a copy to keep.

Why is this study being done?

The purpose of this study is to evaluate the absorption of the antituberculosis medicine PAS when given in the currently recommended dose in patients with drug-resistant TB.

What will happen to me and what will I have to do if I participate in this study?

If you decide to take part in this study, a number of investigations will be done. The absorption of the medicines will be studied by placing a needle in a vein and taking a small amount of blood 10 times soon after the medicines have been given. The amount of the medicine in the blood will then be measured. All of the above investigations will mean taking the volume of about 2 teaspoons of blood from you at two occasions approximately a month apart.

How many patients will take part in the study?

Up to 40 patients will be enrolled in the study.

How long will you be in the study?

The investigations will be completed as soon after being admitted to Brooklyn Chest Hospital as possible and the examination by the doctor and the blood investigations should be completed within a day and will be repeated about a month later.

Why might the doctor not admit me to this study?

The study doctor may decide that you are too sick to take part in the study. The study might also be cancelled by the site's Ethics Committee (the committee that watches over the safety and rights of research subjects).

What are the risks of the study?

Your participation in this study will not increase the risk that you might be affected by PAS, but will increase the chance that such reactions will be detected early. A small amount of bleeding may occur at the site where the blood is taken from and this may leave a bruise which should soon heal.

Are there benefits to taking part in this study?

It is likely that you may derive no benefit from being in this study. Information from this study may, however, will help experts decide what dosage of PAS should be recommended for use.

What other choices do I have besides this study?

Should you decide that you do not want to take part in this study your treatment will be unaffected and will continue as usual. Please talk to your doctor about the study if you should be uncertain as to whether you should take part in the study.

What about confidentiality?

Every effort will be made to keep your personal information confidential, however we cannot guarantee absolute confidentiality. Your personal information may be disclosed if required by law. On our study record we will use codes instead of your name. Only the study staff will know these codes. The study workers will not give out any information about you without written consent from you. Your family's privacy will be respected. The public health authorities will be notified as usual if you have TB. Any publication of this study will not use your name or identify you personally.

Your study records may be inspected by the South African Department of Health, study staff or study monitors. The study records will be kept separate from the normal medical records.

What are the costs to me?

There is no cost to you resulting from participation in the study. All medical care will be provided by the study doctor.

Will I receive any payment?

We will re-imburse your transportation money to enable you to attend the hospital on the day that the study.

What happens if I get injured?

If you get injured as a result of being in this study, you will be given immediate treatment for your injuries at no cost to you.

What are my rights as a research subject?

Participation in this study is completely voluntary. You may choose not to take part in this study or withdraw from the study at any time. You will be treated the same no matter what you decide, there will be no penalty.

We will tell you about new information from this or other studies that may affect your health, welfare or willingness to stay in this study. If you want the results of the study inform the study staff.

What do I do if I have questions or problems?

For questions about this study or a research-related injury, contact either **Prof Bernd Rosenkranz** at the above address or the person who gave you this form.

You can contact the Committee for Human Research at 021-938 9207 if you have any concerns or complaints that have not been adequately addressed by your child's study doctor.

You will receive a copy of this information and consent form for your own records.

Declaration by patient

By signing below, I agree to take part in a research study entitled: **Pharmacokinetics and dosing rationale of terizidone and para-aminosalicylic acid (PAS) in children and the evaluation of the in vitro metabolism of ethionamide, terizidone and PAS.**

I declare that:

- I have read or had read to me this information and consent form and that it is written in a language with which I am fluent and comfortable.
- I have had a chance to ask questions and all my questions have been adequately answered.
- I understand that taking part in this study is **voluntary** and I have not been pressurised to take part.
- I may choose to withdraw from the study at any time and will not be penalised or prejudiced in any way.
- I may be asked to leave the study before it has finished if the study doctor or researcher feels it is in my best interests, or if I do not follow the study plan as agreed to.

Signed at (*place*) On (*date*)

.....

Signature of parent/legal guardian

.....

Signature of witness

Declaration by investigator

I (*name*) declare that:

- I explained the information in this document to
- I encouraged him/her to ask questions and took adequate time to answer them.
- I am satisfied that he/she adequately understand all aspects of the research, as discussed above
- I did/did not use an interpreter (*if an interpreter is used, then the interpreter must sign the declaration below*).

Signed at (*place*) On (*date*)

.....

Signature of investigator

.....

Signature of witness

Declaration by interpreter

I (*name*) declare that:

- I assisted the investigator (*name*) to explain the information in this document to (*name of parent/legal guardian*) using the language medium of Afrikaans/Xhosa.
- We encouraged him/her to ask questions and took adequate time to answer them.
- I conveyed a factually correct version of what was related to me.
- I am satisfied that the parent/legal guardian fully understands the content of this informed consent document and has had all his/her questions satisfactorily answered.

Signed at (*place*) On (*date*)
.....

.....
Signature of interpreter

.....
Signature of witness

PARTICIPANT INFORMATION AND INFORMED CONSENT FORM FOR RESEARCH INVOLVING GENETIC STUDIES

TITLE OF RESEARCH PROJECT: Pharmacokinetics and dosing rationale of terizidone and para-aminosalicylic acid (PAS) in children and the evaluation of the in vitro metabolism of ethionamide, terizidone and PAS.

PRINCIPAL INVESTIGATOR: Prof Bernd Rosenkranz

ADDRESS: Faculty of Health Sciences, Stellenbosch University, Francie van Zijl Drive, Tygerberg, 7505

CONTACT NUMBER: Tel +27-21-938 9331 (**Office**); **Cellular phone:** +27-82-9550017

We would like to invite you to participate in a research study that involves genetic analysis and possible long-term storage of blood or tissue specimens. Please take some time to read the information presented here which will explain the details of this project. Please ask the study staff or doctor any questions about any part of this project that you do not fully understand. It is very important that you are fully satisfied that you clearly understand what this research entails and how your child could be involved. Also, your participation is **entirely voluntary** and you are free to decline to participate. If you say no, this will not affect your child negatively in any way whatsoever. You are also free to withdraw your child from the study at any point, even if you do agree to take part initially.

If you do not wish to participate in this genetic analysis part of the study, they can still be part of the main study of antituberculosis drug levels in the blood. The genetic analysis will then not be performed on your blood.

This research study has been approved by the ethics **Committee for Human Research at Stellenbosch University** and it will be conducted according to international and locally accepted ethical guidelines for research, namely the Declaration of Helsinki, and the SA Department of Health's 2004 Guidelines: *Ethics in Health Research: Principles, Structures and Processes*.

What is Genetic research?

Genetic material, also called DNA or RNA, is usually obtained from a small blood sample. Genes are found in every cell in the human body. Our genes determine what we look like and sometimes what kind of diseases we may be susceptible to. Worldwide, researchers in the field of genetics are continuously discovering new information that may be of great benefit to future generations

and also that may benefit people today, who suffer from particular diseases or conditions.

What does this particular research study involve?

In the main part of the study the amount of antituberculosis drug in the blood will be measured at various time points. A number of factors can affect the amount of drug in a patient's blood. One of these is the ability of the liver to produce enzymes (substances which speed up chemical reactions). These enzymes are responsible for the metabolism (breakdown) of drugs which are then passed out of the body.

Some people produce more powerful enzymes than others and they are therefore able to break down drugs more quickly than other people. This is because different people have differences in the genes which control how these enzymes are made. These variations in the genetic material of individuals are known as polymorphisms

This could possibly help us in the future to predict which patients are likely to develop very high (which may be dangerous) or very low (the drug doesn't work) concentrations, based on their genetic makeup. If we know your genetic makeup, we could perhaps take precautions or use a lower or higher dose of these drugs.

Why have I been invited to participate?

You have been invited to participate because you have shown interest in the main part of our study and so we are going to measure blood concentrations of antituberculous drugs in you. Performing genetic testing on will therefore help us determine if genetic differences (polymorphisms) can lead to high or low blood concentrations as explained above.

What procedures will be involved in this research?

You will not be required to give any additional blood or participate in additional procedures. The blood obtained from the main study will be used to perform genetic testing.

Are there any risks involved in genetic research?

In this study, we will be focusing on those genes which are responsible for the production of enzymes which break down or metabolize the drugs your child is receiving or drugs which broken down by the same kind of enzymes.

This study therefore will not identify any diseases or genetic problems which may lead to diseases later in life.

Are there any benefits to your taking part in this study and will you get told your results?

You will not benefit directly from taking part in this genetic research study. You will not be informed of the results. Blood will be stored and only tested at a later date once more is known about the enzymes responsible for drug breakdown and the genetic polymorphisms possibly involved.

It is possible that in the future we can use the study results to predict which patients are likely to develop very high (which may be dangerous) or very low (the drug doesn't work) concentrations. If we know your genetic composition, we could perhaps take precautions or use a lower or higher dose of these drugs.

How long will your blood be stored and where will it be stored?

The blood will be stored in the Division of Clinical Pharmacology and analysed by the University of Stellenbosch.

If your blood is to be stored is there a chance that it will be used for other research?

Your blood will only be used for genetic research that is directly related to the metabolism of antituberculosis drug PAS. If you do not wish your blood specimen to be stored after this research study is completed you will have an opportunity to request that it be discarded when you sign the consent form.

How will your confidentiality be protected?

The blood for DNA analysis will be labelled with your participation number and not name, prior to storage. It will be labelled because we wish to compare your child's blood concentration to their genetic results and so these must be linked by a participant number.

All study records and samples will be kept confidentially in a sealed and locked area only accessible to the investigators. No one other than the investigators or the ethics committee of Stellenbosch University will have access to your child's personal information. In the publication of results, your child's name or identifying information will not be used

Will you or the researchers benefit financially from this research?

You will not be paid to take part in this study.

Important information: In the unlikely event that this research leads to the development of a commercial application or patent, you or your family will not receive any profits or royalties.

Declaration by participant

By signing below, I agree to take part in a genetic research study entitled (*insert title of study*).

I declare that:

- I have read or had read to me this information and consent form and it is written in a language with which I am fluent and comfortable.
- I have had a chance to ask questions and all my questions have been adequately answered.
- I understand that taking part in this study is voluntary and I have not been pressurised to take part.
- I have received a signed duplicate copy of this consent form for my records.

Tick the option you choose:

I agree that my blood sample can be stored **indefinitely/ stored foryears**, but I can choose to request at any time that my stored sample be destroyed. My sample will be identified with a special study code that will remain linked to my name and contact details. I have the right to receive confirmation that my request has been carried out

OR

Please destroy my blood sample as soon as the current research project has been completed.

Signed at (*place*) on (*date*)

.....

Signature of participant

.....

Signature of witness

Declaration by investigator

I (*name*) declare that:

- I explained the information in this document to
- I encouraged him/her to ask questions and took adequate time to answer them.
- I am satisfied that he/she adequately understands all aspects of the research as discussed above.
- I did/did not use an interpreter. (*If an interpreter is used then the interpreter must sign the declaration below.*)

Signed at (*place*) on (*date*)20.....

.....
Signature of investigator

.....
Signature of witness

Declaration by Interpreter

I (*name*) declare that:

- I assisted the investigator (*name*) to explain the information in this document to (*name of participant*) using the language medium of Afrikaans/Xhosa.
- We encouraged him/her to ask questions and took adequate time to answer them.
- I conveyed a factually correct version of what was related to me.
- I am satisfied that the participant fully understands the content of this informed consent document and has had all his/her question satisfactorily answered.

Signed at (*place*) On (*date*) 20.....

.....
Signature of interpreter

.....
Signature of a witness

Case record form

Title of the project: Pharmacokinetics and dosing rationale of terizidone and para-aminosalicylic acid (PAS) in children and the evaluation of the in vitro metabolism of ethionamide, terizidone and PAS.

Project No.....

Date.....

Patient name.....

Date of birth.....

Sex.....

Race.....

Diagnosis.....

Measurements:

Height (cm).....

Weight (kg).....

Mid upper arm circumference (cm).....

Drug for measurement (name).....Dose (mg).....

Dosage time.....

Sample	Sampling time (min)		Time (received)	Remarks
	Planned	Actual sampling		
1				
2				
3				
4				
5				
6				

Details of the co-administered drugs

Drug (name)	Dose(mg)	Dosage time	Duration of Rx