DEVELOPMENT AND VALIDATION OF SELECTIVE AND SENSITIVE LC-MS/MS METHODS FOR THE DETERMINATION OF PARA-AMINOSALICYLIC ACID AND CYCLOSERINE / TERIZIDONE APPLICABLE TO CLINICAL STUDIES FOR THE TREATMENT OF TUBERCULOSIS

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DISSERTATION SUBMITTED IN FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE

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DECLARATION

It is herewith declared that this dissertation for the degree Master of Science (Clinical Pharmacology) at the University of Cape Town is the independent work of the undersigned and has not previously been submitted by him at any other University or Faculty for a degree. In addition, copyright of this dissertation is hereby ceded in favour of the University of Cape Town.

Signed by candidate

08 June 2018

MJ SMIT DATE

Declaration certifying the candidate's personal contribution towards the research, which is the subject of this M. Sc. (Clinical Pharmacology).

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Title: Development and validation of selective and sensitive LC-MS/MS methods for the determination of *para*-Aminosalicylic Acid and Cycloserine / Terizidone applicable to clinical studies for the treatment of tuberculosis.

Supervisor: Dr Lubbe Wiesner

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We, the undersigned, declare that under our supervision, Mr. Smit performed the development and validation of the two assay methods contained in this dissertation, as well as the sample assays of the said research projects. Under our supervision, Mr. Smit personally compiled and typed the dissertation in its present form.

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Dr Lubbe Wiesner	Dr Sandra Castel
07 June 2018	07 June 2018
Date	Date

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ABSTRACT

A method was validated for the quantification of *para*-aminosalicylic acid (PAS) in human plasma. The technique consisted of a protein precipitation extraction, followed by high performance liquid chromatography with tandem mass spectrometry (LC-MS/MS) detection. Rilmenidine was used as the internal standard (ISTD). Analyte mean extraction yields determined were ~100.3% (CV % = 3.3). The extraction procedure was followed by liquid chromatographic separation using a Phenomenex Synergi Hydro-RP (150 x 2.0 mm, 4µm) analytical column. An isocratic mobile phase containing methanol, water and formic acid (40:59.8:0.2, v/v/v) was used at a flow-rate of 300 µl per minute. The retention times for PAS and rilmenidine were, ~2.4 and ~1.6 minutes, respectively.

An AB Sciex API 3000 mass spectrometer at unit resolution in the multiple reaction monitoring (MRM) mode was used to monitor the transition of the protonated precursor ions m/z 154.1 and m/z 181.2 to the product ions m/z 80.2 and m/z 95.2 for PAS and the ISTD, respectively. Electro Spray Ionisation (ESI) was used for ion production.

Accuracy and precision were assessed over three consecutive, independent runs. The calibration curve fits a quadratic (weighted by 1/x concentration) regression for PAS over the range 0.391 – 100 µg/ml, based on peak area ratios. A 1:1 and 1:4 dilution of the QC Dilution sample showed that concentrations of up to 160 µg/ml of PAS in plasma could be analysed reliably when diluted into the calibration range.

Endogenous matrix components were found to have an insignificant effect on the reproducibility of the method, when human plasma originating from eight different sources were analysed. PAS was found to be stable in human plasma for 21 months kept at ~-80°C, for up to 21 hours at room temperature and when subjected to 3 freeze-thaw cycles. Stock solutions of PAS in methanol were stable for 2 days when stored at ~80°C and for 24 hours when stored at room temperature, ~4°C and ~-20°C. Plasma extracts of the analyte/ISTD ratio were shown to be stable on instrument over a period of ~55 hours. Reinjection reproducibility experiments indicated that an assay batch may be re-injected within 58 hours. Quantification of PAS in plasma was not significantly affected by the presence of haemolysed blood (2%) in plasma and when Lithium Heparin was used as anti-coagulant instead of K_3EDTA.

The best marker for terizidone pharmacokinetics is the analysis of cycloserine, a small polar drug with limited potential for absorbing UV that makes it difficult to analyse. A method

was validated for the quantification of cycloserine in human plasma, and consisted of a protein precipitation extraction and derivatization, followed by high performance liquid chromatography with MS/MS detection. No ISTD was used as no suitable match could be found. The mean extraction yield determined was ~77% (CV% = 10.7). The extraction procedure was followed by liquid chromatographic separation using a Gemini NX C18 (50 x 2.0 mm, 5µ) analytical column. An isocratic mobile phase containing acetonitrile, water and formic acid (30:69.9:0.1, v/v/v) was used at a flow-rate of 300 µl per minute. The retention time for cycloserine was ~ 1.5 minutes.

An AB Sciex API 3000 mass spectrometer at unit resolution in the MRM mode was used to monitor the transition of the protonated precursor ion m/z 335.9 to the product ion m/z 157.2 for cycloserine. ESI was used for ion production.

Accuracy and precision were assessed over three consecutive, independent runs. The calibration curve fits a quadratic (weighted by 1/x concentration) regression for cycloserine over the range 0.313 – 40.0 µg/ml, based on peak areas.

A 1:4 dilution of the QC Dilution sample showed that concentrations of up to 64.0 µg/ml of cycloserine in plasma could be analysed reliably when diluted into the calibration range and no carry over peaks were observed.

Endogenous matrix components were found to have no effect on the reproducibility of the method when human plasma originating from six different sources was analysed.

Cycloserine was found to be stable in human plasma for up to 18 hours at room temperature, and when subjected to 3 freeze-thaw cycles. Stock solutions of cycloserine in water and methanol were stable for 10 days when stored at ~ -80°C and for 18 hours when stored at room temperature, ~ 4°C and ~ -20°C. Long term stability in plasma has been proven for 17 months at -80°C. Plasma extracts of the analyte were shown to be stable on instrument over a period of ~ 29 hours. Reinjection reproducibility experiments indicate that an assay batch may be re-injected within 29 hours. Cycloserine is stable in whole blood (on ice) for up to 30 minutes.

Both validated methods presented performed well on clinical samples generated from a multi drug resistant TB (MDR-TB) research study in children dosed with PAS and terizidone.

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LIST OF ABBREVIATIONS

% Dev (% Deviation) Synonymous to: % Bias	The difference between the true nominal value and the value obtained, expressed as a percentage. $\%\ Dev\ (or\ \%\ Bias) = \left(\frac{Found\ value - Nom\ value}{Nom\ value} \right) \times 100$
% CV (% coefficient of variation)	The coefficient of variation (CV), also known as relative standard deviation (RSD), is a standardized measure of dispersion of a probability distribution or frequency distribution. It is often expressed as a percentage. $\%\ CV = \left(\frac{STDEV}{Mean} \right) \times 100$
~	Approximately
µg	Microgram
µl	Microlitre
ALQ	Above the Limit of Quantification
AIDS	Acquired Immune Deficiency Syndrome
ATT	Antitubercular Treatment
AcPAS	N-acetyl-*p*-Amino salicylic acid
Anti-TB	Anti-tuberculosis
APCI	Atmospheric Pressure Chemical Ionization
AVR	Anti-retroviral
BCG	Bacille Calmette Guerin
BE	Bioequivalent
°C	Celsius
C_{max}	Maximum plasma concentration
CFFAB	Continuous Flow Fast Atom Bombardment
CV	Coefficient of Variation

CSF	Cerebrospinal Fluids
DC	Direct Current
DST	Drug Susceptibility Testing
ECD	Electron Capture Detector
EC	Electrochemical Detector
EFV	Efavirenz
ES	Electrospray
eV	Electron volt
FID	Flame Ionisation Detector
FLD	Fluorescence Detector
FDA	Food and Drug Administration
FTMS	Fourier Transform Mass Spectrometer
FWD	Fixed Wavelength Detector
G	Gram
GCP	Good Clinical Practice
GLP	Good Laboratory Practice
GC	Gas Chromatography
HPLC	High Performance Liquid Chromatography
HIV	Human Immunodeficiency Virus
ICH	International Conference on Harmonisation
IRMPDS	Infra-red multiphoton photo dissociation spectroscopy
ISTD	Internal Standard
Kg	Kilogram
K_3EDTA	Potassium Ethylenediamine tetra acetic acid
kV	Kilovolt

LLE	Liquid-Liquid Extraction
LC-MS/MS	Liquid Chromatography with Tandem Mass Spectrometry
LLOQ	Lower Limit of Quantification
mg/ml	Milligram per millilitre
µmol/L	Micromole per litre
mg/day	Milligram per day
MDR-TB	Multi Drug Resistant Tuberculosis
MNR-ESD outlier test	The **M**aximum **N**ormal **R**esidual test modified by using a backwards elimination algorithm referred to as the **E**xtreme **S**tudentised **D**eviate in order to be able to detect multiple outliers. Hawkins (Hawkins, D.M., Identification of Outliers, Chapman and Hall, 1980) has shown this to be an acceptable method for detecting multiple outliers, as the error is well defined and acceptably small.
MS/MS	Tandem Mass Spectrometers
MSD	Mass Selective Detector
MIC	Minimum Inhibitory Concentration
MDR-TB	Multi drug-resistant tuberculosis
NPD	Nitrogen Phosphorus Detector
N	Number of determinations
N/A	Not applicable
ng	Nanogram
PAS	*para*-Amino salicylic acid
PB	Particle Beam
PDA	Photo Diode Array Detector
pH	Potential of Hydrogen
pKa	Acidic Dissociation Constant
PK	Pharmacokinetics
PD	Pharmacodynamics

PTA	Probability of Target Attainment
PPT	Protein precipitation
QC	Quality Control Standard
RF	Radio Frequency
RR-TB	Rifampicin Resistant Tuberculosis
SPE	Solid Phase Extraction
SOP	Standard Operating Procedure
STD	Calibration Standard
STD DEV	Standard Deviation
SRM	Single Ion Monitoring
TB	Tuberculosis
TDM	Therapeutic Drug Monitoring
TCD	Thermal Conductivity Detector
TOF-MS	Time-of-Flight Mass Spectrometer
Tlag	Time taken for the drug to appear in systemic circulation
ULOQ	Upper Limit of Quantification
UV	Ultraviolet Wavelength Detector
VWD	Variable Wavelength Detector
WHO	World Health Organisation
XDR-TB	Extensively Drug Resistant Tuberculosis

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1. INTRODUCTION

1.1. Brief history of tuberculosis

Tuberculosis (TB) is caused by a variety of mycobacteria strains, typically either *Mycobacterium tuberculosis* or *Mycobacterium bovis.* Although the disease can occur in any organ of the body it is best known as a disease of the lungs. TB has claimed victims throughout much of known human history and may have killed more people than any other microbial pathogen [1]. DNA evidence of TB dates back to 17,000 Before Christ (BC) and palaeopathological evidence to 8,000 BC, evidence of bony TB has been found dating from the Neolithic period in 5,800 BC and in Egyptian mummies dating to 2,400 BC [2, 3].

Although microscopy evidence from autopsies laid the foundation for understanding the pathogenesis of TB at the end of the 18th century, it was the work of Théophile Laennec at the beginning of the 19th century that made us understand that the multiple forms of TB were one single infectious entity [2]. Jean-Antoine Villemin demonstrated the transmissibility of *Mycobacterium tuberculosis* infection in 1865 and Robert Koch identified the tubercle bacillus as the etiologic agent in 1882 [4, 5]. In 1907 Clemens von Pirquet developed the tuberculin skin test and in 1910 used it to demonstrate latent TB infections in children who were carriers of the disease but displayed no symptoms [6].

Nearly fifty years after the successful identification of *Mycobacterium tuberculosis*, a feasible treatment regime for TB was developed by Selman Waksman and Albert Schatz. Selman Waksman worked from 1914-1944 to find a drug with good chemotherapeutic response and low toxicity in humans, which led to the discovery of streptomycin [7]. The next chemotherapeutic breakthrough was isoniazid in 1952, although the drug was already synthesised nearly 50 years earlier [8].

A vaccine against TB, the Bacille Calmette Guerin (BCG) vaccine, was developed and first used in humans in 1921. The vaccine was widely used in Europe during World War I, but never gained popularity in the United States. It's effectiveness against pulmonary tuberculosis vary, but it is still the only vaccine currently available with new vaccines in development [9].

While TB steadily declined during the late 19th and early 20th century, the prevalence remained high in many parts of the world. TB treatment outcomes are diverged along the lines of global economy and it is necessary to understand the different treatment approaches

and implementation in high-incidence, low-income countries [2]. Although TB is a contagious disease its progress is highly influenced by environmental factors and the immunocompetency of the host. Only about one tenth of immunocompetent people infected develop symptoms over their lifetimes [10]. *Mycobacterium tuberculosis* has proved to be a very resilient microorganism, with the ability of developing drug resistant mutants if treated with one drug alone. The modern standard for TB treatment became a combination of four drugs, to keep successful tubercle bacillus mutations at bay [11].

Resurgence of TB, particularly in the developing world, has stimulated research, providing new potential tuberculosis vaccines. This resurgence is not only due to the re-emergence of dense population centres with poor sanitation (a phenomenon that contributed to TB's prevalence historically in Europe), but is also due to the rise of other diseases that compromise immunity such as the human immunodeficiency virus / acquired immune deficiency syndrome (HIV/AIDS). When nearly one third of the world's population harbours asymptomatic infections, being able to identify a particular strain of TB and its drug resistant status is more crucial than ever before [12].

Although TB is still an epidemic and survey data show it is larger than previously estimated, the number of deaths and incident rate globally are decreasing. "In 2015, there were an estimated 10.4 million new (incident) TB cases worldwide, an estimated 1.4 million TB deaths and an additional 0.4 million deaths resulting from TB disease among people living with HIV. People living with HIV accounted for 1.2 million (11%) of all new TB cases. Six countries accounted for 60% of the new cases: India, Indonesia, China, Nigeria, Pakistan and South Africa. Worldwide, the rate of decline in TB incidence remained at only 1.5% from 2014 to 2015. This rate will need to increase to a 4–5% annual decline by 2020 in order to reach the first milestones of the "End TB Strategy" by the World Health Organisation (WHO)" [13].

In 2011 there where an estimated 310 000 incident cases of multidrug resistant (MDR-TB) and that increased in 2015 to an estimated 480 000 incident cases. In 2015, an additional 100 000 people with rifampicin-resistant TB (RR-TB) incidence were reported, they were also eligible for MDR-TB treatment and required second line drugs [13, 14].

Increasing incidence rates for MDR-TB have been recorded in several settings, with South

Africa suffering one of the highest occurrences of MDR-TB and extensively drug resistant TB (XDR-TB). Since 2002, MDR-TB in South Africa has been treated by a standardized combination therapy, which includes ofloxacin, kanamycin, ethionamide, ethambutol and pyrazinamide. Since 2010, ethambutol has been replaced by cycloserine or terizidone [14].

"Although the number of TB deaths fell by 22% between 2000 and 2015, TB remained one of the top 10 causes of death worldwide in 2015. It is estimated that TB care and prevention treatment averted 49 million deaths globally between 2000 and 2015, but important diagnostic and treatment gaps persist" [13].

The emergence of MDR-TB and XDR-TB threatens disease control efforts throughout the world. Drug-resistant tuberculosis may be acquired if bacteria expressing drug resistant mutations are positively selected due to: inadequate treatment regimes, poor drug quality or patient non-compliance. Alternatively, drug resistant tuberculosis may also occur through the transmission of existing resistant strains, termed primary resistance. High rates of primary resistance reflect poor transmission control, often exacerbated by delays in drug susceptibility testing and initiation of appropriate treatment [15].

1.2. Treatment of tuberculosis

The aims of TB treatment are:

- curing patients of TB in order to restore their quality of life and productivity
- preventing relapse of TB
- reducing transmission of TB to others
- preventing the development and transmission of drug resistant TB

TB drug treatment is sometimes referred to as antitubercular treatment (ATT), and involves a combination of the different drugs available. Some TB drugs are only used for the treatment of new patients when there is no suggestion of any drug resistance, also called drug susceptible TB, and others are only used for the treatment of drug resistant TB. More than 90% of people with drug susceptible TB can be cured in six months using a combination of first line TB drugs [16].

First line drugs are those TB drugs that generally have the greatest bactericidal activity when used for TB treatment. The amount of drug that an adult TB patient requires is dependent on

their weight.

For new patients with presumed drug susceptible pulmonary TB, the WHO recommends that they receive six months of treatment. This consists of a two-month intensive TB treatment phase followed by a four-month continuation phase. The WHO recommends isoniazid, rifampicin, pyrazinamide and ethambutol for the intensive treatment phase and isoniazid and rifampicin for the continuation phase.

It is recommended that patients undertake this regime daily for six months, however taking the drugs three times a week is also possible in some circumstances. In the interest of reducing the further development of resistant strains, it is essential that all the recommended TB drugs are taken [17].

Treatment of drug resistant TB is more challenging than the treatment of drug susceptible TB, and the treatment regime is defined by the type and number of drugs each particular strain is resistant to:

- MDR-TB is caused by a strain of *Mycobacterium tuberculosis* that are resistant *in vitro* to rifampicin and isoniazid.
- XDR-TB is caused by a strain of *Mycobacterium tuberculosis* that are resistant *in vitro* to isoniazid, rifampicin and at least one injectable agent (i.e. amikacin, kanamycin or capreomycin) and any of the fluoroquinolones.
- Mono-resistant TB is caused by strains of *Mycobacterium tuberculosis* that are resistant to just one anti-TB drug.
- Polyresistant TB is caused by strains of *Mycobacterium tuberculosis* that are resistant to more than one drug, but not isoniazid and rifampicin together.

Resistant strains require the use of second line or reserve drugs that are costlier, cause more side effects and need to be taken for longer periods of time, sometimes up to two years [17].

The drugs that are used for the treatment of drug resistant TB are grouped according to how effective they are, how much experience people have with their use and the drug class. All first line anti-TB drugs are in Group 1, apart from streptomycin which is classified with the other injectable agents in Group 2 as presented in Table 1.

Table 1. Summary of the TB drug classification.

First line drugs: (*Group 1: First Line Oral Agents*)	Second line drugs *Group 2: Injectable Agents*	*Group 3*: *Fluoroquinolones*	*Group 4*: *Oral Bacteriostatic Second Line Agents*	*Group 5*: Agents with an unclear role
Pyrazinamide	Kanamycin	Levofloxacin	PAS **	Clofazimine
Ethambutol	Amikacin	Moxifloxacin	Cycloserine	Linezolid
Rifampicin	Capreomycin	Ofloxacin	Terizidone	Amoxicillin/clavulanate
Isoniazid	Streptomycin	Gatifloxacin	Thionamide	Thioacetazone
Rifabutin *			Protionamide	Imipenem/cilastatin
Rifapentine *				High dose isoniazid
* New generation rifamycins				Clarithromycin
** para-Aminosalicylic acid				Meropenem/clavulanate
				High dose Isoniazid/Imipenem

New drugs recently added to group 5 are delamanid and bedaquiline. Bedaquiline was approved by the FDA in the United States, for use in the treatment of drug resistant TB, when no alternatives are available. These drugs have some significant side effects [18].

A patient's access to drug susceptibility testing (DST) and the type of DST available, determines the treatment protocol for a patient with drug resistant TB. Clinicians often prescribe regimes compiled by an algorithm, or formula, set by a regional or national TB treatment program in a country. A DST may take many weeks or months, therefore, initially a standard drug regimen is typically provided, until the patient's drugs can be targeted in light of the results. More modern rapid DSTs are available, such as the newer molecular TB tests. This allows the clinician access to at least some drug resistance information, and thereby allowing for a quicker turnaround time in providing an individualized program of TB drug treatment to the patient [19].

A major cause of the current drug resistance problems is the complexity and length of even the "basic" treatment regime for drug sensitive TB. There is an urgent need for new drugs with shorter, simpler regimes as well as new drugs for the treatment of MDR-TB [17].

In areas of minimal or no MDR-TB, TB cure rates of up to 95 per cent can be achieved. Cure rates for multi drug resistant TB are lower, typically ranging from around 50% to 70%. The fact that cure rates for MDR-TB are so low, mean that MDR-TB continues to spread, and globally MDR-TB is becoming an increasingly severe problem [13].

Several countries have been testing a new treatment for MDR-TB. This new regime, sometimes called the "Bangladesh regime", requires far fewer drugs to be taken and a cheaper, shorter treatment period of around nine months. This regimen prescribes gatifloxacin in combination with ethambutol, pyrazinamide, and clofazimine throughout, supplemented by kanamycin, prothionamide and isoniazid during the initial four-month period [20]. In 2016, the WHO recommended that this treatment regime could be used for patients with "uncomplicated MDR-TB". "Uncomplicated MDR-TB" is defined as TB infection by bacteria resistant to rifampicin and isoniazid. This protocol is also recommended for individuals who have not yet been treated with second line drugs [17].

In order to identify patients who are suitable for this treatment regime, it is necessary to do a Line Probe Assay test. This test verifies that the active infection does not already demonstrate resistance to one or more of the second line drugs. If the infection does demonstrate resistance, a shorter regime with fewer drugs, could not only result in the patient not being cured, but also create the selective environment that would encourage the bacterium to develop further resistance. The Line Probe Assay test is, however, usually only available in national TB reference laboratories [21].

All patients receiving TB treatment should be monitored during their treatment to assess their response to the drug treatment. Regular monitoring also helps to ensure that patients complete their treatment. It can also help to identify and manage adverse drug reactions. Patients need to have their weight checked every month, and if the patient's weight changes the drug dosages may need to be adjusted [17].

When patients have pulmonary TB, the patient's response to TB treatment should be monitored using sputum smear microscopy. The recommendation from the WHO is that for smear positive TB patients treated with first line drugs, a smear microscopy should be performed at the end of the two months' intensive phase of treatment [17]. Sputum should be collected when the patient is given the last dose of the intensive phase of treatment. If the patient has a positive sputum smear at the end of the intensive phase, then there should be a patient assessment carried out, because the positive smear could indicate several different

situations. An example is that the patient may have drug resistant TB, and a change in the TB drugs they are taking might be needed. Alternatively, patient adherence may have been poor, and they might not have been taking their drugs correctly. The assessment might result in changes needing to be made to the patient's treatment, or to their support and supervision. Different actions may need to be taken in a variety of other circumstances, such as the patient having received treatment before [22].

The latest guidelines for the treatment of drug-resistant TB were developed by the WHO in 2016, based on the retrospective analysis of patient records from at least 32 observational studies investigating the impact of type, number of drugs and duration of treatment and outcome. The greatest chance for treatment success was with an intensive phase of second-line anti-TB drugs for 7–8.5 months and a total treatment duration of 25–27 months [17]. The initial regimen was composed of at least four active drugs during the intensive phase and three drugs during the continuation phase. Active second line anti-TB drugs include injectable anti-TB drugs such as: kanamycin, amikacin and capreomycin; fluoroquinolones like: moxifloxacin, ofloxacin and gatifloxacin; oral bacteriostatic second line anti-TB drugs like: ethionamide, prothionamide, cycloserine, terizidone and para-aminosalicylic acid and class 5 anti-TB drugs that include: clofazimine, linezolid, amoxicillin clavulanate, clarithromycin, imipenem high-dose isoniazid, meropenem / clavulanate, delamanid and bedaquiline [18, 23].

The drugs which this project will focus on are two oral bacteriostatic second line anti-TB drugs *para*-aminosalicylic acid (PAS) and cycloserine / terizidone.

1.3. Aim of this research

This project aimed to develop and validate selective and sensitive LC-MS/MS methods to determine PAS and cycloserine / terizidone in biological matrixes, applicable to clinical studies where subjects were exposed to therapeutic doses of these drugs.

PAS is a poorly tolerated bacteriostatic agent and cycloserine / terizidone use is hindered by serious central nervous system toxicity. As with other oral bacteriostatic second-line anti-TB agents, little is known about their pharmacokinetics (PK) / pharmacodynamics (PD) and how that correlates to their activities.

In vitro animal models and human pharmacokinetic studies in concert are necessary to define the lowest exposure necessary for maximal bacteriostatic effect, to identify lower and more tolerable doses that retain similar efficacy. For these studies, more sensitive, accurate and efficient analytical methods are needed to generate quality PK data. Quality PK data will lead to better recommendations regarding dosing and duration of treatment needed to maximize efficacy with acceptable safety and tolerability for these oral bacteriostatic second line anti-TB drugs.

2. METHOD DEVELOPMENT

2.1. Introduction

The quantification of chemical entities in different biological matrices, bioanalytical chemistry, is constantly changing and more must be done in shorter time. Bioanalytical chemistry is an important component within medical and pharmaceutical research both for drug discovery and development. The cost of developing drugs has increased significantly and the drive towards higher productivity and shorter development times is crucial in all the areas of the drug discovery process [24]. On the other hand, analytical methods employed for the determination of drugs and their metabolites in biological samples are key determinants in generating reproducible and reliable data. The focus of bioanalysis in the pharmaceutical industry is to provide a quantitative measurement of the active drug and / or metabolite(s) for the accurate assessment of PK data, toxicokinetics, bioequivalence (BE) and exposure-response (PK/PD) relationships in the drug discovery and development process as described in Figure 1 [25, 26].

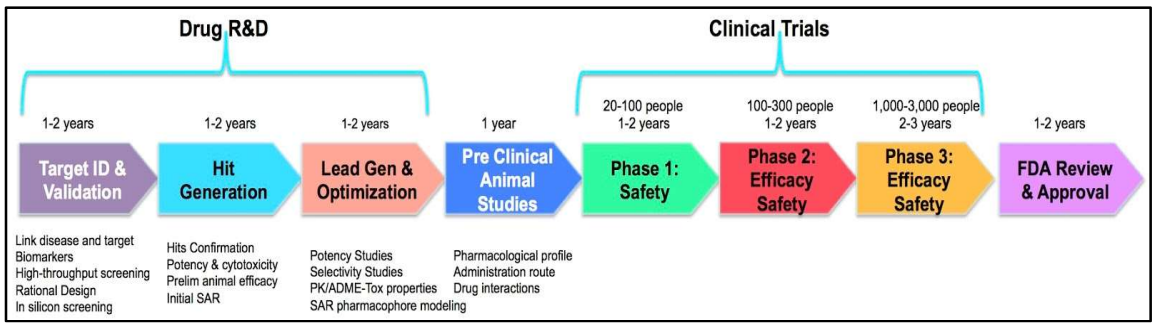

Figure 1. Flowchart of drug discovery and development studies [27].

The quality of these studies, which are often used to support regulatory filings and other evaluations, is directly related to the conduct of the underlying bioanalysis. Therefore, the application of best practices in bioanalytical method development, validation and associated sample analysis is key to an effective discovery and development program leading to the successful registration and commercialization of a drug product [28].

The analytical chain describes the process of method development and includes sampling, sample preparation, separation or extraction techniques, detection and evaluation of the results. Analytical method development is the process of creating a procedure to enable a compound of interest to be identified and quantified in a suitable matrix, against established acceptance criteria for specific characteristics. It is essential to employ well-characterized and fully validated analytical methods to yield reliable results, which can be satisfactorily interpreted [29, 30].

2.2. Literature survey

One of the starting points for method development is a thorough literature survey, a compound can often be measured by several methods. The optimum technique should be identified based on the intended purpose and scope of the analytical method. A survey on published assay methods can save time and money during method development. Analytical information obtained during a literature survey may provide: the chemical properties and stability of the analyte, type of instrumentation, type of column, type of extraction, type of matrices and anticoagulant used, the linear range, the method limit of detection and quantitation [31].

Clinical information is essential for the method development phase, to define what the limit of quantitation and the linear range of the method needs to be. Therefore, dose and sampling times are important information to obtain during the literature survey. Clinical information is usually obtained from the study protocol, a document that describes how a clinical trial will be conducted (the objective(s), design, methodology, statistical considerations and organization of a clinical trial,) and ensures the safety of the trial subjects and integrity of the data collected [32]. After all the information is obtained from the literature survey, a procedure is outlined in an action plan or method development plan.

2.3. Method development plan

The method development plan is the starting point of the method development process. Information from the literature survey is used, but if very little or no information is available, a new method is developed based on the chemical properties of the drug to be assayed. From existing knowledge of our test drugs, the following techniques were identified as potentially useful.

2.3.1. Chromatographic systems

A combination of different factors will determine the type of chromatographic system to be used: availability of instruments, instrumentation used in published assays, selectivity needed, the limit of quantitation and the cost of the analysis.

The most common chromatographic systems available for medical and pharmaceutical research are high performance liquid chromatography (HPLC) and gas chromatography (GC). Historically most of the volatile and more basic drugs were analysed on GC systems and the more non-volatile and acidic drugs were assayed on HPLC systems.

2.3.1.1. High Performance Liquid Chromatography

HPLC (high-pressure liquid chromatography), is a technique in analytical chemistry used to separate, identify, and quantify components in a mixture. A pump passes a liquid solvent, called the mobile phase, containing the sample mixture under high pressure through a column filled with a solid adsorbent material, called the stationary phase. Each component in the sample interacts slightly differently with the adsorbent material, causing different flow rates for the different components and leading to the separation of the components as they elute out of the column. Each component is then “detected” by the detector that converts the component concentrations into an electronic response acquired by a data acquisition system as represented in Figure 2 [33].

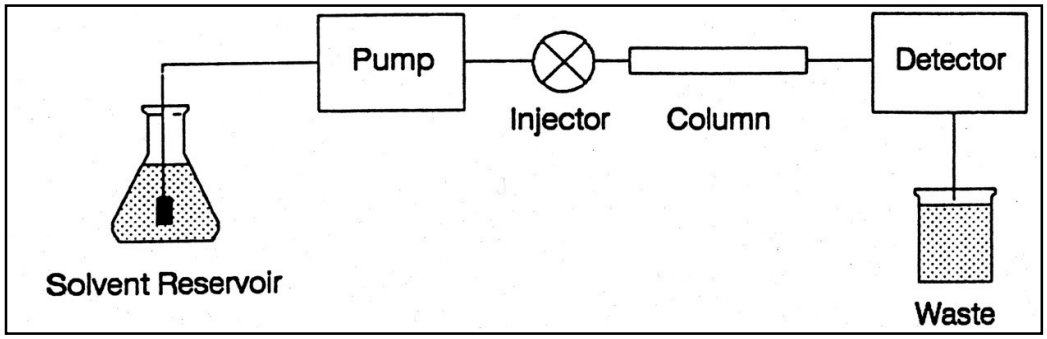

Figure 2. Schematic representation of an HPLC unit

2.3.1.2. Gas Chromatography (GC)

In gas chromatography an inert gas such as helium, or an unreactive gas such as nitrogen is used as the mobile phase or carrier gas. Helium remains the most commonly used carrier gas in about 90% of instruments although hydrogen is preferred for improved separations. The stationary phase is a microscopic layer of adsorbent material, normally a liquid or polymer, inside a piece of glass or metal tubing called a column. The sample mixture is evaporated into gas and moved through the column, the components in the sample mixture would interact with the column and separate. The column normally sits inside a column oven, a heat gradient as well as the flow of the carrier gas elute the components from the column to be detected, as presented in Figure 3. The instrument used to perform gas chromatography is called a gas chromatograph. Conventional GC sometimes involved tedious derivatisation reactions in order to obtain good chromatography and separation [34].

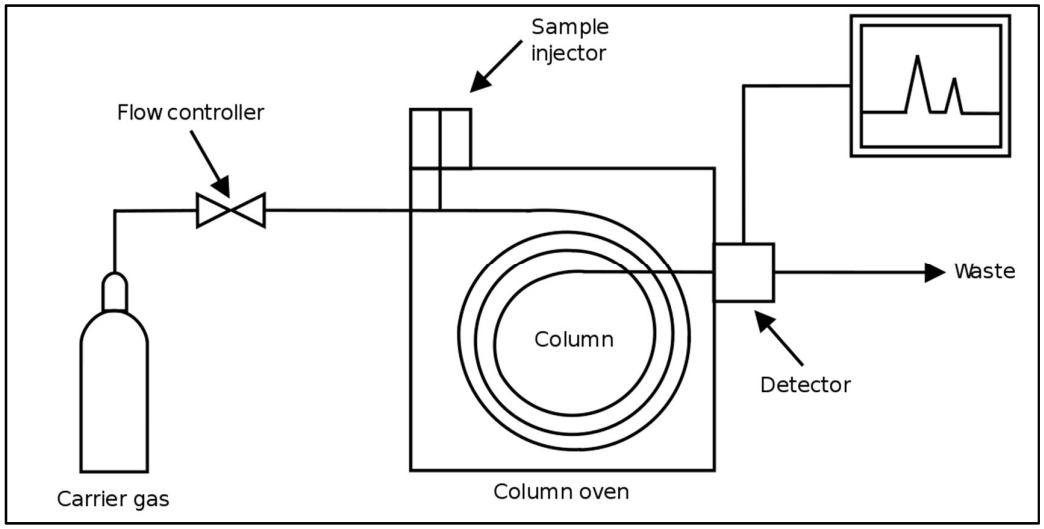

Figure 3. Schematic representation of a gas chromatograph

In the last decade, there have been tremendous advancements in the field of mass spectrometry, with the development of new interfaces, ionisation and detection techniques. These advancements have resulted in the rapid emergence of widespread commercial use of hyphenated liquid chromatography mass spectrometry (LC-MS/MS) based assays, which have largely replaced conventional HPLC and GC assays [35].

Advancement in sample handling and extraction instrumentation has been seen with the use of multi-well plates, automated robotic sample processing and electronic data reporting becoming common place [26].

2.3.2. Detection instrumentation

The most common detectors used in high performance liquid chromatography (HPLC) are variable wavelength (UV), fluorescence (FLD), electrochemical (EC) and mass spectrometer (MS) detectors. In gas chromatography (GC) the most common detectors are nitrogen phosphorus (NPD), electron capture (ECD), flame ionisation (FID) and mass spectrometer (MS) detectors. Each one of these detectors have some advantages and some disadvantages and need to be carefully selected for a specific assay.

2.3.2.1. UV/Vis Detectors

UV detection is one of the most common types of HPLC detectors. It measures the ability of solutes to absorb light at a particular wavelength(s) in the ultraviolet (UV) or visible (Vis) wavelength range.

There are three common types of UV/Vis absorbance detectors:

- Fixed Wavelength Detectors (FWD), the absorbance of only one given wavelength is monitored by the system at all times (usually 254 nm), this is the simplest and cheapest of the UV/Vis detectors, but the types of compounds that can be detected are limited.
- The Variable Wavelength Detector (VWD), monitors a single wavelength at any given time, but any wavelength in a wide spectral range can be selected (190-900 nm). This detector is more expensive and requires more advanced optics, but can be used to detect a wider range of compounds.
- The Photo Diode Array Detector (PDA) operates by simultaneously monitoring absorbance of solutes at several different wavelengths, the detector uses a series or an array of several detector cells within the instrument, with each responding to changes in absorbance at different wavelengths. The entire spectrum of a compound can be read in a minimum amount of time, useful in detecting the presence of poorly resolved peaks or peak contaminants [36].

2.3.2.2. Fluorescence Detectors

Fluorescence detection is a selective HPLC detector that measures the ability of eluting solutes to fluoresce at a given set of excitation and emission wavelengths. Fluorescence can be used to selectively detect any compound that absorbs and emits light at the chosen set of excitation and emission wavelengths. Typical applications are in the analysis of drugs, food additives and environmental pollutants [36].

2.3.2.3. Electrochemical Detectors

Electrochemical detectors are used to monitor any compound in the mobile phase that can undergo an oxidation or reduction, electrochemical detection coupled with liquid chromatography is sometimes referred to as LC/EC. These detectors can be made specific for a given compound or class of compounds by selecting the conditions at the electrodes,

the detector has high selectivity with a low background signal [36].

2.3.2.4. Nitrogen–phosphorus detectors

The nitrogen–phosphorus detector (NPD) is a common GC detector. A form of thermionic detector where nitrogen and phosphorus alter the work function on a specially coated bead, creating a current proportional to sample concentration. It responds selectively to most organic compounds that contain phosphorus or nitrogen down to picogram levels and is mass flow dependant. It is useful for analysis of drugs and pesticides containing phosphorus [37].

2.3.2.5. Electron Capture Detection

Electron capture detection (ECD) is another GC detector, which uses a radioactive beta particle (electron) source to measure the degree of electron capture. ECD is used for the detection of molecules containing electronegative elements and functional groups like halogens, carbonyl, nitriles, nitro groups, and organometallics. In this type of detector, either nitrogen or 5% methane in argon is used as the mobile phase carrier gas. The carrier gas passes between two electrodes placed at the end of the column. Adjacent to the anode (negative electrode) resides a radioactive foil such as 63Ni. The radioactive foil emits beta particles (electrons), which collides with and ionizes the carrier gas to generate more ions resulting in a current. It is a very specific detector, non-destructive in nature and widely used in environmental analysis, e.g. organochlorine pesticide [37].

2.3.2.6. Flame Ionization detector (FID)

In the flame ionization detector (FID), electrodes are placed adjacent to a flame fuelled by hydrogen / air near the exit of the column. When carbon containing compounds exit the column they are pyrolyzed by the flame. This detector works only for organic / hydrocarbon containing compounds due to the ability of the carbons to form cations and electrons upon pyrolysis, which generates a current between the electrodes. The increase in current is translated and appears as a peak in a chromatogram. FIDs have low detection limits (a few picograms per millilitre) but they are unable to generate ions from carbonyl containing carbons. FID compatible carrier gasses include helium, hydrogen, nitrogen, and argon [37].

2.3.2.7. Thermal conductivity detector (TCD)

The thermal conductivity detector is a GC detector less sensitive than the FID, and is used for preparative applications. It is a non-destructive universal detector and response depends on the thermal conductivity difference between the carrier gas and the eluted components. This method also responds to inorganic gases such as CO, CO_2, NH_3, CS_2, N_2, etc [37].

2.3.2.8. Mass Spectrometers (MS)

Mass Spectrometers (MS), also called mass analysers, are compound specific detectors and give unambiguous identification of sample components, often used in conjunction with HPLC and GC. The rich chemical information available from spectra allows identification of unknown components in samples, even when a standard is not available for comparison. Mass spectrometry is ideal for drug metabolism, product stability, natural product elucidation, forensic analysis and all areas of analysis where structural elucidation and identification is critical. Drug discovery and diagnostics are two important areas where rapid identification of sample components are key to successful problem-solving.

The mass spectrometer cannot determine the mass of an uncharged atom or molecule. The uncharged species must first be ionized so that it can be repelled and attracted by the fields generated in the mass analyser. A mass spectrometer determines the mass-to-charge ratio of an ion, this is usually referred to as *m/z* (mass divided by the charge). Since the charge on most small molecule ions created in a mass spectrometer is 1, *m/z* is equivalent to the mass. For the mass of an ion to be determined, the ions must pass through the analyser region of the mass spectrometer on its way to the detector. In order to do this, the ions must be in a gas phase. All mass analysers have the means to separate and detect gas-phase ions and it is through this method that determines the information content of a given experiment. As ion production, mass separation and ion detection are common to all mass analysers, the method in which ions are introduced into an analyser, how ions of different masses are separated and how they are detected can be quite different with each specific mass analyser. Some analysers introduce ions into the mass analysers as a continuous beam (quadrupoles and magnetic sector analysers) of ions and others as a pulse (time of flight, ion trap and Fourier transform analysers). Some spectrometers separate ions in space while others are traps that separate ions in time [35].

2.3.2.8.1. Quadrupole Mass Analysers

Quadrupoles consist of four precisely parallel rods (called poles) spaced around a central axis, represented in Figure 4. An ion source produces ions that moves through the mass analyser along the axis of the quadrupoles by applying a voltage gradient. The voltage needed for the ions to move is as little as 5 – 10 eV and therefore quadrupoles are considered low energy mass analysers.

Applying a specific voltage, with a specific radio frequency (RF) and direct current (DC), and alternating the voltages between opposing sets of quadrupoles creates the "mass filter". By using a specific voltage gradient on opposite sets of quadrupole rods a complete range of masses can be passed through the detector.

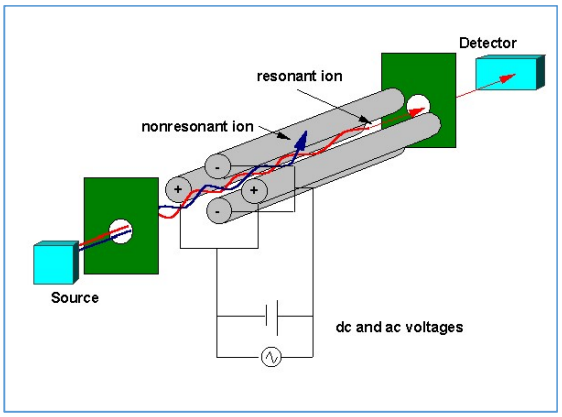

Figure 4. Schematic of a quadrupole MS

Ions with a specific mass-to-charge ratio requires a certain voltage to pass through the filter and be detected. Quadrupoles are versatile mass analysers and suitable for high throughput and production applications. The most common type of quadrupole setup used today are the triple quadrupole instruments which consist of 3 sets of quadrupoles in tandem. The first set of quadrupoles (Q1), analyse the intact charged molecule, the second set of quadrupoles produce fragmentations of the molecule and the third set of quadrupoles (Q3) analyse the fragments. This has become the standard tool used in LC-MS/MS for clinical assays [35].

2.3.2.8.2. Ion Trap Mass Analysers

This mass analyser works by first trapping ions then detecting them based on their *m/z* ratio's. Because an ion trap is a variation on the quadrupole mass filter, it is sometimes referred to as a quadrupole ion trap. By alternating the voltages between the electrodes and applying the same focussing principals as in the quadrupoles setup, ions are "trapped" or

focused in a 3-dimensional volume, as represented in Figure 5.

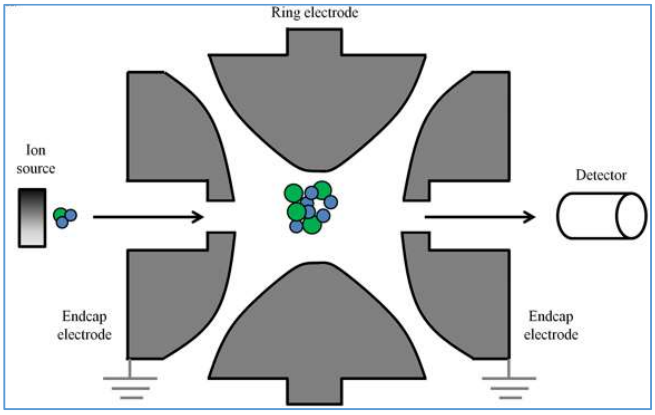

Figure 5. Schematic of an ion trap MS

Ions are detected by placing them in unstable orbits, which causes them to leave the trap and transit to the detector. An ion trap consists of two endcap electrodes and a ring electrode. By adding a gas like helium, the trap encourages ions to migrate to the centre of the trap. The tighter the ions are focused the more efficient their detection. After trapping and focusing the ions, collision dissociation takes place and tandem mass spectrometry can be performed. This capability provides the analyst with rich structure information and therefore applications of ion traps have been found in combinatorial chemistry and drug metabolism [35].

2.3.2.8.3. Time-of-Flight Analysers

One of the simplest mass analysers is called the time-of-flight (TOF) mass analyser. These analysers operate on the principal that an ion's velocity of travel is mass dependant i.e. the smaller the mass of an ion, the higher it's velocity relative to heavier ions. A typical TOF mass analyser consist of an ion source, a flight tube, a reflectron and a detector as represented in Figure 6. An ion source produces ions that are accelerated down the flight tube through the use of a high voltage. Each mass entering the flight tube will travel at different velocities. As the initial bundle of ions travel down the flight tube, they are separated and arrive at the detector at different times. The arrival time of each ion is directly related to its mass or in this case, *m/z*.

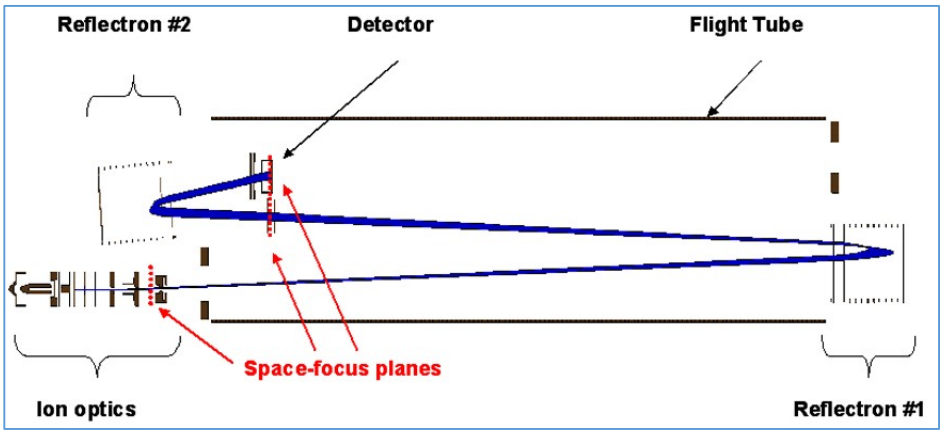

Figure 6. Schematic of a TOF MS

TOF mass analysers are ideal for determining the chemical structure of compounds because of their sensitivity and high-speed scanning. TOF mass analysers are often used in tandem with quadrupoles, which allow for high efficiency in the first analyser and high selectivity scanning in the second analyser. TOF is unrivalled for applications where high molecular weight is important, for example the analysis of polymers and biopolymers, because it has a large mass range and practically no upper limit [35].

2.3.2.8.4. Magnetic Sector Mass Analysers

Organic chemistry and analysis have been dominated by the high-resolution application of the magnetic sector mass analysers for the past 30 years. Ions created in the source are accelerated down the ion beam into the analyser with typical voltages of 4-8 kV, represented in Figure 7. The radius of curvature in a given magnetic field of the sector mass analyser is a function of the *m/z.* Ions will follow different radius curves through the magnetic field based on their mass. Ions of different masses are then separated and scanned by a fixed detector by varying either the magnetic field or the ion source voltage. To achieve a high resolution, magnetic sector analysers are coupled with electromagnetic sectors to generate different kinetic energies and separate mixtures of ions.

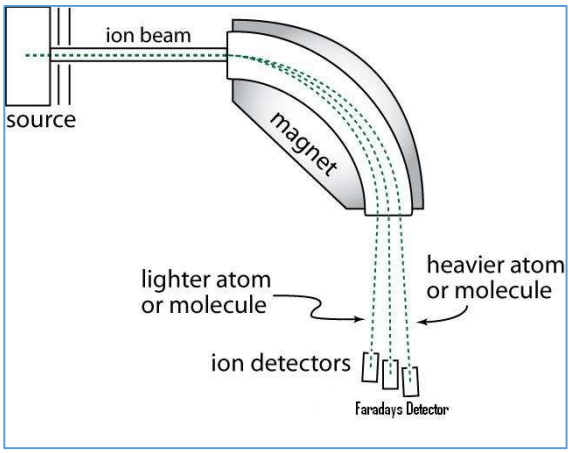

Figure 7. Schematic of a Magnetic Sector MS

Magnetic sector mass analysers are reliability instruments with a high dynamic range, making them a competitive alternative for qualitative analysis [35].

2.3.2.8.5. Fourier Transform-MS

The Fourier transform mass spectrometer (FTMS) consists of a cell formed by excitation plates and detector or trapping plates. A very high vacuum (<10-6 Torr) creates a vacuum chamber inside the cell. As ions pass through the cell they are trapped by a combination of a high magnetic field and electric potentials applied to the detector or trapping plates, as represented schematically in Figure 8. As the magnetic field inside the cell is increased, the ions begin to rotate within the cell around the axis of the magnetic field. The rotation is detected indirectly through an induced current generated on the detector plates as the ions pass near the plates. As the frequency of the rotation is inversely proportional to the mass, the frequency of ion rotation can be converted to mass through what is called Fourier transform.

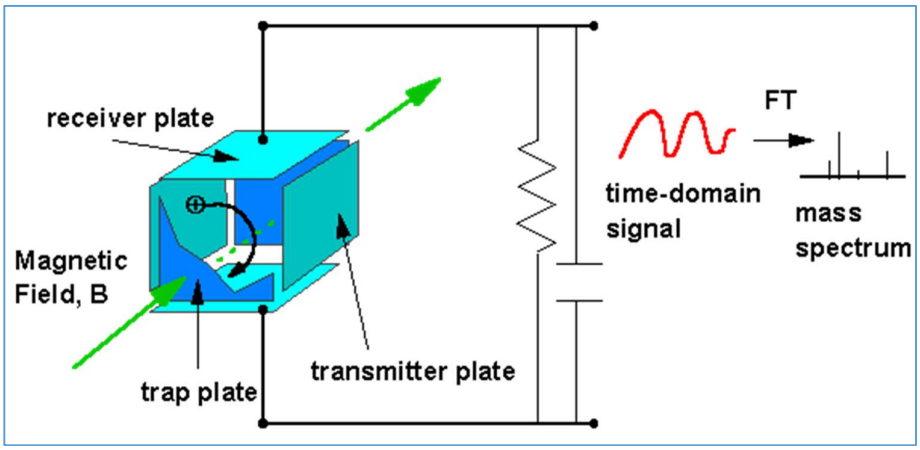

Figure 8. Schematic of a Fourier Transform MS

The advantages of FTMS is high resolution (>500,000), high mass accuracy, simplicity and versatility. This spectrometer is most often used for qualitative and not quantitative analysis. A disadvantage of the FTMS use its high cost [35].

Coupling liquid chromatography to mass spectrometry is called interfacing, and is the conversion process where the liquid phase is evaporated into a gas phase, atmospheric pressure is reduced to high vacuum and ionization takes place. A wide variety of commercial alternative interfaces exist, electrospray (ES), atmospheric pressure chemical ionization (APCI), particle beam (PB), continuous flow fast atom bombardment (CFFAB) and thermo-spray (TS) [35].

2.3.3. Extraction techniques

Extraction techniques have long served as valuable tools to analytical chemists, particularly during the last century when selective and sensitive instrumentation were not generally available. Yet even today, despite the constant development of selective and sensitive detection instrumentation, analytical chemists are constantly challenged to perform determinations in which available instrumentation is inadequate for the task without prior separation steps to provide pre-concentration or the required selectivity. The methods used for treating biological samples prior to their introduction into chromatographic systems generally fall into one of two categories, extraction or direct injection. Three of the main techniques used in the bioanalytical field to separate drugs from very complex biological

fluids like blood, serum, plasma, urine etc., are: protein precipitation (PPT), liquid-liquid extraction (LLE) and solid phase extraction (SPE).

2.3.3.1. Protein precipitation

Drugs can be isolated from biological fluids like whole blood, serum and plasma by deproteination of the specific biological fluid before it is injected into the chromatographic system. Deproteination can be done by the addition of organic solvents like: acetonitrile, acetone, methanol etc., and ionic salts such as, ammonium sulphate, perchloric acid and trifluoracetic acid [38].

2.3.3.2. Liquid-liquid extraction

Liquid-liquid extraction (LLE) is one of the oldest techniques for isolating desired components from a mixture. The principal used is to separate the mixture by using two immiscible solvents, an aqueous solvent or phase and an immiscible organic solvent or organic phase. The compound in the mixture are separated by their ability to partitioned in the two immiscible solvents. The compounds are distributed between the two phases in which one is immiscible to the other. The compound of choice can be selected from the mixture by creating the perfect partitioning environment, by choosing the proper potential of hydrogen (pH) and extraction solvent. The initial solvent needs to be a suitable solvent to dissolve the mixture, for example an aqueous matrix like plasma or serum, a second solvent is added that is immiscible with the first. The two solvents are thoroughly mixed and allowed to separate into layers. The components of the mixture will be distributed among the two layers as determined by their partition coefficient. The less dense solvent will be the upper layer, while the denser solvent will be the lower layer. The compounds in the mixture will separate between the two layers based on their solubility in that specific layer, the more soluble the more likely it is to reside in that layer. The compounds in each layer are isolated when the two immiscible layers are separated. If one of the layers is an aqueous layer, separation can be done by freezing. Most hydrophilic compounds are found in the more polar aqueous phase and the hydrophobic compounds in the organic phase. Compounds extracted into the organic phase are easily recovered by evaporation of the organic solvent, the residue can then be reconstituted in an appropriate solvent. The LLE technique is a very cost-effective extraction procedure compared to solid phase extraction (SLE) and generally a

cleaner extract than PPT [25].

2.3.3.3. Solid phase extraction

Solid phase extraction (SPE) use a stationary sorbent or solid phase to retain the compound of choice from a mixture. SPE can be used to extract a compound from biological matrix as well as concentrate the sample. Different extraction sorbents can be used and knowledge of the mechanism of interaction between the compound and sorbent can be used to select the compound of choice from a complex mixture. Understanding the chemical properties of the compound and sorbent, like polarity, hydrophobicity and solubility helps with retaining the selective compound. The most common retention mechanism in SPE are based on van der Waals forces (non-polar interactions), hydrogen bonding, dipole-dipole forces (polar interactions) and the cation-anion interactions (ionic interactions). Stationary sorbents are packed inside a cartridge or column and is available in different chemistries, this include reverse phase, normal phase and ion exchange. Sample is normally loaded onto the stationary phase and either vacuum of positive pressure is used to force the sample through the cartridge [39].

SPE extraction generally follows the following protocol: Pre-treatment of sample, conditioning of the cartridge, loading of the sample, washing the cartridge and then an elution step.

1. The pre-treatment of the sample includes steps to avoid blocking of the cartridge and better absorption of the compound to the stationary phase, this include dilutions, pH adjustment, deprotonation and centrifugation.
2. Conditioning of the cartridge is very important and this allows the sample solvent to penetrate the pores of the stationary phase and interact with the compound. In reverse phase SPE solvents such as methanol, acetonitrile, isopropyl alcohol or tetrahydrofuran is used and in normal phase solutions like hexane, ethers or chloroform.
3. The loading step involves loading the pre-treated sample onto the conditioned cartridge for the compound to be retained on the stationary sorbent.
    3.1 In reverse phase sorbents, hydrophobic interactions are used to retain the compound and involve a polar sample matrix and a non-polar stationary phase. Retention occurs *via* non-polar interactions between the analyte and the

sorbent functional groups, due to Van der Waals or dispersion forces. When an analyte is in its neutral form, it becomes more hydrophobic and retention strengthens. Therefore, in general, acidic compounds are best retained at a pH value at least 2 pH units below the compound's acidic dissociation constant (pKa) value, and basic compounds are best retained at 2 pH units above the compound's pKa value.

3.2 In normal phase sorbents, hydrophilic interactions are used to retain the compound and involve a polar analyte, a mid to non-polar matrix and a polar stationary phase. Retention of analyte is primary due to interactions between polar functional groups off the analyte and polar groups on the sorbent surface due to hydrogen bonding and dipole-dipole, induced dipole-dipole and *pi-pi* forces.

3.3 In ion exchange, the electrostatic attraction of charged groups on the compound and the charged groups on the sorbent's surface are used to retain the compound. Anionic (negatively charged) compounds can be isolated on an aliphatic quaternary amine group that is bonded to the silica surface and cationic compounds are isolated by using the silica with aliphatic sulfonic acid groups that are bonded to the silica surface.

4. The washing step is the cleaning step, as the compound is retained, the SPE column can be washed with solutions and solvents that do not disrupt these interactions and therefore flush the sample of complex matrixes like proteins and salts.
5. The elution step is used to elute the retained compound from the stationary sorbent or cartridge, a solvent or solution is used that would disrupt the interactions between the stationary sorbent and the compound. The eluted fraction then contains the analyte of choice and can be used as is or further concentred by evaporation and reconstitution steps [25].

2.3.4. Matrix effect

Variability of instrument response due to the differences in the physiological state of matrices is called the matrix effect. Especially with LC-MS/MS based procedures and methods, appropriate steps should be taken to minimise the matrix effect. In general, cleaner

samples, changes to the chromatography or the use of deuterated internal standards are recommended to minimise the matrix effect. Matrix effect should always be tested if the nature of the matrix changes from that used during initial method validation [26].

2.3.5. Robustness of the method

During the early stages of method development, the robustness of methods should be evaluated. Robustness of a method is very important and measures the capability of the method to remain unaffected under different conditions, such testing should be performed during development of the analytical procedure. This is particularly important when methods are transferred between facilities, the method executed by different analysts or a significant time lapse between periods of operation occur [29, 40]. The analytical procedure should be described and documented in a standard operating procedure (SOP), this will allow for the procedure to be reproduced within set acceptance criteria [30].

3. METHOD VALIDATION

Selective and sensitive analytical methods for the quantitative evaluation of drugs and their metabolites (analytes) are critical for the successful conduct of preclinical and/or biopharmaceutics and clinical pharmacology studies [41, 42]. "Bioanalytical method validation includes all of the procedures that demonstrate that a particular method used for quantitative measurement of analytes in a given biological matrix, such as blood, plasma, serum, or urine, is reliable and reproducible for the intended use" [32]. The fundamental tests that need to be included in validating a method include selectivity (specificity), accuracy, precision, recovery, calibration / standard curve reproducibility, stability and ruggedness [40, 43]. Validation involves the documentation of all procedures and measuring it against a set of acceptance criteria, this process continues to evolve. Today more statistical considerations are given to the reliability and suitability of a method and set guidelines are used [44, 45]. The acceptability of analytical data today corresponds directly to the criteria set in the guidelines used to validate the method [28, 46].

3.1. Parameters for the validation process

3.1.1. Selectivity (specificity)

Selectivity is the ability of an analytical method to select the compound of choice from a complex mixture of components. Blanks from the appropriate matrix (plasma, urine, or other matrix) should be obtained from at least six different sources. Selectivity is then proven in each individual matrix at the lower limit of quantification (LLOQ). The method should be free from interferences due to endogenous matrix compounds, metabolites, concomitant medication or decomposition products. This should be tested for each analyte quantified by the method [29, 40, 45].

3.1.2. Accuracy

The accuracy of an analytical method describes the difference between a test results obtained by the method to the true value. Accuracy is determined by replicate analysis of samples containing known amounts of the analyte, normally this is determined by the

quality control standards. Accuracy should be measured using a minimum of five determinations per concentration level, at three concentrations levels representing the expected concentrations range. The mean value should be within 15% of the actual value except at LLOQ, where it should not deviate by more than 20%. The deviation of the mean from the true value serves as the measure of accuracy [26, 41].

3.1.3. Precision

The precision of an analytical method describes the ability to reproduce the same result when the procedure is applied repeatedly to multiple aliquots of a single sample. Precision should be measured using at least five determinations per concentration level. A minimum of three concentrations levels representing the expected concentrations range [29]. The precision determined at each concentration level should not exceed 15% of the coefficient of variation (CV) except for the LLOQ, where it should not exceed 20%. Precision should also be assessed during a single analytical run (intra batch), and between-runs (inter batch), which measures precision over time and may involve different analysts, equipment, reagents, and laboratories [28, 30].

3.1.4. Recovery

The recovery is the comparison of the amount analyte recovered during an extraction by comparing the response of an extracted sample to the response of an un-extracted sample at the same concentration level. Thus, recovery could also be called the extraction efficiency of an analytical method and is expressed as a percentage. Recovery of the analyte need not be 100%, but more important is that the recovery is consistent, precise and reproducible for both analyte and ISTD at all concentration levels. Recovery experiments are performed at three concentrations (low, medium, and high) [45].

3.1.5. Calibration / Standard Curve

A calibration or standard curve is constructed from a set of calibrations standards, samples of known concentrations of the analyte, that span the entire concentration range. According to the FDA guidelines at least 6 non-zero calibrators should be used and be prepared in the

same matrix as the intended samples they will be used for. Concentrations of standards should be chosen based on the concentration range expected in a particular study and this should be generated separately for each analyte in the sample. A calibration curve should consist of a double blank sample (matrix sample processed without ISTD), a blank sample (matrix sample processed with ISTD), and six to eight non-zero samples covering the expected range, including LLOQ" [29].

3.1.5.1. Lower Limit of Quantification

The lower limit of quantification is the lowest standard being used to construct a calibration curve and should meet at least the following conditions:

- The analyte response or peak height at the LLOQ should be at least 5 times higher than the noise around the peak.
- Analyte peak (response) should be reproducible with a precision of 20% and accuracy of 80–120%.

3.1.5.2. Calibration Curve / Standard Curve / Concentration-Response

The simplest regression model that fits the concentration-response relationship should be used. Selection of weighting and use of a complex regression equation must be justified. The following conditions should be met in developing a calibration curve:

- Within 20% deviation of the LLOQ from nominal concentration
- Within 15% deviation of standards other than LLOQ from nominal concentration

At least four out of six non-zero standards should meet the above criteria, including the LLOQ and the calibration standard at the highest concentration. Excluding some of the standards should not change the model used [26, 29].

3.1.6. Stability

Stability of the drug must be tested in the solutions used for the initial stock preparation and working solutions, as well as in the intended matrix, for short and long-term storage conditions. Conditions used in stability experiments should reflect situations likely to be encountered during actual sample handling and analysis. Stability procedures should also evaluate the stability of the analyte in the intended matrix during sample collection (whole

blood stability), long-term storage (frozen at the intended storage temperature) and short-term for handling and extraction (bench top, room temperature and freeze thaw stability).
All stability determinations should use a set of samples prepared from a freshly made stock solution of the analyte in the appropriate analyte-free, interference-free biological matrix. Stock solutions of the analyte for stability evaluation should be prepared in an appropriate solvent at known concentrations [29, 30].

3.1.6.1. Stock Solution Stability

Stock solutions of the drug and ISTD should be evaluated in the appropriate solutions and for the period that at least covers the intended time of use. After completion of the desired storage time, the stability should be tested by comparing the stored solution with a freshly prepared solution at the same concentration [29].

3.1.6.2. Short Term Stability

High and low concentrations of the analyte, in the intended matrix, should be tested at room temperature after being kept at this temperature for a selected period of time, normally between 4 and 24 hours. This time period should cover at least the time it takes to extract the batch for analysis [29].

3.1.6.3. Freeze-Thaw Stability

Analyte stability should be determined after three freeze-thaw cycles. High and low concentrations of the analyte should be stored at the intended storage temperature for at least 24 hours and then thawed completely for the first cycle. The sample is then refrozen for 12 to 24 hours. The freeze-thaw cycle should be repeated two more times, then analysed after the third cycle [45].

3.1.6.4. Long Term Matrix Stability

The long-term stability is tested in the intended matrix and the intended temperature for a selected period of time. The duration of the long-term stability should cover the time from collection of the samples until it is being analysed. Long term stability is done at high and low concentrations with at least 3 replicates at each concentration from 3 different stored aliquots. The concentrations of all the stability samples should be compared to nominal

values for the standards at the appropriate concentrations [45].

3.1.6.5. Post Preparative Stability

The stability of the processed samples should be determined in the injection solution. This is tested during the autosampler stability and reinjection stability test. This will determine the total batch run time and ultimately the batch size. In the FDA guidelines comparing post preparative samples with freshly prepared samples is proposed, but statistical approaches based on confidence limits for evaluation of an analyte's stability in a biological matrix can be used. This should clearly be stated and described in the standard operating procedures (SOPs) [29, 30].

3.2. Validation Process

3.2.1. Bioanalytical Method Validation for Biological Matrix

The fundamental parameters to ensure the acceptability of the performance of a bioanalytical method validation include accuracy, precision, selectivity, sensitivity, reproducibility, and stability [41, 46].

The bioanalytical method should be well documented, in the form of a protocol, study plan, report or SOP. Each step in the method process should be investigated to determine the extent to which environmental, matrix, material, or procedural variables have on the quantification of analyte in the matrix from collection of the sample up to the time of analysis [32].

The same biological matrix that will be used for the test samples should be used for validating the method. The accuracy, precision, reproducibility, response function, and selectivity of the method for the analyte in the presence of endogenous substances, metabolites, and known degradation products should be established for the analyte in this matrix. It is important to consider the potential variability of quantitation in the matrix due to the physiological nature of the sample. With LC-MS/MS-based procedures, appropriate steps should be taken to ensure the absence of matrix effects, especially if the matrix differs from that used during method validation [32].

A bioanalytical method should be validated for its intended use or application. All

experiments supporting the conclusions about the validity of the method should be described in the method validation report.

For selectivity, there should be evidence that the substance being quantified is the intended analyte. The stability of the analyte and its metabolites in the intended matrix during the collection process and the sample storage period should be assessed during the validation. For additional matrix stability testing, the stability of the analyte in the matrix from dosed subjects should be tested [29, 45].

"The concentration range over which the analyte will be determined should be defined in the bioanalytical method, based on evaluation of actual standard samples over the range, including their statistical variation. This defines the standard calibration curve.

In consideration of high throughput analyses, including but not limited to multiplexing, multicolumn, and parallel systems, sufficient quality control (QC) samples should be used to assess variability of the assay. The number of QC samples required for validation is determined by the run size which are interspersed in a predetermined manner in each batch to avoid bias" [29].

With the drive towards better quality method validations, guidance and acceptance criteria set out by the FDA and EMEA guideline are the standards most modern laboratories work towards [28, 29, 30].

3.2.1.1. Specific Recommendations for Method Validation

The number of standards used should be a function of the dynamic range. The larger the range the more standards should be included. A minimum of six non-zero standards is the acceptance criteria. Additional standard concentrations may be required for standard curves that are non-linear.

The standard curve should cover the entire range of expected concentrations. Enough standards should be used to adequately define the relationship between concentration and response. The relationship between response and concentration must be demonstrated to be continuous and reproducible. The ability to dilute samples to concentrations above that of the original upper limit of the standard curve should be demonstrated by accuracy and precision parameters in the validation. Standard curve fitting is determined by applying the simplest model that adequately describes the concentration-response relationship using

appropriate weighting and statistical tests for most appropriate fit. Reported method validation data should include all outliers. Calculations of accuracy and precision may exclude values that are statistically determined as outliers, but all outliers should be reported in the final data [46].

LLOQ is the lowest concentration of the standard curve that can be measured with acceptable accuracy and precision. The LLOQ should be established using at least five samples, independent of standards, and determining the coefficient of variation and/or the appropriate confidence interval. The LLOQ should not be confused with the limit of detection and/or the low QC sample. The highest standard that can be measured with acceptable accuracy and precision defines the upper limit of quantification (ULOQ) of an analytical method.

For validation of the bioanalytical method, accuracy and precision should be determined using a minimum of five determinations per concentration level (excluding blank samples). The mean value should be within 15% of the theoretical value, except at LLOQ, where it should not deviate by more than 20%. The precision around the mean value should not exceed 15% of the CV, except for LLOQ, where it should not exceed 20% of the CV. Other methods of assessing accuracy and precision that meet these limits may be acceptable [30, 32].

The accuracy and precision, with which known concentrations of analyte in biological matrix can be determined, should be demonstrated. This can be accomplished by analysis of replicate sets of analyte samples of known concentrations, such as QC samples from an equivalent biological matrix. At a minimum, three concentrations representing the entire range of the standard curve should be studied: one within 3x the lower limit of quantification (LLOQ) (low QC sample), one near the centre (middle QC), and one near the upper boundary of the standard curve (high QC) [42].

The stability of the analyte in its biological matrix at intended storage temperatures should be established. The influence of freeze-thaw cycles (a minimum of three cycles at two concentrations in triplicate) should be reported. The stability of the analyte in matrix at ambient temperature should be evaluated over a time period equal to the typical sample preparation, sample handling, and analytical run times. Reinjection reproducibility should be evaluated to determine if an analytical run could be reanalysed in the case of instrument failure [46].

The specificity of the assay methodology should be established using a minimum of six independent sources of the same matrix. For hyphenated mass spectrometry-based methods, however, testing six independent matrices for interference may not be important. In the case of LC-MS and LC-MS/MS-based procedures, matrix effects should be investigated to ensure that precision, selectivity, and sensitivity will not be compromised. Method selectivity should be evaluated during method development and throughout method validation and can continue throughout application of the method to actual study samples [46].

Acceptance / rejection criteria for spiked, matrix-based calibration standards and validation QC samples should be based on the nominal (theoretical) concentration of analytes. Specific criteria can be set up in advance and achieved for accuracy and precision over the range of the standards, if so desired [29, 30].

3.2.2. Preparation of calibration standards and quality control samples in biological fluids

There are important factors to consider when calibration standards and quality control samples are prepared in biological fluids. The calibration standards and quality control samples should, where possible, be prepared in the same biological fluid as the intended use of that method, i.e. if the test samples are going to be analysed in plasma then the calibration standards and quality control samples should be prepared in plasma. The anticoagulant reagent used for the biological fluid should be the same [29].

The correct calibration range is important, it should cover the concentrations found in the study samples. To determine the correct range for a specific dose can be done practically by pooling small amounts of all the samples at a specific time point, and then analyse the profile and determine the maximum and minimum values in that profile, but this can only be done if samples are available and the analyte stability allows for such an experiment. Secondly if the samples are not available a thorough literature and clinical protocol review is important, to determine a calibration range for the specific dosing regimen.

The storage of the calibration standards and quality control samples should also be considered and, where possible, be in the same container and at the same temperature as the samples to be analysed [29, 30].

3.3. Performing validations

Calibration standards and quality control samples prepared in the appropriate matrix are extracted and assayed according to a protocol, study plan, report, and/or SOPs.

The calibration standards and quality controls are interspersed in a predetermined manner in each batch to avoid bias. After a batch is run, the chromatograms are inspected and the data evaluated against documented acceptance criteria. The calibration curves are plotted, regression equations determined, and the quality controls calculated as unknowns using the regression equation that had previously been selected, this would constitute a validation batch. For a full validation, three such validation batches should be run consecutively. Full validation batches are necessary when developing and implementing a bioanalytical method for the first time, for a new drug entity, and when metabolites are added to an existing assay for quantification. Partial validations are modifications of validated analytical methods that do not necessarily require full validations. Partial validations can range from as little as one inter assay accuracy and precision determination to a "nearly" full validation, typical changes that fall in this category, but are not limited to method transfer between laboratories, instrument and software changes, change in species matrix (e.g. rat plasma to mouse plasma), changes in matrix within a species (e.g. human plasma to human urine), rare matrixes and a change in the anti-coagulant. Another type of validation is a cross validation, which is performed to compare bioanalytical methods and is necessary when two or more methods are used to generate data within the same study [26].

4. METHOD DEVELOPMENT AND VALIDATION OF AN ANALYTICAL ASSAY METHOD FOR THE DETERMINATION OF *PARA*-AMINOSALICYLIC ACID IN HUMAN PLASMA

4.1. Objective

A sensitive and specific liquid chromatography-tandem mass spectrometry method was developed for the quantification of *para*-aminosalicylic acid (PAS) in human plasma. The objective was to develop a method to analyse clinical samples and contribute to the better understanding of the pharmacokinetics of PAS.

4.2. Literature survey

4.2.1. Clinical information

PAS, also known as 4-aminosaliccylic acid, has been used as an anti-TB drug since 1946, and for nearly 15 years, starting in 1955, PAS was considered a first-line drug in a combination regimen with isoniazid and streptomycin [47]. Currently PAS is considered an oral bacteriostatic second line anti-TB drug used for MDR-TB [48, 49].

PAS is a bacteriostatic drug against *Mycobacterium tuberculosis* which also inhibits the onset of bacterial resistance to streptomycin and isoniazid. There are two mechanisms responsible for PAS's bacteriostatic action against *Mycobacterium tuberculosis*. Firstly, PAS interfere with bacterial folic acid synthesis. It binds to pteridine synthetase with greater affinity than *para*-aminobenzoic acid (PABA), effectively inhibiting the synthesis of folic acid [50]. As bacteria are unable to use external sources of folic acid, cell growth and multiplication slows. Secondly, PAS inhibits the synthesis of the cell wall component, mycobactin, thus reducing iron uptake by *Mycobacterium tuberculosis* [47, 51].

The minimum inhibitory concentration (MIC) of PAS for *Mycobacterium tuberculosis* is 1 µg/ml. There are two major considerations in the clinical pharmacology of PAS. Firstly,

the prompt production of a toxic inactive metabolite under acidic conditions and secondly, the short serum half-life of one hour for the free drug, after 4–5 hours the plasma concentration of the drug is minimal, which justifies the need for doses of 10–12 g to maintain bacteriostatic activity. PAS is metabolised in the intestines and liver via acetylation into N-acetyl-*para*-aminosalycylic acid (AcPAS). More than 80% of the drug is excreted by the kidney through glomerular filtration and tubular secretion [47]. PAS acts preferentially on extracellular bacilli. New delayed-release formulations produce higher PAS exposures, overcoming the rapid metabolism and clearance of early formulations. The drug can currently be administered in granules stored in 4 mg envelopes, replacing the former 500 mg capsules. The delayed-release granules contain an acid-resistant coating to protect against degradation in the stomach; the granules are designed to escape the usual restriction on gastric emptying of large particles. The coating dissolves promptly (within 1 minute) at the neutral pH found in the small intestine or in neutral foods. This granular slow-release form of PAS, also called *para*-aminosalicyclic acid delayed-release granules (PACER granules) has been available for nearly two decades and has been found to cause less intolerance by several studies in TB patients and healthy volunteers [52]. The use of PAS is limited by its toxicities ranging from gastrointestinal irritation, myxoedema, hypokalaemia, life threatening hypersensitive reactions and vitamin B12 deficiency [53]. PAS is readily absorbed from the gastro-intestine tract and the median time to peak serum / plasma concentrations is 6 hours following a single 4-g dose in healthy adults. The ingestion of 4 g of PAS granules lead to a maximum serum concentration of 20–60 µg/ml after 4–6 hours [47].

Co-administration of other TB drugs have a minimal effect on the pharmacokinetics of PAS in adults. However, co-administration of food delays the time to maximum concentration (Tmax). High-fat food significantly increases the maximum drug concentration (Cmax) and area under the curve ($AUC_{0-\infty}$) by 1.5 and 1.7-fold, respectively, and extended the finite time taken for the drug to appear in systemic circulation (tlag). Concomitant dosing of orange juice and anti-acids has minimal effect on both rate and extent of drug absorption. The administration of PAS with appropriate food therefore, increases the time that plasma concentrations remain above the target concentration and should be taken with a high-fat meal whenever practical [54].

Co-administration of other drugs may impact the pharmacokinetics of PAS in adults. One

study compared PAS pharmacokinetics between HIV-uninfected and HIV-coinfected TB patients [52]. The study reported that PAS concentrations in HIV-infected patients was reduced and that the co-administration of Efavirenz (EFV) played a role in this deficit, a 52% increase in PAS clearance was seen when co-administered with EFV. An overall reduction of approximately 30% PAS exposure should be expected in HIV-infected TB patients who are on concurrent EFV. When ARV therapy contains EFV, once-daily dosing up to 12 g PAS in these patients is not recommended. If maintenance of PAS concentrations above the MIC is desired a regimen of 4 g twice daily provides sufficient pharmacodynamic coverage of a probability of target attainment (PTA) above 90% over dosing interval, and a PTA of 100% is achieved with trice-daily regimen of 12g/day [52].

Resistance to PAS is associated with mutations of mycobacterial *thyA*, but this mechanism accounts for only 6% of phenotyping resistance [53].

4.2.2. Analytical information

4.2.2.1. Chemical information

4.2.2.1.1. *para*-Aminosalicylic acid (PAS)

Synonym: 4-Aminosalicylic acid

Molecular formula: $C_7H_7NO_3$

Molecular weight: 153.14

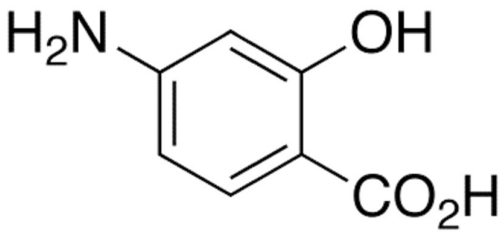

Figure 9. Chemical Structure of PAS

4.2.2.1.2. Rilmenidine

Molecular formula: $C_{10}H_{16}N_2O$

Molecular weight: 180.25

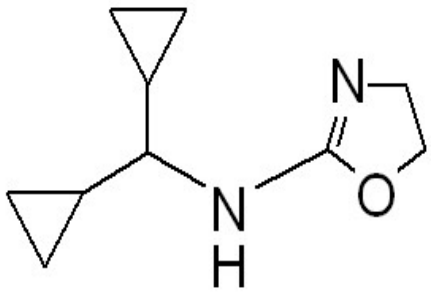

Figure 10. Chemical Structure of Rilmenidine

4.2.2.2. Analytical information

An analytical method has been developed for PAS in urine [55]. It is used as the pharmacokinetic marker to determine the *p*-amino benzoic acid excretion index. The technique analyses urine samples on a fluorescence spectrometer. PAS is measured as ternary complexes with terbium and tri-*n*-ocylphosphine oxide in weakly acidic aqueous solutions. Sensitising of fluorescence of lanthanide ions, especially europium and terbium ions, by organic donors have been studied extensively in recent years, with the aim of developing spectrofluorometric methods for quantifying organic analytes. A limit of detection of 0.02 µmol/L, and a calibration range of 0–40 µmol/L was obtained for PAS in the urine samples. The main disadvantage of this method is interference from certain endogenous and extraneous compounds through severely overlapping spectra [55].

A capillary zone electrophoresis method has been developed for the determination of PAS and its metabolite AcPAS in urine [56]. A linear relationship is observed between time normalized peak area and the concentration of the analyte and metabolite with correlation coefficients greater than 0.9990. The method could be applied to measure PAS and its metabolite in urine without any sample pre-treatment. Good separation of the analytes can be achieved in a run of 12 minutes with an added 3 minutes for the capillary wash. The range and concentration of the standard concentration curves presented was 6.6–800 µg/ml for PAS and 70.0–4000 µg/ml for AcPAS [56].

An ion pair reagent reversed-phase high performance liquid chromatography method has been described to determine anti-tuberculosis drugs and their metabolites in biological fluids [57]. The method is based on the ion interaction reagents technique and uses a mobile phase

of 5 mM octylammonium o-phoshate with HPLC and a UV/VIS detector. The reported limit of detection for PAS was 68 µg/L. The sample preparation made use of ultra-filtration and the assay ran in 25 minutes [57].

Another HPLC method presented describes the analysis of PAS and its metabolites in plasma and in cerebrospinal fluid (CSF) and brain tissue samples, using fluorescence detection [48]. The technique involves a one-step protein precipitation, with the sample fractionated on a reverse phase C18 column with a gradient mobile phase. The optimised method used gradient chromatography with 17.5 mM potassium phosphate buffer and methanol over a 25 minutes run time. The calibration range reported was 0.05–500 µg/ml in plasma, and 0.017–166.7 µg/g in a CSF and brain tissue samples. Studies on the freeze-thaw stability over 3 cycles, as well as long term stability over 2 weeks revealed no significant decrease in concentrations of both PAS and AcPAS [48].

A UPLC-MS/MS method has been described for the simultaneous analysis of nine second-line anti-TB drugs to be used as a therapeutic drug monitoring (TDM) assay [58]. The method makes use of a 3M hydrochloric acid (HCl) acidified methanol extraction, neutralized by sodium hydroxide (NaOH). The samples were separated on a HSS T3 (Waters, 50.0 x 2.1mm, 5µ) column using gradient chromatography with 10 mM Ammonium formate in 0.1% formic acid in water and 0.1% formic acid in acetonitrile. The run time was 3 minutes. The single ion reaction monitoring (SRM) transition used for PAS was *m/z* of 154.1 →119.0, with a calibration range of 5–100 µg/ml [58].

Liwa *et al.,* described a HPLC-MS/MS method using an Agilent Zorbax column (150 x 2.1mm, 5µm) with a gradient mobile phase of water and methanol both containing 0.1% formic acid [59]. The PAS concentrations were determined on a AB Sciex API 2000 tandem mass spectrometer equipped with an atmospheric turbo ion spray source. SRM transitions were used, with a *m/z* of 237.1→119.9 and 154.2→136.2 for PAS and ISTD (thiacetazone), respectively [59].

Pastorini *et al.*, described a method for the determination of 5-aminosalicylic acid and its major metabolite N-acetyl-5-aminosalycylic acid in human plasma [60]. PAS was used as the ISTD. The method uses protein precipitation with methanol as extraction solvent. The analytes were separated on a C18 column with a mobile phase consisting of 17.5 mmol/L acetic acid (pH 3.3) and acetonitrile (85:15, v/v) at a flow rate of 0.2 ml/min. Negative ionisation was used with a transition of *m/z* of 152 →108 for PAS [60].

The analytical information is summarised and presented in Table 2.

Table 2. PAS literature survey summary

Reference	Detection	Concentration range	Sample volume (µl)	Column	Mobile phase	Flow rate (ml/min)	Run time (min)	Matrix and extraction
Lianidou *et al.*, 1996	Spectrofluorometric	0 – 40 µmol/L	50	Not mentioned	Not mentioned	Not mentioned	Not mentioned	Urine, deproteinised with acetonitrile
Cummins *et al.,* 1997	Capillary electrophoresis	70 – 800 µg/ml	Not mentioned	Polymicro (44 cm, 50µm)	Buffer – mixture of 75mM mono and dibasic sodium phosphate.	Not mentioned	15	Urine, diluted.
Gennaro *et al.*, 2001	UV/VIS	82 µg/L - 2.0 mg/L	Not mentioned	Merck Lichrocart (250 x 4.6 mm)	50 mM octylammonium / o-phosphate, pH8	1.5	15	Plasma, ultra-filtration.
Hong *et al*., 2011	FLD	0.05-500 µg/ml plasma, 0.017 – 166.7 µg/g CSF and brain tissue	200	Econosphere (250 x 4.6 mm)	17.5 mM potassium phosphate buffer and methanol (gradient)	1.0	25	Plasma, CSF and brain tissue (protein precipitation)
Han *et al.*, 2013	MS/MS pos (*m/z* 154.1 →119.0)	5-100 µg/ml	50	Waters HSS T3 (50 x 2.1 mm)	Ammonium formate and acetonitrile with 0.1%formic acid (Gradient)	0.2	3.0	Serum, acidified methanol protein precipitation.
Liwa *et al.,* 2013	MS/MS pos *(m/z* 154.2 →136.2)	Not mentioned	Not mentioned	Agilent Zorbax (150 x 2.1 mm)	Methanol and 0.1% formic acid (gradient)	Not mentioned	8.0	Plasma, methanol protein precipitation.
Pastroni *et al.,* 2008	MS/MS neg (*m/z* 152 →108)	Used as internal standard	Not mentioned	Synergi Hydro-RP (150 x 2.0 mm)	17.5 mmol/L acetic acid and acetonitrile (gradient)	Not mentioned	25	Plasma, methanol protein precipitation

4.3. Method development

The literature and clinical protocol review revealed that a LC-MS/MS method was needed to cover a possible Cmax of up to 60 µg/ml and a LLOQ of at least 5 half-lives, therefore a calibration range of 0.391–100 µg/ml was chosen. LC-MS/MS was used, because it is more specific and sensitive than conventional HPLC using UV or fluorescence detection. Most conventional HPLC methods use mobile phases that contain high concentrations of non-volatile buffer salts that are incompatible with LC-MS/MS methods. Based on the literature survey, most of the mobile phases used for LC-MS/MS analysis contained low concentrations formic acid, acetic acid or ammonium acetate and either methanol or acetonitrile as the organic solvent. A stock solution of PAS was prepared in methanol. From the prepared stock solution, three different diluted solutions were prepared for infusion, one

in methanol : water (50:50, v/v), the second in methanol : water : formic acid (50:49.9:0.1, v/v/v) and a third in methanol : water : ammonium hydroxide (50:49.9:0.1, v/v/v). Each were then infused separately into the mass spectrometer and the Q1 mass filter optimised for the best protonated ionisation response for the precursor ion with a *m/z* of 154.0. The best ionisation response for the precursor ion was achieved with a mixture of methanol : water : formic acid (50:49.9: 0.1, v/v/v). The protonated precursor ion was fragmented and the product ions optimised for a specific and sensitive MRM transition, the *m/z* of 154.1→80.2 was monitored. The product ion mass spectrum of PAS is presented in Figure 11.

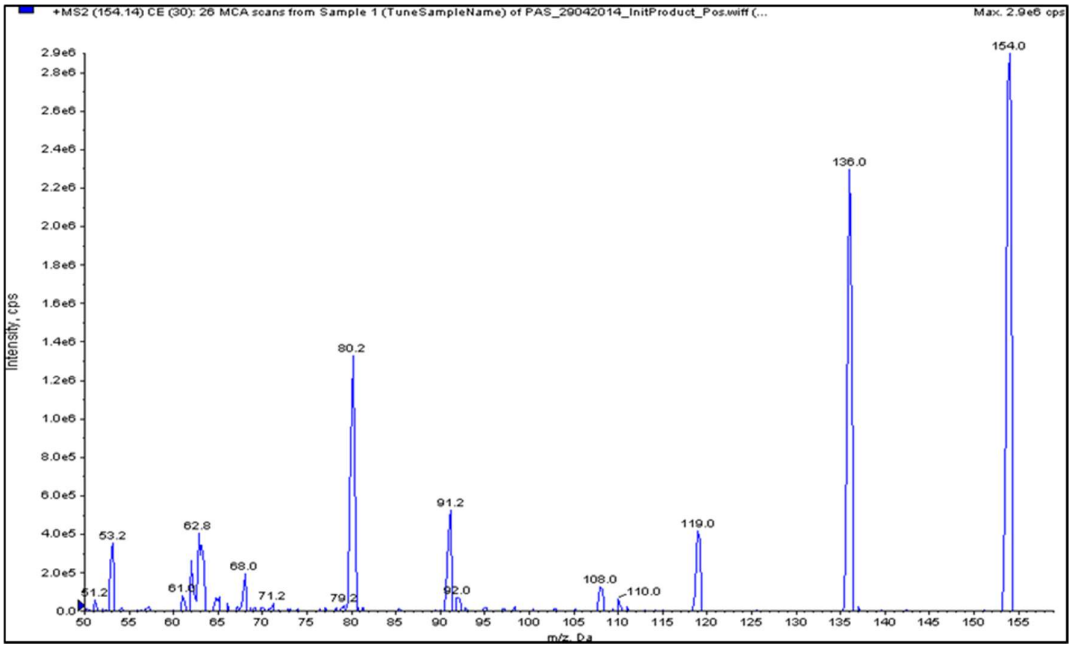

Figure 11. Product ion mass spectrum of *para*-aminosalicylic acid (PAS) after collision induced dissociation in the fragmentation cell, showing the PAS precursor ion at m/z 154 as well as the product ions

Chromatography development was started with a Phenomenex C18 (150 x 2.0 mm, 5µm) column, because this was the most commonly used column found in the literature. An isocratic mobile phase of 0.1% formic acid in water and methanol (50:50, v/v) was used as the starting point, because of its good ionisation properties. Different ratios of this mobile phase were tested, but even with the best ratio between formic acid and methanol for good retention, good peak shapes could not be obtained. Possible solutions considered were to use a gradient mobile phase or to use different types of stationary phases with different selectivity. A range of columns was tested and the best chromatography, as well as good retention was obtained with a Phenomenex Synergi Hydro-RP (150 x 2.0 mm, 4µm)

column. Different concentrations of formic acid were tested in the mobile phase and the best sensitivity was obtained with a mobile phase containing 0.2% formic acid. In pure solutions, good linearity was obtained over the concentration range of 0.391–100 µg/ml. The next step was to develop an extraction method from human plasma. Literature suggested protein precipitation as extraction method, and because methanol was used in the mobile phase, this was tested as the first protein precipitation reagent. This extraction method worked well over the concentration range, but an ISTD was needed to follow the analyte during extraction and the ionisation process. Normally, an isotopically labelled standard or analogue of the analyte are used as ISTDs, but were not available. Instead, different analytes with similar retention times and ionisation properties were evaluated as ISTDs for this assay. This method's intended use was for clinical sample analysis, therefore TB drugs or anti-retrovirals were excluded. Different drugs were tested and rilmenidine was found to be the best ISTD. The product ion mass spectrum of rilmenidine is presented in Figure 12.

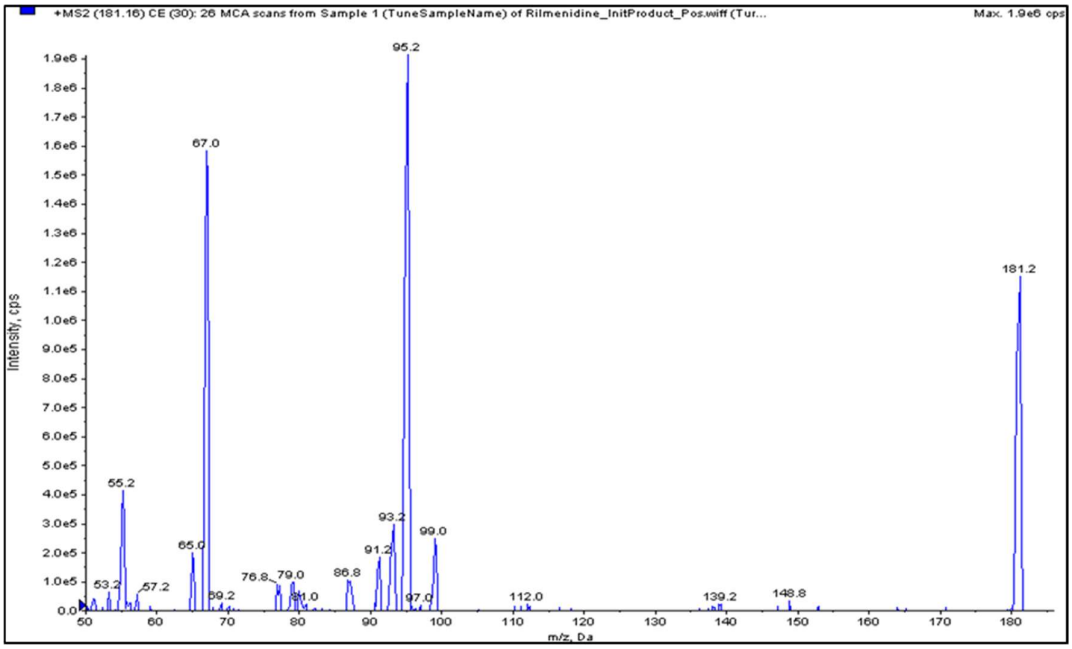

Figure 12. Product ion mass spectrum of the rilmenidine, ISTD, after collision induced dissociation in the fragmentation cell, showing the rilmenidine precursor ions at m/z 181 as well as the product ions

The extraction method was optimised to include the ISTD and the final extraction method, HPLC conditions and MS/MS settings included in a SOP.

4.3.1. The final SOP extraction procedure

An analytical batch is defined as follows: A single extraction procedure applied to a series of unknown samples, which must include a system performance verification (SYS) sample (of sufficient volume to inject at least six times) a minimum of eight calibration standards analysed in duplicate, three levels of quality controls analysed in duplicate, a blank and a double blank sample. Calibration standards are spread throughout the run in order to capture instrument drift. Quality control samples are also spread over the run on order to control the analysis appropriately. Double blanks and blanks are run after the highest calibration standard, consecutively to ascertain the level of carryover for the batch.

Extraction procedure:

Thaw plasma samples, including calibration standards, quality control samples, blanks and unknown samples, are thawed at room temperature and vortexed briefly.

Aliquot 20 µl plasma into 1.5 ml polypropylene micro-centrifuge tubes.

Add 200 µl precipitation reagent containing the ISTD at 500 ng/ml to the samples (not more than 4 samples at a time).

Vortex for 30 seconds and remember to extract the double blank with an aliquot of ISTD-free acetonitrile.

Centrifuge the samples at 16 000 rcf for 5 minutes.

Transfer 150 µl of the supernatant into a 96-well plate and add 50 µl 0.2% formic acid in water.

Inject 5 µl into LC-MS/MS instrument for PAS analysis.

For dilutions: Only a 1:4 (sample : plasma, v:v) has been validated. Dilutions are performed singly and the following procedure is followed:

Pipette 20 µl of sample into a labelled polypropylene tube.

Add 80 µl of blank plasma to this sample and vortex to mix.

Pipette 50 µl of this mix into a clean labelled polypropylene microcentrifuge tube and extract as per normal.

4.3.2. Final chromatography and mass spectrometry conditions

The final HPLC and chromatographic conditions and MS/MS detector settings are presented in Tables 3-5.

Table 3. Instrumentation and Chromatographic conditions for *para*-aminosalicylic acid (PAS)

Instrument used	API 3000-1
Project (Analyst)	PAS_Study 2013_146
Acquisition method	PAS_1 Method
Analytical Column	Phenomenex Synergi Hydro-RP (2.0 x 150 mm, 4µm)
Column Temperature	Room temperature (~24°C)
Mobile Phase	Methanol : 0.2% Formic acid (40:60, v/v)
Pump Type	Agilent 1200 Series Binary Pump
Flow Rate	300 µl/min
Autosampler Type	HTC PAL
Sample arrangement	96 well plate
Injection Volume	5 µl
Autosampler Temperature	10 °C

Table 4. *Para*-aminosalicylic acid (PAS) Electrospray Ionisation Settings

Mass Spectrometer Identity	**API3000-1**
APCI/ESI	**ESI**
Parameter	**Value**
Nebuliser gas (Gas 1) (arbitrary unit)	6
Turbo gas (Gas 2) (arbitrary unit)	8
CUR (curtain gas) (arbitrary unit)	6
CAD (collision gas) (arbitrary unit)	4
TEM (Source Temperature) (°C)	500
IS (Ion Spray Voltage) (V)	5000

Table 5. *Para*-aminosalicylic acid (PAS) and Rilmenidine (ISTD) MS/MS Settings

	PAS (4-Aminosalicylic Acid)	**Rilmenidine**
Protonated molecular ion mass (m/z) $[M+H]^+$	154.1	181.2
Product ion mass (m/z) Quantifier	80.2	95.2
Product ion mass (m/z) Qualifier	136.2	67.0
Dwell time (ms)	150	150
Declustering potential (V)	30	31
Focus potential (V)	200	150
Entrance potential (V)	10	10
Collision energy (eV)	37	23
Collision cell exit potential (V)	4	6
Scan Type	MRM	MRM
Polarity	Positive	Positive
Pause Time (ms)	5	5

Pre-validation extractions of standards and controls in plasma showed good accuracy, precision and recoveries for PAS and the method was validated according to the FDA and EMA guidelines [30], [29].

4.4. Validation of the Assay Method

4.4.1. Procedure

Validation of an assay method is a process performed to objectively demonstrate and document the accuracy, precision, specificity, sensitivity and reproducibility of the assay method and the stability of the analyte for the purposes of assaying samples of unknown concentrations.

To demonstrate acceptable within- and between-day accuracy and precision of the method over the desired concentration range, calibration standards and quality control samples were prepared and assayed in three consecutive runs. A full set of calibration standards and quality controls was prepared and stored frozen, and the required aliquots were thawed and

assayed in each run. Each run consisted of all the calibration standards in duplicate to produce one calibration curve and six replicates of the prepared quality control samples. A quality control spiked to a concentration above the upper limit of quantification was diluted (1:1 and 1:4) with blank plasma to validate the dilution of samples for which the concentrations potentially do not fall within the validated range.

Standard curve fitting was determined by applying the simplest model that adequately describes the concentration-response relationship using appropriate weighting and statistical tests for goodness-of -fit. A calibration curve based on a well selected regression model must consist of at least six calibration levels covering the entire calibration range from the lower limit to the upper limit of quantification. The regression model selected during the validation is used for the quantification of the study samples. In the case of a re-instatement (or partial) validation, the regression model that was used for the full validation must be used for the re-instatement validation. Table 6 summarises the regression and quantitation parameters used for PAS.

Table 6. *Para*-aminosalicylic acid (PAS) quantitation parameters, ions monitored and retention times

Software	Analyst 1.5.2
Algorithm	Analyst Classic
Calibration Fit Type	Quadratic
Parameter	Area
Curve Weighting	1/x
	PAS (4-Aminosalicylic Acid)
Bunching factor	3
Number of smoothes	3
Precursor ion	154.1
Product ion	80.2
Retention Time (min)	2.4
	Rilmenidine
Bunching factor	2
Number of smoothes	3
Precursor ion	181.2

Product ion	95.2
Retention Time (min)	1.6

4.4.2. Calibration standard and quality control preparation

4.4.2.1. Preparation of Stock Solutions of PAS and Rilmenidine

Stock solutions (SS1, SS2) of PAS and (ISS1, ISS2) of rilmenidine were prepared by weighing a mass of the analyte or ISTD into a container and dissolving it in the desired volume of solvent. The weighed mass of the analyte is also adjusted where applicable (purity, salt, etc.). Stock solutions SS1 and SS2 (5000 µg/ml) and ISS1 and ISS2 (1000 µg/ml) were prepared in methanol. All stock solutions were kept at ~ -80°C until required. These stock solutions were prepared to spike blank biological matrix as required as presented in Tables 7–10.

Table 7. Preparation of *Para*-aminosalicylic acid (PAS) stock solution (SS1)

Solvent used	Volume Solvent (ml)	Weighed mass of analyte (mg)	Adjusted* mass of analyte (mg)	SS1 concentration (µg/ml)
Methanol	6.921	35.31	34.60	5000

** Reason for Adjustment (e.g. purity, salt, hydrate): Purity*
** Calculation: 35.31 x 0.98 = 34.60*

Table 8. Preparation of *Para*-aminosalicylic acid (PAS) stock solution (SS2)

Solvent used	Volume solvent (ml)	Weighed mass of analyte (mg)	Adjusted* mass of analyte (mg)	SS2 concentration (µg/ml)
Methanol	2.256	11.51	11.28	5000

** Reason for Adjustment (e.g. purity, salt, hydrate): Purity*
** Calculation: 11.51 x 0.98 = 11.28*

Table 9. Preparation of Rilmenidine stock solutions (ISS1)

Solvent used	Volume solvent (ml)	Weighed mass of analyte (mg)	Adjusted* mass of analyte (mg)	ISS1 concentration (µg/ml)
Methanol	2.133	3.28	2.13	1000.00

** Reason for Adjustment (e.g. purity, salt, hydrate): Salt*
** Calculation: (3.28 x 180.25)/277.24 = 2.13*

Table 10. Preparation of Rilmenidine stock solutions (ISS2)

Solvent used	Volume solvent (ml)	Weighed mass of analyte (mg)	Adjusted* mass of analyte (mg)	ISS2 concentration (µg/ml)
Methanol	0.860	1.32	0.86	1000.00

** Reason for Adjustment (e.g. purity, salt, hydrate): Salt*
** Calculation: (1.32 x 180.25)/277.24 = 0.86*

4.4.2.2. Preparation of Calibration Standards

Calibration standards were prepared volumetrically in plasma (anticoagulant K_3EDTA) at room temperature by spiking 100 µl of the analyte stock solution (SS1) into 4.90 ml of normal blank plasma (STD 1), which was then serially diluted with normal blank plasma to attain the desired concentrations of calibration standards (STD 2 - STD 9) as presented in Table 11. Multiple 60 µl aliquots of each calibration standard are stored in individual 1.5 ml polypropylene tubes at approximately -80 °C to allow for duplicate 20 µl extractions from each tube. Stability has been shown at -80°C for up to 2 weeks [48].

Table 11. Preparation of calibration standards

Standard	Blank Plasma volume (ml)	Volume SS1 spiked (µl)	Dilution Source	Dilution Source Volume (ml)	Total Volume of dilution (ml)	PAS Conc. (µg/ml)
STD 1 - ULOQ	4.90	100			5.00	100
STD 2	2.50		*STD 1*	*2.50*	5.00	50.0
STD 3	2.50		*STD 2*	*2.50*	5.00	25.0
STD 4	2.50		*STD 3*	*2.50*	5.00	12.5
STD 5	2.50		*STD 4*	*2.50*	5.00	6.25
STD 6	2.50		*STD 5*	*2.50*	5.00	3.13
STD 7	2.50		*STD 6*	*2.50*	5.00	1.56
STD 8	2.50		*STD 7*	*2.50*	5.00	0.781
STD 9 -LLOQ	2.50		*STD 8*	*2.50*	5.00	0.391

4.4.2.3. Preparation of Quality Control Samples

Quality controls were prepared volumetrically in plasma (anticoagulant K_3EDTA) at room temperature using the same methodology that was used for the calibration standards; spike 160 µl of the analyte stock solution (SS2) into 4.84 ml of normal blank plasma to obtain QC Dilute. This quality control is serially diluted with normal blank plasma to attain the desired concentrations (QC 1–6) as presented in Table 12. Multiple 60 µl aliquots of each quality control were stored in individual 1.5 ml polypropylene tubes at approximately -80 °C to

allow for duplicate 20 µl extractions from each tube. Stability has been previously been demonstrated at –80°C for up to 2 weeks [48].

Table 12. Preparation of quality control samples

Standard	Blank Plasma volume (ml)	Volume SS2 spiked (µl)	Dilution Source	Dilution Source Volume (ml)	Total Volume of dilution (ml)	PAS (µg/ml)
QC Dilute	4.840	160			5.000	160
QC 1	4.000		*QC Dilute*	*4.00*	8.00	80.0
QC 2	4.000		*QC 1*	*4.00*	8.00	40.0
QC 3	4.000		*QC 2*	*2.00*	6.00	13.3
QC 4	4.000		*QC 3*	*1.50*	5.50	3.64
QC 5	5.845		*QC 4*	*1.60*	7.45	0.781
QC 6	2.000		*QC 5*	*2.00*	4.00	0.391

Note: The QC Dilute is used during a validation batch to quality control the dilution process.*

4.4.2.4. Preparation of ISTD Working Solution

A stock solution (ISS1, 1000 µg/ml) of rilmenidine was prepared in methanol and multiple 500 µl aliquots of ISS1 were stored in individual 1.5 ml polypropylene tubes and stored at approximately -80 °C. ISS1 was used to prepare the precipitation reagent (IWS1) in methanol when needed as represented in Table 13.

A volume of 200 µl of this ISTD working solution (IWS1) was added to each sample (excluding double blank samples), as described in the assay procedure.

Table 13. Working solution – Internal Standard (Rilmenidine)

	Source Solution	Solvent Used	Solvent Volume (ml)	Volume Spiked (µl)	Conc. (ng/ml)	Date Prepared	Storage Temp. (°C)
IWS1	*ISS1*	Methanol	20	5	250	20 May 2014	-20°C
IWS1	*ISS1*	Methanol	20	5	250	26 May 2014	-20°C

4.4.3. Validation Results

Acceptance criteria: Accuracy is expressed as the concentration of analyte found as a percentage of the nominal concentration (% Accuracy) while precision is expressed as the coefficient of variation (% CV) seen in a batch of assays.

The calculated calibration curve should fit the plot of measured responses *vs*. nominal concentrations of the calibration standards adequately, giving a r^2 fit parameter of as close to one as possible. For a valid method, the within- and between-batch accuracy is required to be within 15 % (i.e. % Accuracy should be between 85–115%) over the entire calibration

range and within 20% of nominal concentration at the LLOQ. For a valid method, the within- and between-batch precision is required to be less than 15 % (i.e. % CV should be less than 15 %) over the entire calibration range and less than 20 % at the LLOQ. Duplicate standards are analysed at each calibration point. Each one of these standards is used to define the calibration equation, unless one of those points does not meet the above criteria. In this instance, the invalid point would be excluded and only a single standard would be used at that level. This allows for a single standard to fail at either the LLOQ or the ULOQ without effecting the resulting calibration range.

4.4.3.1. Validation 1 (Day 1)

Within-batch accuracy and precision are assessed by assaying all the calibration standards in duplicate, to produce one calibration curve, and 6 replicates of each quality control level in a single batch of assays. The regression equation used was Quadratic (weighted by 1/x concentration), $f(x) = a + bx + cx^2$, as presented in Figure 13 and Table 14.

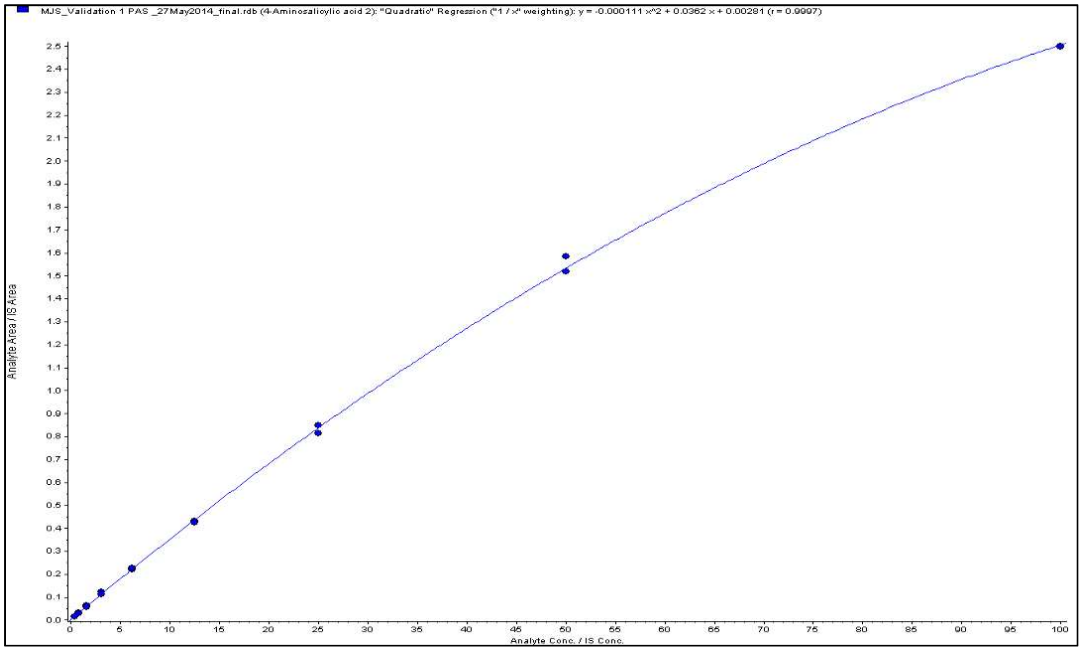

Figure 13. Representative calibration curve for PAS - validation 1, day 1.

Table 14. Regression equation (weighted by 1/x concentration)

Validation Batch	Quadratic Calibration Curve Parameters			
	a	B	C	r
1	0.00281	0.0362	-1.11E-04	0.9997

The within-batch accuracy and precision of the assay procedure are assessed by calculating the regression equation and constructing the calibration curve based on peak area ratios of analyte to ISTD, as presented in Table 15 and Table 16.

Table 15. *Para*-aminosalicylic acid (PAS) Calibration Standards Accuracy and Precision - validation 1

Sample ID	Nominal Conc. (µg/ml)	Mean Observed Conc. (µg/ml)	Std Dev	% CV	% Accuracy	*N*
S9	0.391	0.381	0.00728	1.9	97.3	2 of 2
S8	0.781	0.772	0.0458	5.9	98.9	2 of 2
S7	1.56	1.61	0.176	10.9	103.1	2 of 2
S6	3.13	3.22	0.159	4.9	102.9	2 of 2
S5	6.25	6.25	0.126	2.0	99.9	2 of 2
S4	12.5	12.2	0.199	1.6	97.7	2 of 2
S3	25.0	24.8	0.747	3.0	99.1	2 of 2
S2	50.0	50.8	1.85	3.6	101.5	2 of 2
S1	100	100	0.0298	0.03	99.5	2 of 2

Table 16. Summary of *Para*-aminosalicylic acid (PAS) intra-validation quality control samples

Sample ID	Nominal Conc. (µg/ml)	Mean Observed Conc. (µg/ml)	Std Dev	% CV	% Accuracy	*N*
QC 6	0.391	0.434	0.0491	11.3	111.1	6 of 6
QC 5	0.781	0.844	0.0456	5.4	108.1	*5 of 6
QC 2	40.0	37.2	1.66	4.5	92.9	6 of 6
QC 1	80.0	78.1	2.35	3.0	97.7	6 of 6

** = Outlier with MNR-ESD Outlier Test*

4.4.3.2.Validation 2 and 3 (Days 2 and 3)

Between-batch accuracy and precision are assessed by assaying an additional two separate consecutive batches, each consisting of a double set of calibration standards designated for use in the assay of samples of unknown concentrations and 6 replicates of each of the quality control samples as presented in figures 14 – 15.

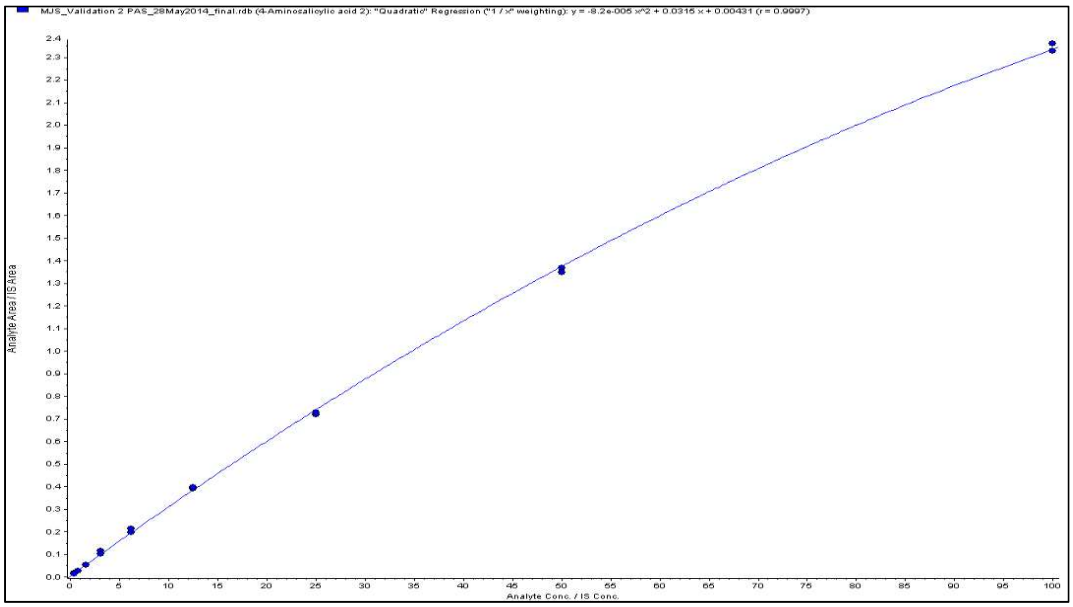

Figure 14. Representative calibration curve for *Para*-aminosalicylic acid (PAS) - validation 2**,** day 2.

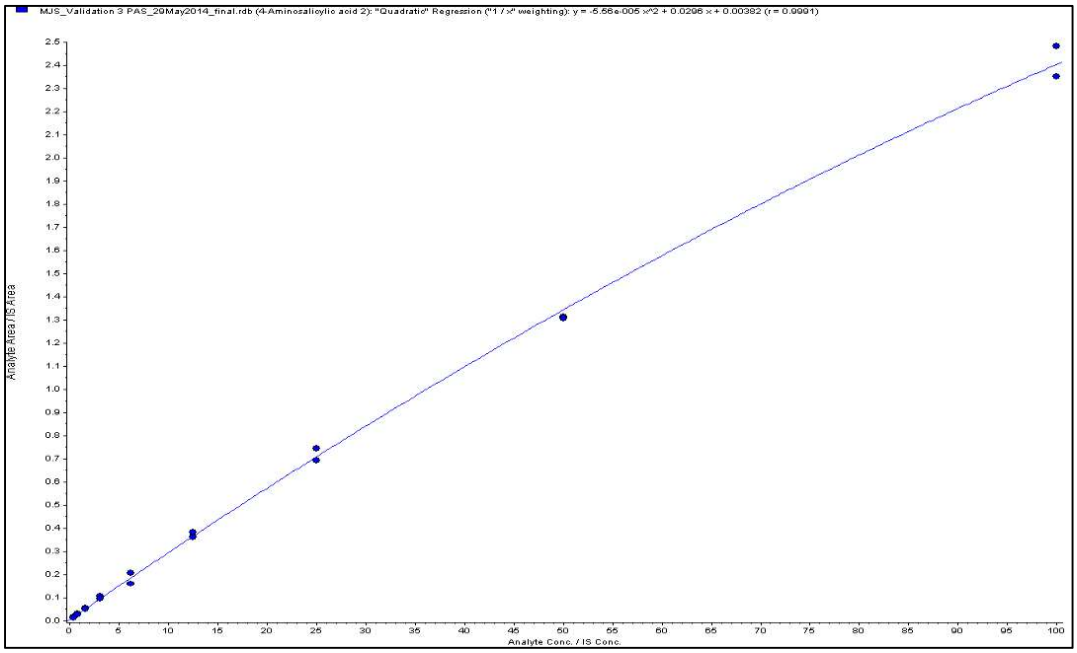

Figure 15. Representative calibration curve for *Para*-aminosalicylic acid (PAS) - validation 3, day 3.

The regression equation was again applied (weighted by 1/x concentration), $f(x) = a + bx + cx^2$, as presented in Tables 17 and 18.

Table 17. Regression equation (weighted by 1/x concentration)

Validation Batch	Quadratic Calibration Curve Parameters			
	a	B	C	r
2	0.00431	0.0315	-8.2E-05	0.9997

Table 18. Regression equation (weighted by 1/x concentration)

Validation Batch	Quadratic Calibration Curve Parameters			
	a	B	C	r
3	0.00382	0.0296	-5.56E-05	0.9991

The between-batch accuracy and precision of the assay procedure are assessed by constructing a calibration curve based on analyte/ISTD peak area ratios and calculating the regression equations as represented in Tables 19, 20, 21 and 22.

Table 19. Summary of *Para*-aminosalicylic acid (PAS) calibration standards accuracy and precision - validation 2

Sample ID	Nominal Conc. (µg/ml)	Mean Observed Conc. (µg/ml)	Std Dev	% CV	% Accuracy	*N*
S9	0.391	0.366	0.0260	7.1	93.7	2 of 2
S8	0.781	0.739	0.0157	2.1	94.6	2 of 2
S7	1.56	1.60	0.00587	0.4	102.3	2 of 2
S6	3.13	3.32	0.298	9.0	106.1	2 of 2
S5	6.25	6.49	0.356	5.5	103.9	2 of 2
S4	12.5	12.8	0.192	1.5	102.5	2 of 2
S3	25.0	24.4	0.259	1.1	97.7	2 of 2
S2	50.0	49.3	0.531	1.1	98.6	2 of 2
S1	100	101	1.42	1.4	100.7	2 of 2

Table 20. Summary of *Para*-aminosalicylic acid (PAS) intra-validation quality control samples - validation 2

Sample ID	Nominal Conc. (µg/ml)	Mean Observed Conc. (µg/ml)	Std Dev	% CV	% Accuracy	*N*
QC 6	0.391	0.350	0.0192	5.5	89.5	6 of 6
QC 5	0.781	0.841	0.0176	2.1	107.7	6 of 6
QC 2	40.0	35.645	1.28	3.6	89.1	6 of 6
QC 1	80.0	74.087	3.06	4.1	92.6	6 of 6

Table 21. *Para*-aminosalicylic acid (PAS) calibration standards accuracy and precision - validation 3

Sample ID	Nominal Conc. (µg/ml)	Mean Observed Conc. (µg/ml)	Std Dev	% CV	% Accuracy	*N*
S9	0.391	0.345	0.0191	5.5	88.2	2 of 2
S8	0.781	0.805	0.0730	9.1	103.1	2 of 2
S7	1.56	1.64	0.0549	3.4	104.8	2 of 2
S6	3.13	3.26	0.222	6.8	104.1	2 of 2
S5	6.25	6.15	1.18	19.2	98.3	2 of 2
S4	12.5	12.7	0.493	3.9	101.9	2 of 2
S3	25.0	25.4	1.34	5.3	101.6	2 of 2
S2	50.0	48.7	0.164	0.3	97.3	2 of 2
S1	100	101	4.99	5.0	100.7	2 of 2

Table 22. Summary of *Para*-aminosalicylic acid (PAS) intra-validation quality control samples - validation 3

Sample ID	Nominal Conc. (µg/ml)	Mean Observed Conc. (µg/ml)	Std Dev	% CV	% Accuracy	*N*
QC 6	0.391	0.370	0.0401	10.8	94.6	6 of 6
QC 5	0.78	0.855	0.0125	1.5	109.5	6 of 6
QC 2	40.00	39.1	0.850	2.2	97.9	6 of 6
QC 1	80.00	77.0	2.74	3.6	96.3	6 of 6

4.4.3.3. Summary of the combined calibration standard and quality control results

The overall accuracy and precision of the assay procedure is assessed by calculating the accuracy and precision statistics over the within- and between-batch validation batches (3 in total). Accuracy is expressed as the concentration of the analyte found as a percentage of the nominal concentration (% Accuracy), while precision is expressed as the coefficient of variation (CV %). The combined regression, calibration standards and quality control results (all 3 validations) of PAS are summarised in tables 23, 24 and 25.

Table 23. Overall Summary of calibration curve parameters - validation 1–3

Validation Batch	Quadratic Calibration Curve Parameters			
	a	B	C	r
1	0.00281	0.0362	-1.11E-04	0.9997
2	0.00431	0.0315	-8.2E-05	0.9997
3	0.00382	0.0296	-5.56E-05	0.9991

Table 24. Overall summary of calibration standard accuracy and precision - validations 1–3

Sample ID	Nominal Conc. (µg/ml)	Mean Observed Conc. (µg/ml)	Std Dev	% CV	% Accuracy	*N*
S9	0.391	0.364	0.0219	6.0	93.1	6 of 6
S8	0.781	0.772	0.0492	6.4	98.9	6 of 6
S7	1.56	1.61	0.0843	5.2	103.4	6 of 6
S6	3.13	3.27	0.186	5.7	104.3	6 of 6
S5	6.25	6.29	0.58	9.2	100.7	6 of 6
S4	12.5	12.6	0.386	3.1	100.7	6 of 6
S3	25.0	24.9	0.82	3.3	99.5	6 of 6
S2	50.0	49.6	1.296	2.6	99.2	6 of 6
S1	100	100	2.40	2.4	100.3	6 of 6

Table 25. Overall Quality Control Accuracy and Precision Estimation

Sample ID	Nominal Conc. (µg/ml)	Mean Observed Conc. (µg/ml)	Std Dev	% CV	% Accuracy	*N*
QC 6	0.391	0.385	0.0516	13.4	98.4	18 of 18
QC 5	0.781	0.847	0.0266	3.1	108.5	*17 of 18
QC 2	40.0	37.3	1.918	5.1	93.3	18 of 18
QC 1	80.0	76.4	3.11	4.1	95.5	18 of 18
QC DIL 1:1	160	162	11.6	7.1	101.2	6 of 6
QC DIL 1:4	160	156	8.7	5.6	97.3	6 of 6

* = *Outlier with MNR-ESD Outlier Test*

4.4.3.4.Calibration Range

Results from the validation assays above indicate a valid calibration range of 0.391–100 µg/ml for PAS. The lower limit of quantification (LLOQ) was set at the concentration of the lowest validated standard for PAS, namely 0.391 µg/ml.

4.4.3.5.Quantification Method

The results show that the method provides sufficient accuracy and precision over the entire range based on analyte/ISTD peak area ratios with a quadratic calibration curve (weighted by 1/x concentration).

4.4.4. Stability assessment

Various stability experiments were performed to ensure that the analyte concentrations were not affected by the assay procedure or associated conditions. The assay of study samples could be completed within the period for which the analyte is shown to be stable.

4.4.4.1.Stock solution stability and accuracy

Stock solutions SS1-4-aminosalicylic acid (prepared on the 14 May 2014) and ISS1-rilmenidine (prepared on the 20 May 2014) were prepared in methanol and kept at -80°C. Test and control samples of these stock solution was left at room temperature, ~ 4°C and ~ -20°C for 24 hours, respectively. Reference stock solutions, SS2-4-aminosalicylic acid (prepared on the 16 May 2014) and ISS2-rilmenidine (prepared on the 30 May 2014), were prepared after the storage period of the test and control samples. The reference, test and control samples were diluted with injection solvent to a concentration of 100 µg/ml and 0.250 µg/ml, for the analyte and ISTD, respectively. These were analysed as per the method procedure. Peak areas of the test and control sample assays compared to the reference assays are presented in Tables 26 and 27, for the analyte and ISTD, respectively. Peak areas of the initial stock solution (kept at -80°C) and reference analyte stock solutions was compared for stability at -80°C and accuracy and are presented in Table 28.

Table 26. Stock solution stability of *Para*-aminosalicylic acid (PAS)

	Reference	Control (~- 20 ºC)	Control (~ 4 ºC)	Test (Room temp)
Peak area 1	5540000	5960000	5920000	5680000
Peak area 2	5530000	5410000	6340000	5420000
Peak area 3	5500000	5720000	5460000	5520000
Peak area 4	5610000	5760000	5450000	5420000
Peak area 5	5420000	5370000	5390000	5450000
Peak area 6	5280000	5180000	5110000	5400000
Average	5480000	5566667	5611667	5481667
STDEV	115758	292689	441697	105909
% CV	2.1	5.3	7.9	1.9
% Difference		1.6	2.4	0.03

Table 27. Stock solution stability of rilmenidine

	Reference	Control (~- 20 °C)	Control (~ 4 °C)	Test (Room temp)
Peak area 1	4290000	4090000	4090000	3990000
Peak area 2	4130000	4070000	3910000	4040000
Peak area 3	3940000	3990000	3910000	4090000
Peak area 4	4130000	4060000	3880000	3920000
Peak area 5	4070000	3920000	3810000	3780000
Peak area 6	3700000	3580000	3490000	3700000
Average	4043333	3951667	3848333	3920000
STDEV	202550	192605	198436	152447
% CV	5.0	4.9	5.2	3.9
% Difference		-2.3	-4.8	-3.1

Table 28. Stock solution accuracy of *Para*-aminosalicylic acid (PAS)

	Reference (SS1)	Test (SS2)
Peak area 1	3850000	3820000
Peak area 2	3440000	3570000
Peak area 3	3820000	3770000
Peak area 4	3530000	3670000
Peak area 5	3870000	3690000
Peak area 6	3410000	3600000
Average	3653333	3686667
STDEV	216025	96056
% CV	5.9	2.6
% Difference		0.9

Acceptance Criteria: A high % CV (higher than 15%) of the measured values and a difference of more than 15 % from the reference solution could indicate instability in the stock/working solution. For accuracy, the % difference between two stock solutions should not be more than 5%.

Conclusion: From the peak areas tabulated above it can be concluded that PAS stock solutions prepared in methanol are stable for 24 hours stored at room temperature, ~ 4ºC and ~ -20°C. The initial stock solution kept at ~ – 80°C compared to the reference showed to be stable for 2 days and the % difference of 0.9 showed good accuracy.

4.4.4.2.Storage stability in matrix

Samples for the long-term matrix stability assessment of PAS in human plasma have been prepared in-house on 21 May 2014 and was kept at ~ -80°C until 12 Mar 2016 when it was extracted and analysed. The assessment should cover, at a minimum, the length of time from when the first sample was drawn during the clinical phase to the date of final sample analysis during the analytical phase of a study. Long term matrix stability is presented in Table 29.

Table 29. Long term matrix stability of *Para*-aminosalicylic acid (PAS) in plasma

	High Concentration		Low Concentration	
	Nominal conc. (µg/ml)	Observed conc. (µg/ml)	Nominal conc. (µg/ml)	Observed conc. (µg/ml)
Sample 1	80.0	66.3	0.781	0.738
Sample 2		72.9		0.828
Sample 3		79.1		0.766
Sample 4		74.0		0.778
Sample 5		71.8		0.792
Sample 6		69.7		0.795
	Average	72.3	**Average**	0.783
	STDEV	4.3	**STDEV**	0.0303
	% CV	5.9	**% CV**	3.9
	% Difference	-9.6	**% Difference**	0.2

Acceptance criteria: A high % CV and a high % Difference (higher than 15%) of the measured values compared to nominal concentrations could indicate matrix instability.

Discussion: The % CV and % Difference for PAS are reported to be within 15% and therefore the long term matrix stability for PAS in plasma is indicated for at least 21 months.

4.4.4.3. "Fresh" vs. "Frozen" stability

In order to qualify the "fresh" vs. "frozen" effect, a fresh set of calibration standards were prepared in plasma and prior to aliquoting and freezing, tested against stored QC's. The fresh curve is used to quantify the QC's. The calibration standards and quality control samples accuracy and precision are presented in Tables 30 and 31, respectively.

Table 30. Calibration standards accuracy and precision – "fresh" vs. "frozen" stability of *Para*-aminosalicylic acid (PAS)

Sample ID	Nominal Conc. (µg/ml)	Mean Observed Conc. (µg/ml)	Std Dev	% CV	% Accuracy	*N*
S9	0.391	0.354	0.00340	1.0	90.6	2 of 2
S8	0.781	0.812	0.0182	2.2	104.0	2 of 2
S7	1.56	1.50	0.0256	1.7	96.1	2 of 2
S6	3.13	3.29	0.0517	1.6	105.1	2 of 2
S5	6.25	6.55	0.292	4.5	104.8	2 of 2
S4	12.5	12.7	0.746	5.9	101.4	2 of 2
S3	25.0	24.9	0.957	3.8	99.5	2 of 2
S2	50.0	49.0	0.0729	0.1	97.9	2 of 2
S1	100	101	2.45	2.4	100.7	2 of 2

Table 31. Quality control samples accuracy and precision – "Fresh" vs. "Frozen" stability of *Para*-aminosalicylic acid (PAS)

Sample ID	Nominal Conc. (µg/ml)	Mean Observed Conc. (µg/ml)	Std Dev	% CV	% Accuracy	n
QC 6	0.391	0.419	0.0232	5.5	107.1	6 of 6
QC 5	0.78	0.854	0.0503	5.9	109.3	6 of 6
QC 2	40.00	37.2	1.03	2.8	93.0	6 of 6
QC 1	80.00	73.8	5.10	6.9	92.3	6 of 6

Acceptance criteria: The normal acceptance criteria for quality controls apply. The accuracy is required to be within 15% (i.e. % Accuracy should be between 85–115%) and the precision is required to be < 15% (i.e.CV % should be < 15%).

Discussion: The % CV and % Accuracy for the analyte are reported to be within 15%, which indicates that sample freezing does not influence the accuracy and precision of the assay. It also indicates that the analyte is stable for at least 8 days when stored at ~ -80 °C.

4.4.4.4. Freeze-thaw stability

The freeze and thaw stability evaluation should mimic the intended sample handling conditions to be used during sample analysis. To ascertain freeze-thaw stability, low and high quality controls were frozen at ~ - 80ºC, and put through three consecutive freeze-thaw cycles. Sample aliquots were prepared and frozen for at least 24 hours prior to starting this experiment. Each cycle consisted of sufficient thawing time at room temperature followed

by 12–24 hours freezing time. These samples were analysed against a valid calibration curve and compared to controls from the batch, analysed at the same concentration. The measured concentrations and calculated differences after three cycles for the two sets of quality control samples are presented in Table 32.

Table 32. Freeze and thaw stability of *Para*-aminosalicylic acid (PAS)

	High Concentration		Low Concentration	
	Observed Q1 Mean (µg/ml)	**Observed F/T High (µg/ml)**	**Observed Q5 Mean (µg/ml)**	**Observed F/T Low (µg/ml)**
Sample 1	74.1	74.4	0.841	0.772
Sample 2		73.5		0.790
Sample 3		71.5		0.846
Sample 4		73.1		0.832
Sample 5		79.7		0.797
Sample 6		81.5		0.885
	Average	75.6	**Average**	0.820
	STDEV	4.01	**STDEV**	0.0419
	% CV	5.3	**% CV**	5.1
	% Difference	2.0	**% Difference**	-2.5

Acceptance criteria: A high % CV and high % Difference (higher than 15 %) of the measured values could indicate freeze-thaw instability.

Conclusion: The % CV and % Difference for the analyte are reported to be within 15% which indicates that the analyte is stable in plasma for at least three freeze-thaw cycles.

4.4.4.5. Benchtop stability

To ascertain benchtop stability, low and high quality control samples were frozen at ~ -80ºC, and subsequently left on the bench for approximately 21 hours (maximum anticipated time that future study samples will be left thawed until extracted). Samples were removed on 27 May 2014 at 12H00 and extracted on 28 May 2014 at 09H00. These samples were analysed against a valid calibration curve. The measured concentrations and calculated accuracies for the two sets of quality control samples are presented in Table 33.

Table 33. On bench stability of *Para*-aminosalicylic acid (PAS)

	High Concentration		Low Concentration	
	Observed Q1 Mean (µg/ml)	**Observed BT High (µg/ml)**	**Observed Q5 Mean (µg/ml)**	**Observed BT Low (µg/ml)**
Sample 1	**74.1**	76.0	**0.841**	0.843
Sample 2		78.2		0.840
Sample 3		74.2		0.814
Sample 4		81.3		0.760
Sample 5		76.7		0.912
Sample 6		84.3		0.918
	Average	78.9	**Average**	0.849
	STDEV	3.95	**STDEV**	0.0670
	% CV	5.0	**% CV**	7.9
	% Difference	6.5	**% Difference**	0.9

Acceptance criteria: A high % CV and a high % Difference (higher than 15%) of the measured values could indicate on-bench instability.

Conclusion: The % CV and % Difference for PAS are reported to be within 15%. On-bench stability for PAS in plasma is indicated for at least 21 hours.

4.4.4.6. Reinjection Reproducibility

Reinjection reproducibility should be evaluated to determine if an analytical run can be reanalysed by reinjection in the case of instrument interruptions. Following the injection of the first validation run (Validation 1), the extracted samples (96-well plate) remain in the autosampler at the method defined temperature. The extracts were reinjected following the injection of the second validation run (Validation 2) and third validation run (Validation 3). This provides evidence of reinjection reproducibility as presented in Tables 34–37.

Table 34. Calibration standards accuracy and precision – validation 1 reinjection 1

Sample ID	Nominal Conc. (µg/ml)	Mean Observed Conc. (µg/ml)	Std Dev	% CV	% Accuracy	*N*
S9	0.391	0.384	0.0234	6.1	98.3	2 of 2
S8	0.781	0.719	0.00821	1.1	92.1	2 of 2
S7	1.56	1.63	0.0830	5.1	104.3	2 of 2
S6	3.13	3.22	0.182	5.6	103.0	2 of 2
S5	6.25	6.35	0.129	2.0	101.7	2 of 2
S4	12.5	12.8	0.386	3.0	102.3	2 of 2
S3	25.0	25.0	0.167	0.7	99.9	2 of 2
S2	50.0	48.9	1.46	3.0	97.8	2 of 2
S1	100	101	1.33	1.3	100.8	2 of 2

Table 35. Quality control samples accuracy and precision – validation 1, reinjection 1

Sample ID	Nominal Conc. (µg/ml)	Mean Observed Conc. (µg/ml)	Std Dev	% CV	% Accuracy	*N*
QC 6	0.391	0.396	0.0699	17.7	101.2	6 of 6
QC 5	0.78	0.800	0.0566	7.1	102.4	6 of 6
QC 2	40.00	38.9	1.38	3.5	97.3	6 of 6
QC 1	80.00	80.4	4.02	5.0	100.5	6 of 6

Table 36. Calibration standards accuracy and precision – validation 1, reinjection 2

Sample ID	Nominal Conc. (µg/ml)	Mean Observed Conc. (µg/ml)	Std Dev	% CV	% Accuracy	*N*
S9	0.391	0.377	0.0397	10.5	96.5	2 of 2
S8	0.781	0.752	0.01380	1.8	96.2	2 of 2
S7	1.56	1.60	0.0611	3.8	102.4	2 of 2
S6	3.13	3.19	0.067	2.1	101.9	2 of 2
S5	6.25	6.41	0.162	2.5	102.6	2 of 2
S4	12.5	12.8	0.323	2.5	102.5	2 of 2
S3	25.0	24.6	0.440	1.8	98.3	2 of 2
S2	50.0	49.6	0.30	0.6	99.1	2 of 2
S1	100	100	1.38	1.4	100.4	2 of 2

Table 37. Quality control samples accuracy and precision – validation 1, reinjection 2

Sample ID	Nominal Conc. (µg/ml)	Mean Observed Conc. (µg/ml)	Std Dev	% CV	% Accuracy	*N*
QC 6	0.391	0.414	0.0381	9.2	105.8	6 of 6
QC 5	0.78	0.831	0.0499	6.0	106.4	6 of 6
QC 2	40.00	38.2	1.23	3.2	95.6	6 of 6
QC 1	80.00	77.4	4.30	5.6	96.7	6 of 6

Acceptance criteria: The normal acceptance criteria for quality controls apply. The accuracy is required to be within 15% (i.e. % Accuracy should be between 85–115%) and the precision is required to be < 15% (i.e. CV % should be < 15%).

Conclusion: The % CV and % Accuracy for the analyte is reported to be within 15% which indicates that a batch may be reinjected within 58 hours.

4.4.4.7. Autosampler stability

The high and low control standards that were extracted during the first validation batch (Validation 1), were reinjected after Validation 2 in Reinjection-Validation 1 and again after Validation 3 as Reinjection 2-Validation 1, having been kept in the autosampler throughout this period. The analytes/ISTD peak area ratios of the injections in Reinjection 1-Validation 1 and Re-injection 2-Validation 1 were compared to the analytes/ISTD peak area ratios of the samples injected in Validation 1 to demonstrate autosampler stability. The data is presented in Tables 38 and 39.

Table 38. Autosampler stability for extracted samples - high concentration

High Concentration (80.0 µg/ml)			
First Injection: 2014/05/27 16:11 PM			
Validation 1, Batch Start	Peak area	ISTD peak area	Ratio
QC 1	9580000	4360000	2.2
QC 1	9760000	4450000	2.2
QC 1	9500000	4390000	2.2
QC 1	9020000	4280000	2.1
QC 1	8990000	4290000	2.1
QC 1	9020000	4240000	2.1
Average	9311667	4335000	2.1
STDEV	341199	78677	0.04
% CV	3.7	1.8	2.0
Time lapse from first sample injection: (2014/05/27 19:30 PM) ~3 hours			

Validation 1 Re-injection	Peak area	ISTD peak area	Ratio
QC 1	7970000	3480000	2.3
QC 1	8330000	3530000	2.4
QC 1	8080000	3520000	2.3
QC 1	7740000	3530000	2.2
QC 1	7830000	3550000	2.2
QC 1	7690000	3590000	2.1
Average	7940000	3533333.333	2.2
STDEV	239666	36148	0.081
% CV	3.0	1.0	3.6
		% Difference after Validation 1 Re-injection	4.7
Time lapse from first sample injection: (2014/05/29 05:03 AM) ~ 32 hours			

Validation 3	Peak area	ISTD peak area	Ratio
QC 1	5990000	2740000	2.2
QC 1	6060000	2770000	2.2
QC 1	6130000	2760000	2.2
QC 1	5630000	2740000	2.1
QC 1	5620000	2740000	2.1
QC 1	5680000	2860000	2.0
Average	5851667	2768333	2.1
STDEV	233360	46655	0.10
% CV	4.0	1.7	4.5
		% Difference after Validation 1 Re-injection 2	-1.5
Time lapse from first sample injection: (2014/05/30 04:14 AM) ~ 55 hours			

Table 39. Autosampler stability for extracted samples - low concentration

Low Concentration (0.781 µg/ml)			
First Injection: 2014/05/27 16:23 PM			
Validation 1, Batch Start	Peak area	ISTD peak area	Ratio
QC 5	150000	4580000	0.0328
QC 5	146000	4570000	0.0319
QC 5	153000	4410000	0.0347
QC 5	132000	4200000	0.0314
QC 5	149000	4230000	0.0352
QC 5	127000	4310000	0.0295
Average	142833	4383333	0.0326
STDEV	10685	165368	0.0021
% CV	7.5	3.8	6.6
Time lapse from first sample injection: (2014/05/27 19:42 PM) ~3 hours			

Validation 1 Re-injection	Peak area	ISTD peak area	Ratio
QC 5	114000	3550000	0.0321
QC 5	127000	3700000	0.0343
QC 5	134000	3760000	0.0356
QC 5	106000	3490000	0.0304
QC 5	117000	3440000	0.0340
QC 5	111000	3550000	0.0313
Average	118167	3581667	0.0330
STDEV	10458	123518	0.0020
% CV	8.9	3.4	6.1
% Difference after Validation 1 Re-injection			1.1
Time lapse from first sample injection: (2014/05/29 05:16 AM) ~ 32 hours			

Validation 3	Peak area	ISTD peak area	Ratio
QC 5	80900	2840000	0.0285
QC 5	83700	2850000	0.0294
QC 5	85300	2720000	0.0314
QC 5	75800	2500000	0.0303
QC 5	76200	2730000	0.0279
QC 5	88600	2770000	0.0320
Average	81750	2735000	0.0299
STDEV	5104	127240	0.00161
% CV	6.2	4.7	5.4
% Difference after Validation 1 Re-injection 2			-8.2
Time lapse from first sample injection: (2014/05/30 04:27 AM) ~ 55 hours			

Acceptance criteria: A high % CV and a high % Difference (higher than 15%) of the measured values could indicate autosampler instability.

Conclusion: The % CV and % Difference for the analyte concentrations are reported to be within 15 %. On-instrument stability for the analyte was indicated for at least 55 hours.

4.4.5. Specificity

The very high specificity of the LC-MS/MS assay procedure precludes the detection of any compounds that do not possess the capability to produce the specific parent ion followed by formation of the specific product ion produced and monitored in the mass spectrometer. A representative chromatogram of STD 1 is presented in Figure 16.

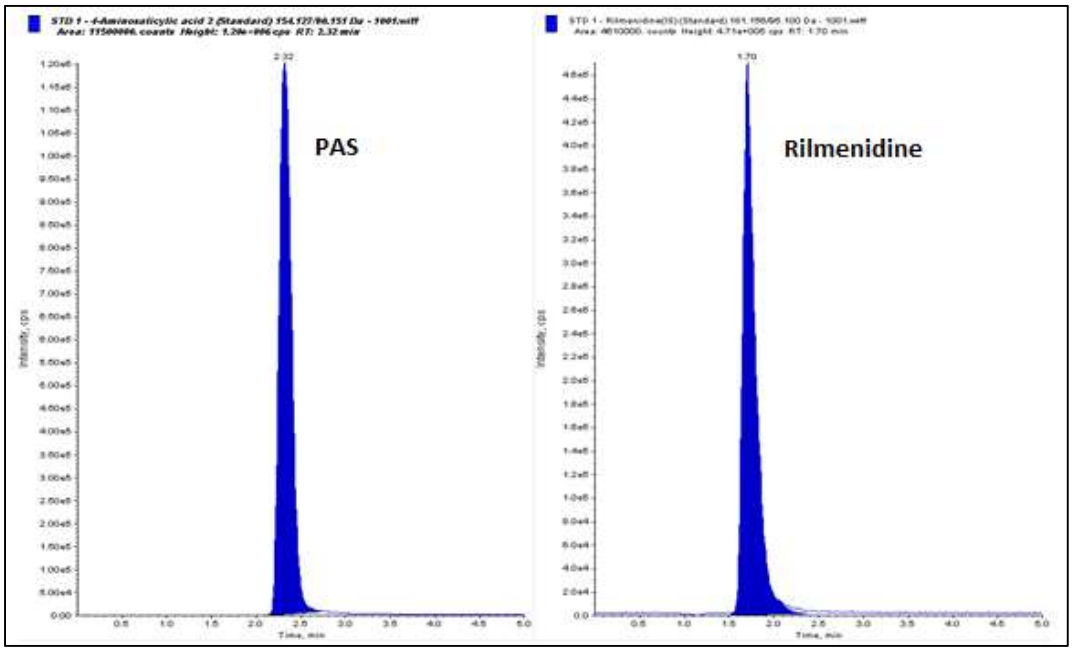

Figure 16. Representative chromatogram of STD 1

4.4.6. Carry-over

A double blank sample (without analyte and ISTD) was positioned in the injection sequence immediately after the highest calibration standard to assess possible carry-over effects. A chromatogram of a double blank sample is presented in Figure 17.

A blank sample (without analyte) was also included to determine the possible contamination of the analyte by the ISTD without an additional carry-over effect. A chromatogram of a blank sample is presented in Figure 18.

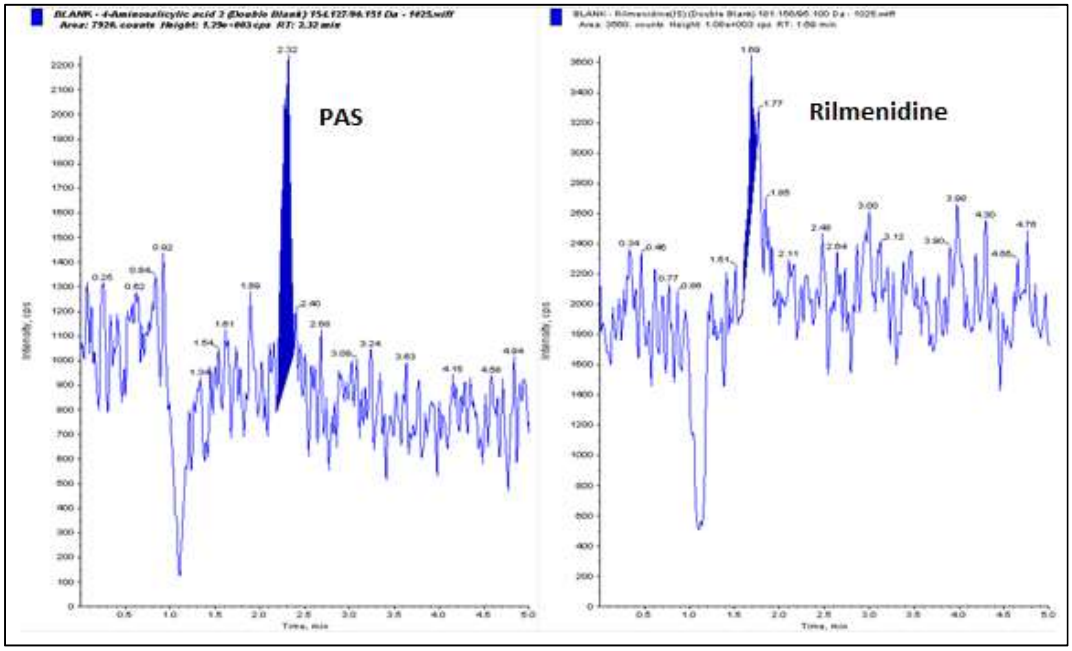

Figure 17. Chromatogram of a double blank plasma sample

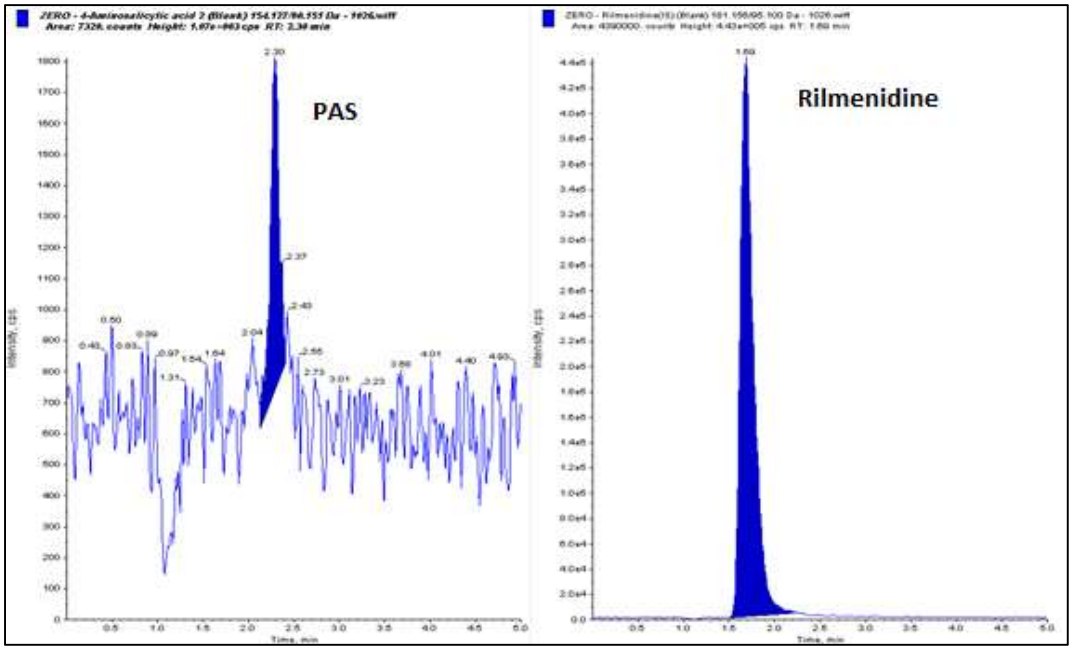

Figure 18. Chromatogram of a blank plasma sample

Acceptance criteria:

Double blank assessing carry-over: A peak that is observed for the analyte should not be > 20% of the area of the peak obtained at the LLOQ. A peak that is observed for the ISTD should not be > 5% of the peak observed for the ISTD at the working concentration.

Blank assessing contribution: A peak that is observed for the analyte when ISTD is present at the working concentration should not be > 20% of the area of the peak obtained at the LLOQ.

Conclusion: Small carry over peaks were observed but the peak areas was less than 20% of the LLOQ peak areas of the analyte and less than 5% of the ISTD peak areas.

4.4.7. Sensitivity

The LLOQ of this method is 0.391 µg /ml, representative chromatograms are presented in Figure 19 and the signal-to-noise chromatogram in Figure 20.

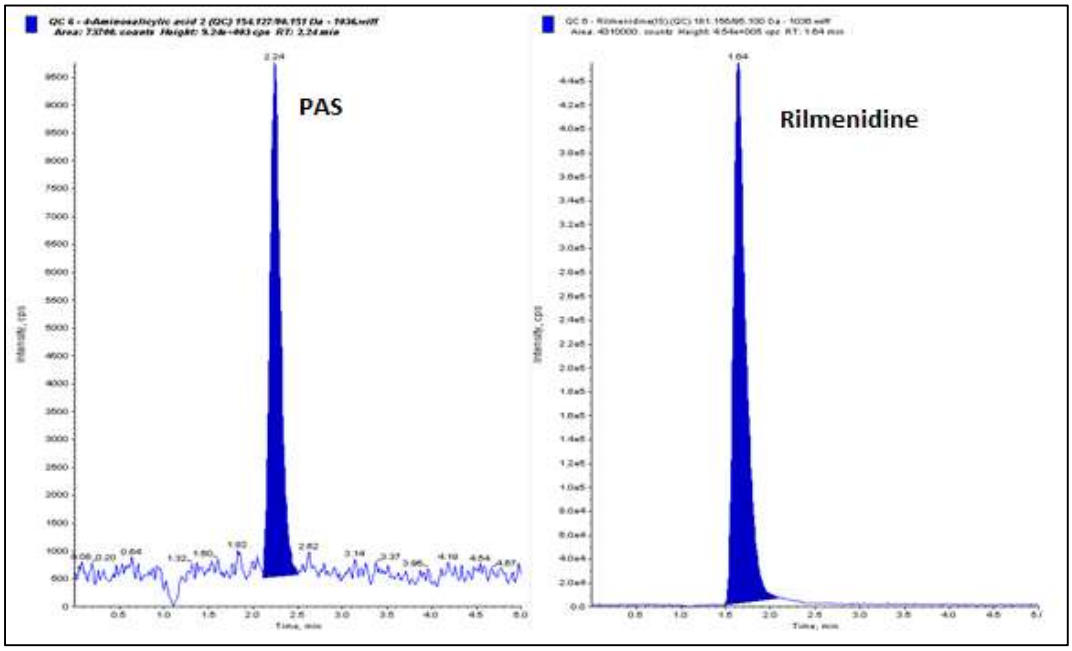

Figure 19. Chromatogram of a lower limit of quantification (LLOQ) sample

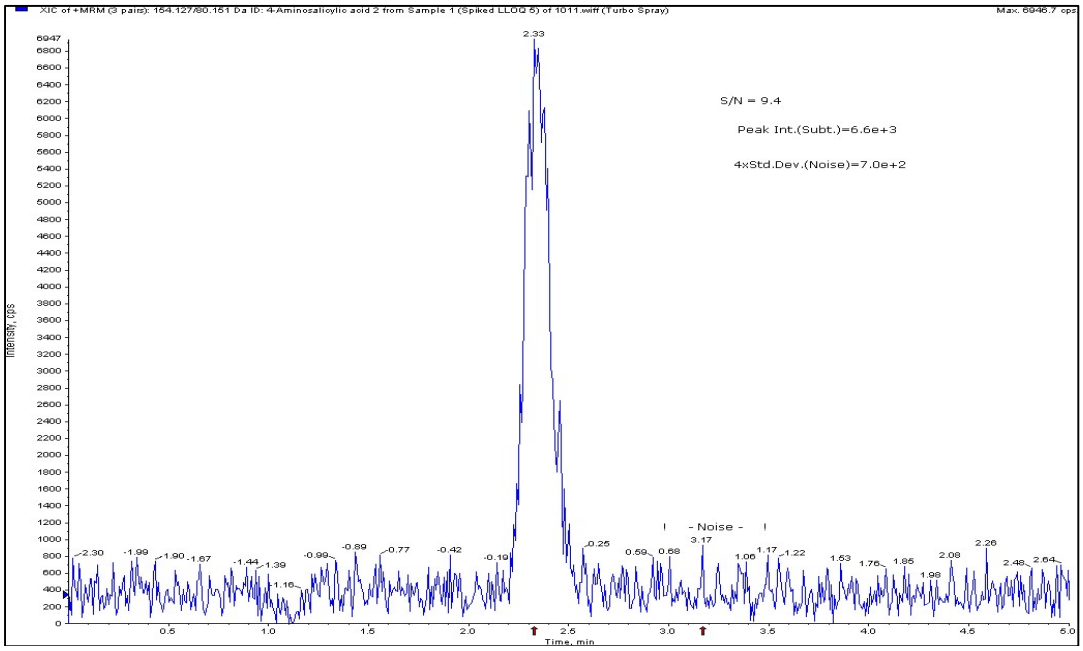

Figure 20. Chromatogram of the S/N ratio of the lower limit of quantification (LLOQ) sample

Acceptance criteria: The mean analyte signal/noise response at LLOQ should be at least > 5 times the response compared to blank response at the retention time of interest.

Conclusion: The raw LLOQ sample chromatograms showed acceptable intensities for the analyte with an average signal/noise ratio of 10:1 for PAS (N=6).

4.4.8. Recovery

The extraction recovery pertains to the extraction efficiency of the analytical process within the limits of variability. It is determined by comparing the analytical response of blank matrix spiked with the analyte and extracted with the response of the blank matrix first extracted and then spiked with analyte (theoretical, represents 100% recovery). No recovery of the ISTD is calculated.

a. Extracted (test) samples: a minimum of six QCs at each concentration level (low, medium and high) are extracted as per the analytical method.

b. Theoretical samples: samples are spiked at each concentration level (relative to the final concentration of the corresponding extracted QC's level) in six-fold using extracted blank matrix.

The analyte peak areas found after extraction compared to the theoretical peak area expressed as a percentage recovery, the data is presented in Table 40.

Table 40. Recovery for *Para*-aminosalicylic acid (PAS)

	High Concentration (100 µg/ml)		Medium Concentration (50 µg/ml)		Low Concentration (0.391 µg/ml)	
	Precipitation: Peak Area	Solution: Corrected Peak Area	Precipitation: Peak Area	Solution: Corrected Peak Area	Precipitation : Peak Area	Solution: Corrected Peak Area
Sample 1	18600000	19500000	10900000	11200000	118000	121000
Sample 2	18000000	18600000	11300000	10600000	119000	123000
Sample 3	17600000	18300000	10900000	10800000	[266000]*	117000
Sample 4	17100000	17600000	10400000	10400000	113000	102000
Sample 5	16800000	16800000	10100000	10300000	120000	109000
Sample 6	16600000	17600000	9750000	9300000	[258000]*	112000
Average	17450000	18066667	10558333	10433333	117500	114000
STDEV	763544	941630	578288	640833	3109	7899
% CV	4.4	5.2	5.5	6.1	2.6	6.9
% Recovery		96.6		101.2		103.1
				Average % Recovery		100.3
				Average % CV		3.3

* = *Outlier with MNR-ESD Outlier Test*

Acceptance Criteria: The mean recovery of a quantitative drug assay should be consistent and the precision of the measured recovery, expressed as percentage coefficient of variation, should not exceed 15% for any particular concentration of the analyte at which it is determined. Recovery reproducibility between concentration levels should not be > 15%.

Conclusion: The mean recovery of 4-aminosalicylic acid (PAS) from plasma over the calibration range is 100.3% with a %CV of 3.3.

4.4.9. Matrix effect

In biological chemical analysis, matrix refers to the components of a sample other than the analyte. Matrix effects are of particular importance when dealing with LC-MS analyses, and may only become evident once novel clinical samples are analysed. The presence of co-extracted matrix background components may impact analyte and ISTD ionization. Appropriate steps should be taken to minimize the influence of matrix components. A matrix effect method was used that quantifies the effect across the calibration range of the assay [61].

A minimum of eight different blank sources of the appropriate biological matrix and anticoagulant type was extracted. Each individual matrix was spiked at low, medium and high concentration levels (taking into account any calculations for dilutions in the analytical method) and at one concentration of the ISTD. The results of the overall %CV's of the slopes calculated, are presented in Table 41.

Table 41. *Para*-aminosalicylic acid (PAS) / internal standard (ISTD) peak areas ratios

	High Conc. 100 µg/ml Peak Area Ratio	Medium Conc. 50.0 µg/ml Peak Area Ratio	Low Conc. 0.391 µg/ml Peak Area Ratio	Area Ratio v Conc. Regression Slope
K3EDTA 14/10/2011-01	4.0	2.2	0.0202	0.0399
K3EDTA 14/10/2011-29	4.0	2.0	0.0218	0.0401
K3EDTA 08/11/2013-05	3.9	2.0	0.0213	0.0390
K3EDTA 07/12/2011-09	4.0	2.1	0.0208	0.0396
K3EDTA 07/04/2014-50E	3.6	2.2	0.0226	0.0362
K3EDTA 07/04/2014-46E	4.1	2.0	0.0223	0.0408
K2EDTA 07/12/2011-10	3.9	2.1	0.0231	0.0390
LiHep 923 SEP 2009	3.9	1.9	0.0222	0.0394
Average	3.9	2.1	0.0218	0.0393
STDEV	0.134	0.104	0.000978	0.00136
% CV	3.4	5.0	4.5	3.5

Acceptance criteria: The peak area ratios of the analyte/ISTD for each level in each matrix source are used to generate regressions for each individual matrix. The slope variability (%CV) for the 8 different matrix sources should not be > 5%.

Conclusion: The slope variability (%CV) for 8 different plasma samples was 3.5 for PAS, which indicates that matrix effects do not adversely influence the precision of the assay.

4.4.10. Effect of Haemolysis

The presence of haemolysed blood in samples could affect the ionization of the analyte and ISTD during analysis. Evidence should be provided that haemolysis has no effect on analyte quantification. If ionisation suppression is observed for the ISTD, and it is comparable to the extent of ionisation suppression observed for the analyte, the ISTD is regarded as providing sufficient compensation for the analyte. Haemolysis was tested at 2% haemolysed blood in plasma.

The influence of haemolysed blood was assessed by the assay of 6 haemolysed samples at high and low PAS concentrations and compared to 6 normal human plasma samples prepared at the same high and low concentrations.

The response ratios observed for PAS / ISTD in the haemolysed plasma samples were compared with the response ratios observed in the normal plasma samples. The ability of the ISTD to provide sufficient compensation for PAS determination were assessed by comparing normal and haemolysed plasma response ratios at high and low concentrations as presented in Table 42.

Table 42. Effect of 2% haemolysis on *Para*-aminosalicylic acid (PAS)

	High Concentration (80 µg/ml)		Low Concentration (0.7805 µg/ml)	
	Observed Normal Peak Area Ratio	Observed Haemolysed Peak Area Ratio	Observed Normal Peak Area Ratio	Observed Haemolysed Peak Area Ratio
Sample 1	1.85	1.52	0.0224	0.0199
Sample 2	1.54	1.54	0.0221	0.0172
Sample 3	1.51	1.63	0.0200	0.0187
Sample 4	1.68	1.37	0.0202	0.0206
Sample 5	1.63	1.43	0.0230	0.0168
Sample 6	1.52	1.60	0.0192	0.0196
Average	1.62	1.52	0.0211	0.0188
STDEV	0.130	0.101	0.00153	0.00152
% CV	8.0	6.7	7.3	8.1
% Difference		-6.6		-11.1

Acceptance criteria: A high % Difference (higher than 15%) between the peak area ratios observed in haemolysed samples and normal samples and a high % CV (higher than 15%) indicates that haemolysis has an effect on the assay of the analyte and that the ISTD does not sufficiently compensate for the analyte.

Conclusion: The results reported above show that the % Difference are within 15 % for PAS in 2% haemolysed plasma samples and has no significant effect on analyte quantification.

4.4.11. Dilutions

To determine if samples originally reported as above the upper limit of the standard curve (ALQ) may be diluted to within the calibration range with accuracy and precision, six extra

high QC samples were prepared at a concentration 2 times higher than the highest QC (160 µg/ml) for PAS. These were then diluted 1:1 and 1:4 with blank plasma. The concentration was determined and compared with the nominal concentration to determine the percentage accuracy as represented in Table 43.

Table 43. Sample Dilution

Sample ID	Nominal Conc. (µg/ml)	Mean Observed Conc. (µg/ml)	Std Dev	% CV	% Accuracy	*N*
QC DIL 1:1	160	162	11.6	7.1	101.2	6 of 6
QC DIL 1:4	160	156	8.71	5.6	97.3	6 of 6

Acceptance criteria: The final mean calculated concentration (incorporating the dilution factor) is determined from the calibration curve and compared to the nominal concentration. The accuracy of the diluted samples is required to be within 15% (i.e. % Accuracy should be between 85–115%) and the precision is required to be less than 15% (i.e. CV% should be less than 15%).

Conclusion: The resulted precision and accuracy fall within the accepted values.

4.5. Discussion

4.5.1. Validation data summary

Accuracy and precision were assessed over three consecutive, independent runs and the calibration curve fits a quadratic (weighted by 1/x concentration) regression over the range 0.391–100 µg/ml for PAS.

A 1:1 and 1:4 dilution of the QC Dilution sample showed that concentrations of up to 160 µg/ml of PAS in plasma could be analysed reliably when diluted into the calibration range. The method was specific for PAS, small carry over peaks were observed, but the peak areas were less than 20% of the LLOQ peak areas.

Endogenous matrix components were found to have an insignificant effect on the reproducibility of the method when human plasma originating from eight different sources were analysed. Also, quantification of PAS in plasma was not significantly affected by the

presence of haemolysed blood (2%) or when Lithium Heparin was used as anti-coagulant instead of K_3EDTA.

PAS was found to be stable in human plasma for 21 months kept at ~ -80°C, for up to 21 hours at room temperature and when subjected to 3 freeze-thaw cycles. Stock solutions of PAS are stable in methanol for 2 days when stored at ~ -80°C and for 24 hours stored at room temperature, ~ 4 ºC and ~ -20 ºC, respectively. PAS is shown to be stable on-instrument over a period of 55 hours. Reinjection reproducibility experiments indicate that a batch may be re-injected within 58 hours. In summary, the method is suited for the analysis of PAS in plasma.

4.6. Clinical application of the method.

This method was successfully used for the assay of samples in a clinical study.

4.6.1.1. Summary of study protocol

TITLE: PHARMACOKINETICS AND TOXICITY OF SECONDLINE ANTITUBERCULOSIS DRUGS IN HIV-INFECTED AND UNINFECTED CHILDREN
NIH R01: 069169-01
The project aims to improve the health of children with drug-resistant tuberculosis (DR-TB) in need of second-line anti-TB drugs, through examining the pharmacokinetics (PK), safety profile and toxicity of commonly used second-line anti-TB drugs in children, with and without HIV co-infection. The specific aims were:
1) to compare the PK of second-line anti-TB drugs in children (≤ 15 years) by age;
2) to compare the plasma concentrations of anti-retroviral (ARV) drugs in HIV-infected children (≤ 15 years) on second-line anti-TB drugs to those not on anti-TB therapy; and
3) to characterize the tolerability and toxicity of second-line anti-TB drugs in HIV-infected and uninfected children.

Childhood TB represents 15–20% of the disease burden in settings where TB and HIV infection is prevalent. MDR-TB; i.e. TB resistance to both rifampin and isoniazid, is an emerging epidemic, with an estimated 489 000 global cases annually [13]. MDR-TB patients often experience prolonged diagnostic delay, resulting in transmission of these

strains especially to young children, with a sharp increase in the numbers of children on treatment and prophylaxis for MDR-TB. This hospital-based study was implemented in Cape Town, Western Cape Province, South Africa at 2 referral hospitals – Brooklyn Hospital for Chest Diseases and Tygerberg Children's Hospital. The prevalence of MDR-TB amongst children with culture-confirmed TB was 8.6% and the prevalence of HIV infection in children with TB was 25-30% during 2009. The most frequently used second-line anti-TB drugs in children in this setting was ethionamide, fluoroquinolones, amikacin and terizidone. Capreomycin, linezolid and PAS were typically used for treatment of extensively drug-resistant TB. Although second-line anti-TB drugs are routinely given and recommended in children, there is limited information available to inform the accurate dosing in young HIV-infected children, who may have altered drug metabolism and unique drug-drug interactions. The literature provides limited data on toxicity of these anti-TB drugs in children, who are typically treated for 18–24 months for DR-TB.

This study provides a longitudinal, hospital-based, observational PK observation of children aged 0–15 years who received routine chemotherapy or chemoprophylaxis for the treatment or prevention of DR-TB. Three hundred and eighteen children were enrolled for observation consecutively over 3.5 years, undergoing intensive PK sampling of second-line anti-TB drugs at baseline. The subjects were undertaking routine treatment for DR disease until treatment completion for clinical outcomes, including TB treatment response and drug adverse effects.

Approximately 30% of the sample were co-infected with HIV. An equal number of HIV-infected children with and without anti-TB therapy (concurrent controls; 42 on efavirenz and 22 on lopinavir) were enrolled, based on the required age strata to allow for comparison of the effect of second-line TB drugs on ARV levels in HIV-infected children with and without TB treatment. HIV-infected children had ARV PK sampling done at baseline. Enrolment was balanced by HIV status and age to ensure adequate numbers of children 0–2 years of age and adequate numbers of HIV-infected children.

The TB medication regimen was based on local and international recommendations for routine care. The PAS local and international dose recommendations for children with TB is 150 mg/kg body weight up to a maximum of 1200 mg/day. This was therefore, not an interventional study. No new medications or new dosages were assessed.

4.6.1.2.Ethical approval of the study.

The study was formally approved by the Faculty of Health Science Research Ethics Committee, see Appendix 2 for approval letter.

4.6.1.3. Accuracy and Precision statistics of the study sample batches

The method described in section 4.3 was applied to this study protocol. The method was accurate and precise, showing a coefficient of variation and bias within 15% at all levels of quality controls analysed during 5 production batches of study samples, data presented in Table 44.

Table 44. Summary of quality control data from 5 sample batches

	High (80.0 µg/ml)	Medium (40.0 µg/ml)	Low (0.781 µg/ml)
Mean % Accuracy	95.0	102.2	102.0
STDEV	6.05	5.76	6.73
RSD (%)	6.4	5.6	6.6
n	10/10	*9/10	10/10

** = One outlier with MNR-ESD Outlier Test, excluded from table.*

The mean accuracy for the high, medium and low quality control concentrations were 95.0%, 102.2% and 102.0% with a coefficient of variation of 6.4%, 5.6% and 6.6%, respectively.

4.6.1.4. Suitability

The calibration range of 0.391–100 µg/ml was appropriate for the quantification of PAS samples generated in this study, only 4% of the total number of sample (182) was below the limit of quantification and no sample was higher than the upper limit of the calibration range, the highest concentration found in this study was 59.6 µg/ml.

4.6.1.5. Sensitivity

The method was sensitive and the lower limit of quantification (LLOQ) of this method of 0.391 µg/ml, enabled the determination of PAS concentrations over the total drug profile for at least 11 hours. With a half-live of 1 hour it was possible to follow the drug elimination for approximately 4 to 5 half-lives in the total 11 hour drug profile.

4.7. Drug levels measured in patient samples.

The minimum inhibitory concentration (MIC) of PAS for *Mycobacterium tuberculosis* is 1 µg/ml and two major considerations in the clinical pharmacology of PAS are: the prompt production of a toxic inactive metabolite under acid conditions and the short serum half-life of one hour for the free drug [47]. After 4–5 hours the plasma concentration of the drug is minimal, which justifies the need for doses of 10–12 g to maintain bacteriostatic activity. There was a high interpopulation variation for the Cmax of PAS in the study subjects, with the lowest at 5.14 µg/ml and the highest at 59.6 µg/ml and an average Cmax of 13.9 µg/ml. Even with this high variation a 1 µg/ml MIC was achieved in all the subjects. The Tmax also varied between 4 and 8 hours for most of the subjects as can be seen in the average concentration (standard deviation) versus time profile, as presented in Figure 21.

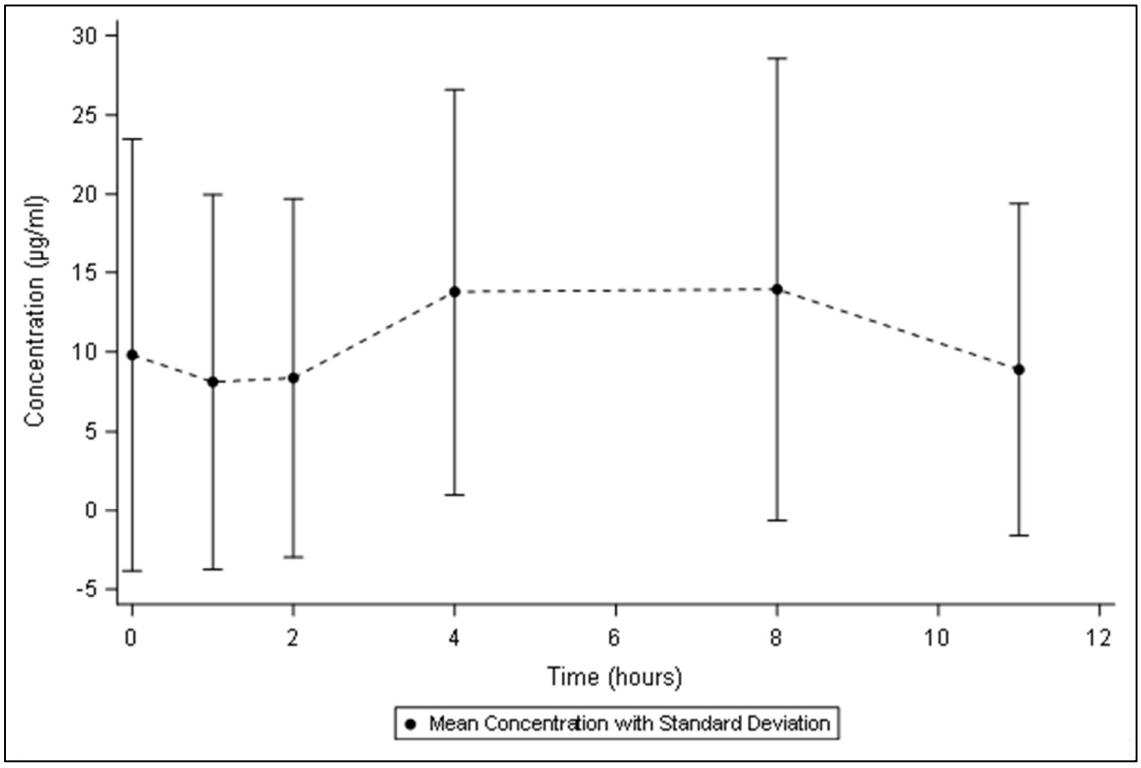

Figure 21. Average concentration (standard deviation) vs time profile from 28 children receiving a 150 mg/kg body weight dose of *Para*-aminosalicylic acid (PAS)

4.8. Conclusion

The developed LC–MS/MS method for PAS was successfully validated according to FDA guidance for industry (2001) and EMA guidelines on bioanalytical method development (2012) [29, 30]. The method was employed for quantitative determination of PAS concentrations in a clinical study following the oral administration of 150 mg/kg body weight dose of PAS in children with drug-resistant tuberculosis (DR-TB), in need of second-line anti-TB drugs. The proposed method is affordable, reliable, simple, selective, rugged and reproducible. The method produced good peak shapes and low base line noise was observed. The mobile phase was optimised in simple isocratic mode, and one of the major advantages of this method is that only 20 µl of plasma is needed for analysis, which greatly facilitates the collection of blood samples in children. The method produced reliable data that contributed to the pharmacokinetics and understanding of PAS as a second line anti-TB drug. PK data generated with this assay will hopefully lead to better recommendations regarding dosing and duration of treatment needed to maximize efficacy with acceptable safety and tolerability for PAS as an oral bacteriostatic second line anti-TB drug in children.

4.9. Publication in peer reviewed scientific journal

An assay manuscript was submitted to the Analytical and Bioanalytical Chemistry on 29 November 2017, and the submission letter are presented below and the manuscript is presented in Appendix 1.

Date: 29-Nov-2017

Manuscript No. ABC-02095-2017
Title: Development, validation and application of a LC-MS/MS method to accurately quantify para-aminosalicylic acid in 20 µl human plasma
Authors: Smit, Michiel; Wiesner, Lubbe; Castel, Sandra; Norman, Jenifer; Strydom, Natasha; Schaaf, Simon; Hesseling, Anneke

Dear Mr. Smit,

Thank you for the submission of your above-mentioned manuscript. Your manuscript has been received in the Analytical and Bioanalytical Chemistry Editorial Office and is in the process of being forwarded to the handling Editor.

Please be sure to mention the manuscript number in all future correspondence or when calling the office with questions. If there are any corrections to your e-mail address or street address, please let us know.

Yours sincerely,

Dr. Nicola Oberbeckmann-Winter

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5. METHOD DEVELOPMENT AND VALIDATION OF AN ASSAY METHOD FOR THE DETERMINATION OF CYCLOSERINE / TERIZIDONE IN HUMAN PLASMA

5.1. Objective

The objective was to develop a method for the analysis of cycloserine in human plasma that is sensitive and specific, and can be used to analyse samples generated in clinical studies after dosing with terizidone. Terizidone is not measurable in plasma, because it is hydrolysed completely into cycloserine pre-systemically [62].

The project aims to contribute to better understanding the pharmacokinetics and pharmacodynamics of cycloserine / terizidone as a second line anti-TB drug.

5.2. Literature survey

5.2.1. Clinical information

A literature and clinical protocol review revealed a number of different techniques available for quantifying cycloserine. Cycloserine is produced by *Streptomyces orchidaceous* and was successfully synthesized in 1952 [47]. It is a structural analogue of D-alanine, an amino acid that is important to the formation of the bacterial cell wall. The mode of action of terizidone is similar to cycloserine and composes of two cycloserine molecules, which are linked with a phenyl ring. Cycloserine/terizidone acts by competition, inhibiting the enzymes D-alanyl-D-alanine synthetase, alanine racemase, and alanine permease, which are indispensable for the synthesis of the peptidoglycan that confers rigidity and stability to the *Mycobacterium tuberculosis* cell membrane [53]. Although the mechanisms of *Mycobacterium tuberculosis* resistance to cycloserine have yet to be fully clarified, it is presumably due to genetic mutations in the aforementioned enzymes [63].

Terizidone pharmacokinetic properties are poorly described in literature, mainly because it was development in the 1970's when bioanalytical methods were limited. In these studies,

terizidone was not directly measured, but estimated, based on its active metabolite cycloserine, which was measured with colorimeter methods. It was not known if terizidone was systemically available [64]. The best marker for terizidone pharmacokinetics is the analysis of cycloserine, a small polar drug with limited potential for absorbing UV that makes it difficult to analyse [65]. Cycloserine was primarily used for the treatment of active pulmonary and extra-pulmonary tuberculosis, and currently cycloserine and terizidone are used as oral bacteriostatic second line anti-TB drugs for multidrug resistant tuberculosis [47].

According to all information currently available to the WHO prequalification of medicine team, terizidone is not measurable in plasma and consequently it seems to be hydrolysed completely into cycloserine pre-systemically [62].

Cycloserine/terizidone are rapidly absorbed after oral administration, the bioavailability of the drugs ranges from 70% to 90% and the plasma levels of the drug peak within 3–4 hours after ingestion. At the usual doses, cycloserine has a bacteriostatic effect [47]. The half-life of cycloserine is 10 hours, the drug does not bind to plasma proteins, and only a small proportion of cycloserine is metabolized in the liver. Most of the dose (70%) is excreted by the kidney, unaltered, within 72 hours and a small proportion of the drug is excreted in faeces. There is no cross-reaction between cycloserine and other anti-tuberculosis drugs and the MIC of cycloserine for *Mycobacterium tuberculosis* is 5–20 µg/mL [47].

As an N-methyl-D-aspartate partial receptor agonist, cycloserine competes with γ-aminobutyric acid in the brain, causing central nervous system side effects [53]. Dosing terizidone instead of cycloserine has advantages, the adverse effects are lowered, therefore it is better tolerated, which leads to better compliance and treatment outcomes [66].

5.2.2. Analytical information

5.2.2.1.Chemical properties

5.2.2.1.1. Terizidone

Molecular formula: $C_{14}H_{14}N_4O_4$

Molecular weight: 302.29

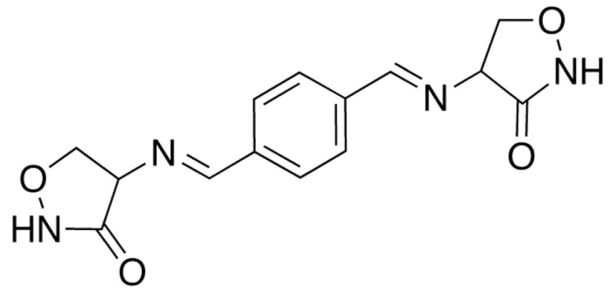

Figure 22. Chemical structure of Terizidone

5.2.2.1.2. Cycloserine

Molecular formula: $C_3H_6N_2O_2$

Molecular weight: 102.09

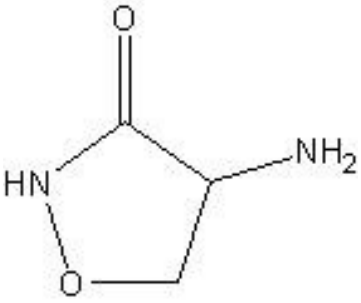

Figure 23. Chemical structure of Cycloserine

5.2.2.2. Analytical information

The first analytical method by R.L. Jones were described in 1956, cycloserine was then already determined by colorimetric determination as a marker for terizidone [64]. David *et al.*, described a method to determine cycloserine in human plasma by high performance liquid chromatography with fluorescence detection, using derivatization with p-benzoquinone [65]. The derivatisation take place at the same time as the deproteinization with ethanol, the ratio between plasma volume and reagent solution used was 1:2 for a benzoquinone concentration of 1000 µg/ml. For chromatography, an isocratic mobile phase was used that consist of formic acid : water : methanol : acetonitrile (0.1:84.9:7.5:7.5, v/v/v/v) with a flow rate of 1 ml/min at 25°C on a XDB C18 column (250 x 4.6 mm, 5µm). Determination by fluorescence detection were achieved with an excitation wavelength of 381 nm and an emission wavelength of 450 nm. The detection limit was 10 ng/ml for an

injection volume of 5 µl and a calibration curve with a range of 0.25 – 10 µg/ml was validated.

Pesek *et al*., described a new approach to evaluate the analysis of cycloserine, a strong hydrophilic and very polar drug [67]. The method utilized aqueous normal phase chromatography with a silica hydride-base stationary phase and mass spectrometry detection. The column used was a Cogent Diamond Hydride column (150 x 2.1mm, 5µm) and significant retention could be observed for underivatized cycloserine under isocratic conditions, using a mobile phase consisting of 50: 50 (v/v) mobile phase A, consisting of water : 2-propanol : acetic acid (49.5:50:0.5, v/v/v), and mobile phase B, consisting of acetonitrile : water : acetic acid (97:2.9:0.1, v/v/v). Detection was in positive ion mode using the ion $(M + H)^+$ at *m/z* of 103.05. While peak shape was acceptable with the isocratic mobile phase it was drastically improved with gradient elution. This method was used to determine impurities and degradation products of a cycloserine solution. The gradient method used a 5 minute column equilibration. A linear relationship was obtained over the concentration range 0.2 – 1.0 µg/ml with a limit of detection of 0.1 µg/ml.

Polagani *et al.,* described a simple, rapid and sensitive LC-MS/MS assay for the determination of cycloserine in plasma using carbamazepine as ISTD. Cycloserine was extracted from 50 µl human plasma via protein precipitation with acetonitrile [68]. The chromatographic separation was achieved on a Hypurity Advance C18 column (50 x 4.6 mm, 5µm) by using a mixture of acetonitrile : water : formic acid (60:39.5:0.5, v/v/v) as the mobile phase at a flow rate of 0.8 ml/min. Stock solutions was prepared in methanol and found to be stable for 14 days at 2–8°C. The calibration curve obtained was linear over the concentration range 50–15 000 ng/ml. The detection was in positive ion mode, using a transition of *m/z* of 103.0 → 75.0 for cycloserine and *m/z* of 237.0 → 194.1 for the ISTD. The runtime of 2.5 minutes made it possible to analyse many samples in a short time. The analyte was stable in plasma for 47 days kept at -70°C, for 9 hours at room temperature, for 3 freeze-thaw cycles and in extract solutions for 44 hours at room temperature.

Patel *et al.*, describe a selective and sensitive LC-MS/MS method for the determination of cycloserine in human plasma, using niacin as the ISTD. The analyte and ISTD were extracted from 500 µl human plasma via solid phase extraction on a Waters Oasis MCX cartridges [63]. Chromatographic separation was achieved on a Peerless Basic C18 (100 x 4.6 mm, 5µm) column under isocratic conditions. Detection was done by LC-MS/MS,

operating in positive ion and multiple reaction monitoring acquisition mode. The protonated precursor to product ion transition monitored for cycloserine and niacin were at *m/z* 103.1→75.0 and 124.1→80.1, respectively. Stock solutions of cycloserine in water were stable for 8 hours at room temperature and 27 days at 4°C. Plasma stability at -20°C was found for a period of 91 days. The limit of detection and LLOQ was 0.0013 µg/ml and 0.2 µg/ml, respectively, with a linear dynamic range of 0.20 – 30.0 µg/ml for cycloserine.

Fraschetti *et al.*, described the role of solvents on the stability of cycloserine under ESI-MS conditions [69]. The effects of methanol and acetonitrile on the stability of cycloserine was studied. Infra-red multiphoton photo dissociation (IRMPD) spectroscopy of the ionic species from electrospray ionization tandem mass spectrometry of the different solutions points to dimerization of cycloserine to cis-3,6-bis(amino oxymethyl)-2,5 piperidinedione. 1D and 2D magnetic resonance experiments confirm these findings by showing the dimerization of cycloserine actually take place at room temperature in acetonitrile even before ESI-MS analysis. The analytical information provided by the literature review is summarised in Table 45.

Table 45. Literature summary for cycloserine

Reference	Concentration range (µg/ml)	Sample volume (µl)	Column	Mobile phase	Flow rate (ml/min)	Run time (min)	Matrix and extraction
David *et al.,* 2001.	0.25-10	1000	XDB C18 (250 x 4.6 mm)	(Isocratic) Formic acid : water : methanol : acetonitrile (0.1: 84.9:7.5:7.5, v/v/v/v)	1.0	15	Plasma, protein precipitation and derivatisation.
Pesek *et al*., 2012.	0.2-1.0	Not mentioned	Cogent Diamond Hydride (50 x 2.1 mm)	Water : 2-propanol : acetic acid (49.5:50:0.5, v/v/v) and acetonitrile : water : acetic acid (97:2.9:0.1, v/v/v)	0.2	7 (with 5 minute equilibration time)	Cycloserine solutions.
Polagani *et al*., 2013.	0.05-15	50	Hypurity Advance (50 x 4.6 mm)	Acetonitrile : water :formic acid (60:39.5:0.5, v/v/v)	0.8	2.5	Plasma with ACN protein precipitation
Patel *et al*., 2011.	0.2-30.0	500	Peerless Basic C18 (100 x 4.6 mm)	Acetonitrile : water : formic acid (70:29.95:0.05, v/v/v)	0.8	5.0	Plasma with SPE extraction

5.3. Method development

The literature and clinical protocol review revealed that a LC-MS/MS method was needed to cover a possible Cmax of up to 40 µg/ml and a LLOQ of at least 5 half-lives of this Cmax, therefore a calibration range of 0.313–40µg/ml was chosen. LC-MS/MS was chosen because it is more specific and sensitive than conventional HPLC using UV or fluorescence detection. Most conventional HPLC methods use mobile phases that contain high concentrations and non-volatile buffer salts that is incompatible with LC-MS/MS methods. Based on the literature survey for cycloserine most of the mobile phases used for LC-MS/MS analysis contained low concentrations formic acid or acetic acid and either methanol or acetonitrile as the organic solvent. From the literature review, we concluded that cycloserine is not stable in acetonitrile and making stock solutions in acetonitrile should be avoided [69].

A stock solution of cycloserine was prepared in methanol. From the prepared stock solution three different diluted solutions were prepared for infusion, one in methanol : water (50:50, v/v), the second in methanol : water : formic acid (50:49.9:0.1, v/v/v) and a third in methanol : water : ammonium hydroxide (50:49.9:0.1, v/v/v). Each were then infused separately into the mass spectrometer and the Q1 mass filter optimised for the best protonated ionisation response for the precursor ion with a *m/z* of 103.0. The best ionisation response for the precursor ion was achieved with a mixture of methanol : water : formic acid (50:49.9:0.1, v/v/v). The protonated precursor ion was fragmented and the product ions optimised for a specific and sensitive MRM transition, the *m/z* of 103.0→75.2 was monitored. The product ion mass spectrum of cycloserine is presented in Figure 24.

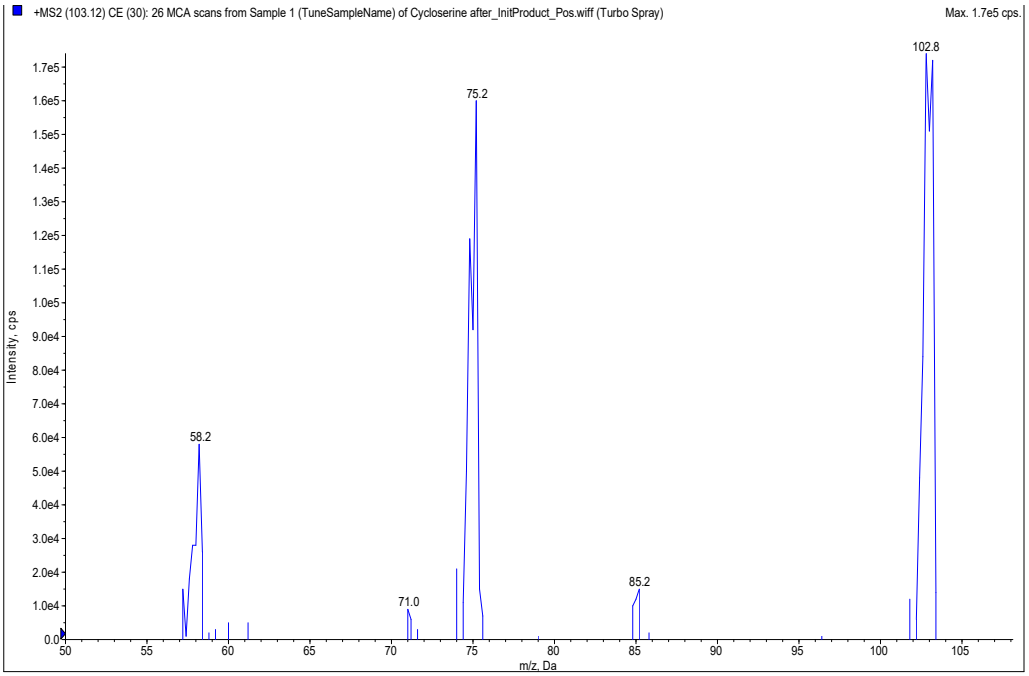

Figure 24. Product ion mass spectrum of cycloserine after collision induced dissociation in the fragmentation cell, showing the cycloserine precursor ion at m/z 103 and the productions

To start with the chromatography a Synergi Hydro RP C18 (150 x 2.0, 4µm) column was chosen, from the literature we could conclude that a more specific column would be needed for this very small and basic drug. An isocratic mobile phase of methanol : water and formic acid (50:49.9:0.1, v/v/v) was used as the starting point. Different ratios of this mobile phase were tested, settling for the best ratio between formic acid and methanol with the most retention and the best peak shape. This optimum mobile phase conditions were found to be methanol: water : formic acid at a ratio of 30:70:0.1 (v/v/v). The extraction method, protein precipitation with methanol yielded high recoveries and precision. In pure solutions as well as with the methanol precipitated plasma calibration standards, good linearity was obtained with a signal-to-noise ratio of more than 1:5 for the LLOQ.

This chromatography and extraction method was used for pre-validation testing. During the pre-validation testing of the matrix effects, the experiment failed to meet acceptance criteria and was repeated. The repeated results were still unacceptable, indicating that background matrix components have an inconsistent impact on the ionisation of cycloserine. Possible solutions to this were to use a different extraction technique or to change the chromatography to gain more retention and separate the analyte peak from the matrix. A

range of columns and different mobile phases was tested but no better retention could be obtained. In the literature, a derivatisation method was described with *p*-benzoquinone in plasma, this was to increase retention and sensitivity of cycloserine [65]. A more mass spectrometry "friendly" derivatisation reagent was chosen, dansyl chloride, and cycloserine was derivatised during the protein precipitation step. The derivatisation reaction is presented in Figure 25.

Dansyl chloride derivative
Molecular formula: $C_{15}H_{17}N_3O_4S$
Molecular weight: 335.38

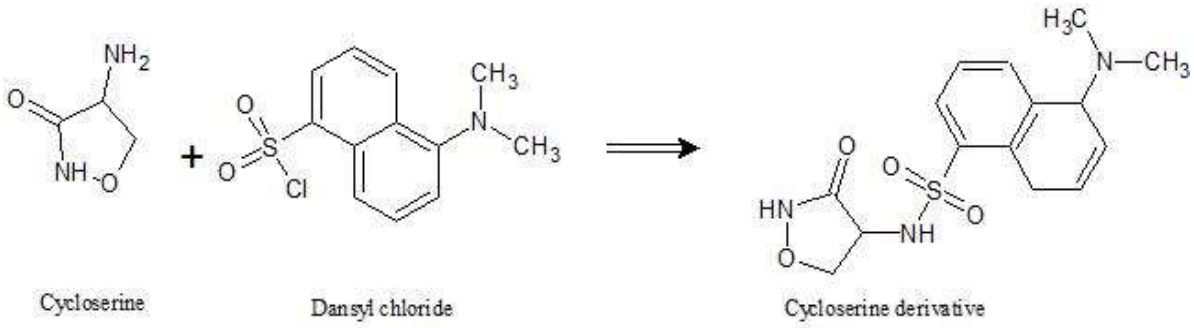

Figure 25. Structure of the cycloserine derivative

New chromatography was needed for the derivative, and as with the analyte, the derivative was infused in different solutions as a starting point. The product ion mass spectrum of cycloserine derivative is presented in Figure 26.

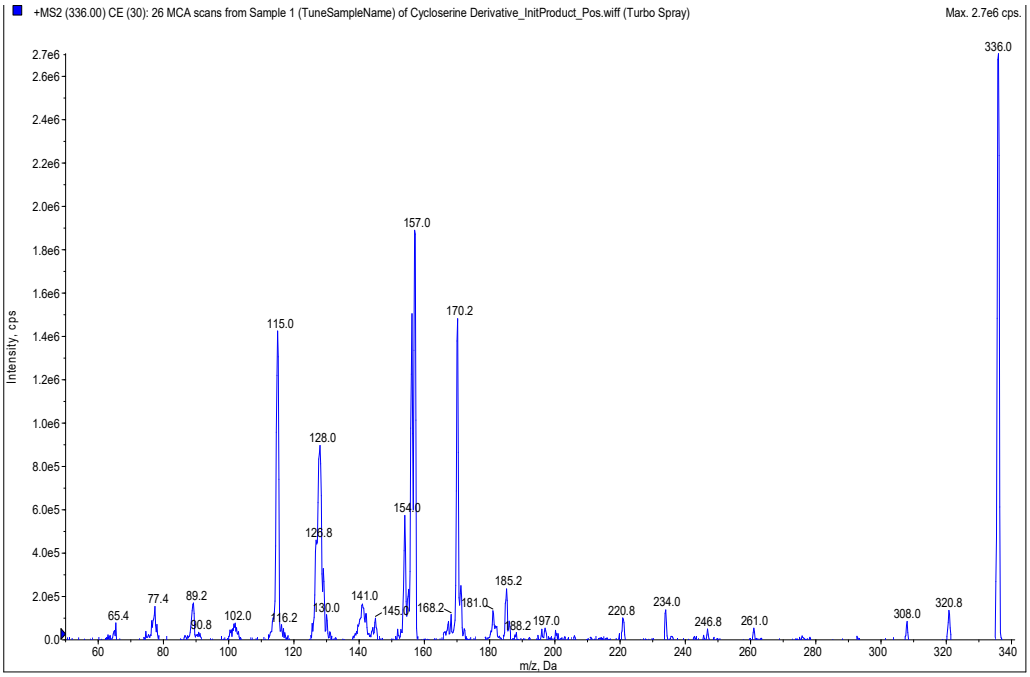

Figure 26. Product ion mass spectrum of the cycloserine derivatised product after collision induced dissociation in the fragmentation cell, showing the cycloserine derivative precursor ion at m/z 336 as well as the product ions

The best chromatography was obtained with a Gemini NX C18 (50 x 2.1mm, 5µm) column and an isocratic mobile phase containing water : formic acid : acetonitrile (69.9: 0.1:30, v/v/v). The derivative with dansyl chloride increase cycloserine sensitivity, so lower concentrations could be detected, and increased retention time on the analytical column. Validation experiments were repeated, including the matrix-effect experiment, and all criteria were met. The extraction method was optimised and the final extraction method, HPLC conditions and MS/MS settings included in a SOP.

5.3.1. The SOP extraction and derivatization procedure for cycloserine

Thaw the plasma samples, all calibration standards, quality control samples, blanks and unknown samples, at room temperature and vortex briefly.

Aliquot 20 µl of the plasma by pipette into 1.5 ml polypropylene micro-centrifuge tubes.

Add the precipitation and derivatisation reagent (methanol, 200 µl) containing dansyl chloride (2 mg/ml) to the samples (not more than 4 samples at a time).

Vortexed for approximately 30 seconds and centrifuge at approximately 16 000 rcf for 5 minutes.

Transfer 150 µl of the supernatant to a new clean 1.5 ml polypropylene micro-centrifuge tube and added and mix ammonium bicarbonate buffer (100 µl, pH 9.5).

Incubate the sample on a heating block for 2 hours at ~ 50°C.

Add formic acid (50 µl, 2%) to each sample to stop the derivatisation reaction and vortex briefly.

Cool the samples at ~5°C for 15 minutes and centrifuge at approximately 16 000 rcf for 5 minutes.

Transfer 200 µl of the supernatant into a clean 96-well plate.

Inject 5 µl of the extracted sample onto the analytical column for LC-MS/MS analysis.

5.3.2. Final Cycloserine conditions and MS/MS settings

The final HPLC and chromatographic conditions and MS/MS detector settings used are included in the final SOP method and are summarised in Tables 46 - 48.

Table 46. Cycloserine instrumentation and chromatographic conditions

Instrument used	API 3000-2
Project (Analyst)	Cycloserine_Study 2014_166
Acquisition method	Method 11
Analytical Column	Phenomenex Gemini NX C18, 2.0 x 50 mm, 5µm
Column Temperature	Room temperature (~24°C)
Mobile Phase	Acetonitrile : Water: Formic acid (30:69.9,0.1 v/v/v)
Pump Type	Agilent 1100 Quarternary Pump
Flow Rate	300 µl/min
Autosampler Type	HTC PAL
Sample arrangement	96 well plate
Injection Volume	5 µl
Autosampler Temperature	10°C

Table 47. Cycloserine electrospray ionisation settings

Mass Spectrometer Identity	**API3000-2**
APCI/ESI	**ESI**
Parameter	**Value**
Nebuliser gas (Gas 1) (arbitrary unit)	10
Turbo gas (Gas 2) (arbitrary unit)	8
CUR (curtain gas) (arbitrary unit)	6
CAD (collision gas) (arbitrary unit)	8
TEM (Source Temperature) (°C)	450
IS (Ion Spray Voltage) (V)	3500

Table 48. Cycloserine MS/MS Settings

	Cycloserine derivative
Protonated molecular ion mass (m/z) $[M+H]^+$	335.9
Product ion mass (m/z) Quantifier	157.2
Product ion mass (m/z) Qualifier	170.1
Dwell time (ms)	150
Declustering potential (V)	61
Focus potential (V)	370
Entrance potential (V)	10
Collision energy (eV)	41
Collision cell exit potential (V)	8
Scan Type	MRM
Polarity	Positive
Pause Time (ms)	5

Pre-validation extractions of standards and controls in plasma showed good accuracy, precision and recoveries for the cycloserine derivative and the method was validated according to the FDA and EMA guidelines [29, 30].

5.4. Validation of the Assay Method

5.4.1. Procedure

Validation of an assay method is a process performed to objectively demonstrate and document the accuracy, precision, specificity, sensitivity and reproducibility of the assay method and the stability of the analyte for the purposes of assaying samples of unknown concentrations.

In order to demonstrate acceptable within- and between-day accuracy and precision of the method over the desired concentration range, calibration standards and quality control samples were prepared and assayed in three consecutive runs. A full set of calibration standards and quality controls was prepared and stored frozen, and the required aliquots were thawed and assayed in each run. Each run consisted of all the calibration standards in duplicate to produce one calibration curve and six replicates of the prepared quality control samples. A quality control spiked to a concentration above the upper limit of quantification was diluted (1:4) with blank plasma to validate the dilution of samples of which the concentrations potentially do not fall within the validated range.

Standard curve fitting was determined by applying the simplest model that adequately describes the concentration-response relationship using appropriate weighting and statistical tests for goodness-of -fit. A calibration curve based on a well selected regression model must consist of at least six calibration levels, covering the entire calibration range, from the LLOQ to ULOQ. The regression model selected during the validation was used for the quantification of the study samples, summarised in Table 49. In the case of a re-instatement (or partial) validation, the regression model that was used for the full validation must be used for the re-instatement validation.

Table 49. Cycloserine quantitation parameters, ions monitored and retention times

Software	Analyst 1.5.2
Algorithm	Analyst Classic
Calibration Fit Type	Quadratic
Parameter	Area
Curve Weighting	1/x
	Cycloserine derivative
Bunching factor	1
Number of smoothes	3
Precursor ion	335.9
Product ion	157.2
Retention Time (min)	1.5

5.4.2.Calibration standard and quality control preparation

5.4.2.1. Preparation of Stock Solutions (Cycloserine)

Stock solutions (SS1 - Cycloserine) were prepared volumetrically. The weighed mass of the analyte was also adjusted where applicable (purity, salt, etc.). Stock solution SS1 and SS2 (1000 µg/ml) were prepared in water : methanol (1:1, v/v) as represented in Tables 50 and Table 51. All stock solutions were kept at ~ - 80°C until required. These stock solutions are used to prepare by spiking blank biological matrix as required.

Table 50. Preparation of Cycloserine stock solution (SS1-Cycloserine)

Solvent Used	Volume solvent (ml)	Weighed mass of analyte (mg)	Adjusted* mass of analyte (mg)	SS1 concentration (µg/ml)
Water : MeOH (1:1)	7.987	8.15	7.987	1000

** Reason for Adjustment (e.g. purity, salt, hydrate): Purity*
** Calculation: 8.15 x 0.98 = 7.987 mg.*

Table 51. Preparation of Cycloserine stock solution (SS2-Cycloserine)

Solvent Used	Volume solvent (ml)	Weighed mass of analyte (mg)	Adjusted* mass of analyte (mg)	SS2 concentration (µg/ml)
Water : MeOH (1:1)	12.142	12.39	12.142	1000

** Reason for Adjustment (e.g. purity, salt, hydrate): Purity*
** Calculation: 12.39 x 0.98 = 12.142mg*

5.4.2.2. Preparation of Calibration Standards

Calibration standards were prepared volumetrically in plasma (anticoagulant K_3EDTA) on 09 December 2014 at room temperature by spiking 240 µl of the analyte stock solution (SS1) into 5.760 ml of normal blank plasma (STD 1), which is then serially diluted with normal blank plasma to produce the desired concentrations for calibration standards (STD 2–8) as presented in Table 52. Multiple 60 µl aliquots of each calibration standard were stored in individual 1.5 ml polypropylene tubes at approximately - 80°C to allow for duplicate 20 µl extractions from each tube. Stability has been shown at - 20°C for at least 91days [63].

Table 52. Preparation of calibration standards

Standard	Blank Plasma volume (ml)	Volume SS1 spiked (µl)	Dilution Source	Dilution Source Volume (ml)	Total Volume of dilution (ml)	Cycloserine (µg/ml)
STD 1 – ULOQ	5.760	240			6.00	40.0
STD 2	3.00		*STD 1*	*3.00*	6.00	20.0
STD 3	3.00		*STD 2*	*3.00*	6.00	10.0
STD 4	3.00		*STD 3*	*3.00*	6.00	5.00
STD 5	3.00		*STD 4*	*3.00*	6.00	2.50
STD 6	3.00		*STD 5*	*3.00*	6.00	1.25
STD 7	3.00		*STD 6*	*3.00*	6.00	0.625
STD 8 – LLOQ	3.00		*STD 7*	*3.00*	6.00	0.313

5.4.2.3.Preparation of Quality Controls

Quality controls were prepared volumetrically in plasma (anticoagulant K_3EDTA) on 09 December 2014 at room temperature using the same methodology that was used for the calibration standards. Spike 320 µl of the analyte stock solution (SS2) into 4.680 ml of normal blank plasma to obtain QC Dilute. This quality control is serially diluted with normal blank plasma to attain the desired concentrations (QC High – QC LLOQ) as presented in table 53. Multiple 60 µl aliquots of each quality control are stored in individual 1.5 ml polypropylene tubes and stored at approximately -80°C to allow for duplicate 20 µl extractions from each tube. Stability has been shown at -20°C for at least 91 days [63].

Table 53. Preparation of quality controls

Standard	Blank Plasma volume (ml)	Volume SS2 spiked (µl)	Dilution Source	Dilution Source Volume (ml)	Total Volume of dilution (ml)	Cycloserine (µg/ml)
QC Dilute	4.680	320			5.00	64.0
QC 1 High	4.00		*QC Dilute*	*4.00*	8.00	32.0
QC 2 Med	3.00		*QC 1*	*3.00*	6.00	16.0
QC SYS	5.00		*QC 2*	*1.00*	6.00	2.67
QC 3 Low	5.00		*QC 3*	*2.08*	7.08	0.783
QC 4 LLOQ	3.00		*QC 4*	*2.00*	5.00	0.313

Note: The QC Dilute is used during a validation batch to quality control the dilution process.*

5.4.3. Validation Results

Acceptance criteria: Accuracy is expressed as the concentration of analyte found as a percentage of the nominal concentration (% Accuracy) while precision is expressed as the coefficient of variation (CV %) seen in a batch of assays.

The calculated calibration curve should fit the plot of measured responses *vs*. nominal concentrations of the calibration standards adequately, giving a r^2 fit parameter of as close to one as possible.

For a valid method, the within- and between-batch accuracy is required to be within 15% (i.e. % Accuracy should be between 85–115%) over the entire calibration range and within 20% of nominal concentration at the LLOQ. For a valid method, the within- and between-batch precision is required to be less than 15 % (i.e. CV %) should be less than 15%) over the entire calibration range and less than 20% at the LLOQ. Duplicate standards are analysed at each calibration point. Each one of these standards is used to define the calibration equation, unless one of those points does not meet the above criteria. In this instance, the invalid point would be excluded and only a single standard would be used at that level. This allows for a single standard to fail at either the LLOQ or the ULOQ without having an effect on the resulting calibration range.

5.4.3.1.Validation 1 (DAY 1)

Within-batch accuracy and precision are assessed by assaying all the calibration standards in duplicate, to produce one calibration curve, and 6 replicates of each quality control level in a single batch of assays as presented in Figure 27.

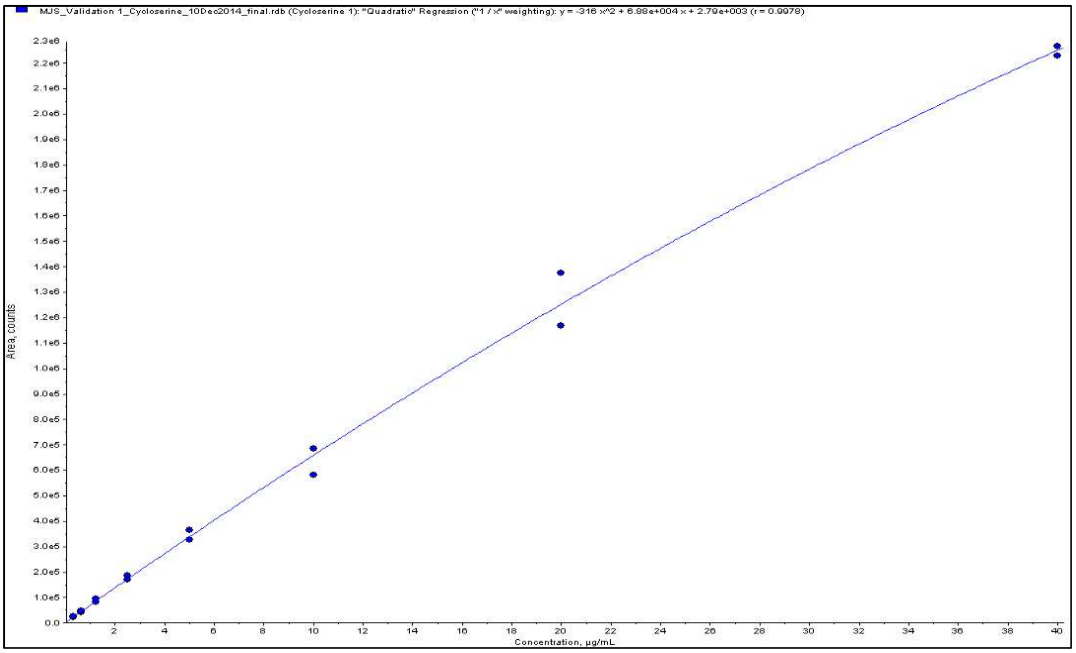

Figure 27. Representative calibration curve for cycloserine- validation 1, day 1

The regression equation used was Quadratic (weighted by 1/x concentration), $f(x) = a + bx + cx^2$, as presented in Table 54.

Table 54. Regression equation, $f(x) = a + bx + cx^2$

Validation Batch	Quadratic Calibration Curve Parameters			
	a	b	c	r
1	2.79E+03	6.88E+04	-316	0.9978

The within-batch accuracy and precision of the assay procedure are assessed by calculating the regression equation and constructing the calibration curve based on peak areas of analyte, as represented in Table 55 and Table 56.

Table 55. Cycloserine Calibration Standards Accuracy and Precision – validation 1

Sample ID	Nominal Conc. (µg/ml)	Mean Observed Conc. (µg/ml)	Std Dev	% CV	% Accuracy	*N*
S8	0.313	0.312	0.0159	5.1	99.6	2 of 2
S7	0.625	0.607	0.0712	11.7	97.1	2 of 2
S6	1.25	1.26	0.141	11.2	100.7	2 of 2
S5	2.50	2.58	0.143	5.6	103.1	2 of 2
S4	5.00	5.11	0.423	8.3	102.3	2 of 2
S3	10.0	9.59	1.18	12.3	95.9	2 of 2
S2	20.0	20.3	2.62	12.9	101.6	2 of 2
S1	40.0	39.9	0.595	1.5	99.8	2 of 2

Table 56. Summary of Cycloserine intra-validation quality controls

Sample ID	Nominal Conc. (µg/ml)	Mean Observed Conc. (µg/ml)	Std Dev	% CV	% Accuracy	*N*
QC LLOQ	0.313	0.293	0.0384	13.1	93.7	6 of 6
QC L	0.783	0.797	0.0673	8.5	101.8	6 of 6
QC M	16.0	16.6	1.35	8.1	103.7	6 of 6
QC H	32.0	34.1	4.06	11.9	106.6	6 of 6

5.4.3.2.Validation 2 and 3 (DAYS 2 and 3)

Between-batch accuracy and precision are assessed by assaying an additional two separate consecutive batches, each consisting of a double set of calibration standards designated for use in the assay of samples of unknown concentrations and 6 replicates of each of the quality control samples, represented in figure 28 and 29.

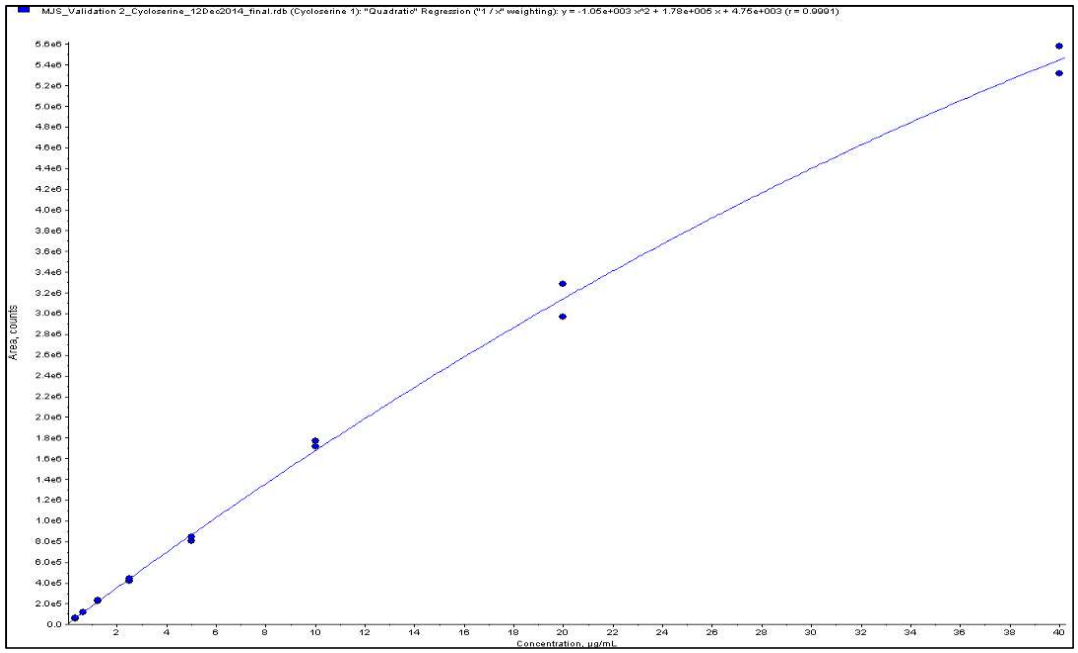

Figure 28. Representative calibration curve for Cycloserine- validation 2, day 2

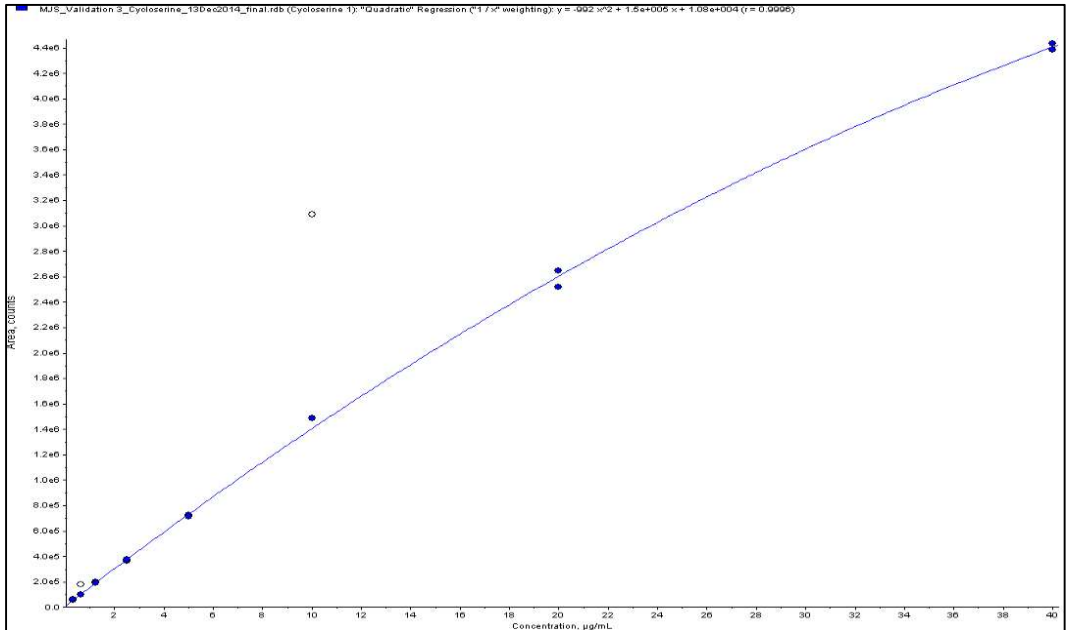

Figure 29. Representative calibration curve for Cycloserine- validation 3

The regression equation used was Quadratic (weighted by 1/x concentration), $f(x) = a + bx + cx^2$, as presented in Tables 57 and 58.

Table 57. Regression equation, $f(x) = a + bx + cx^2$

Validation Batch	Quadratic Calibration Curve Parameters			
	a	b	c	r
2	4.75E+03	1.78E+05	-1.05E+03	0.9991

Table 58. Regression equation, $f(x) = a + bx + cx^2$

Validation Batch	Quadratic Calibration Curve Parameters			
	a	b	c	r
3	1.08E+04	1.50E+05	-992	0.9996

The between-batch accuracy and precision of the assay procedure are assessed by constructing a calibration curve based on analyte peak areas and calculating the regression equations, as represented in Tables 59 - 62.

Table 59. Cycloserine calibration standards accuracy and precision – validation 2

Sample ID	Nominal Conc. (µg/ml)	Mean Observed Conc. (µg/ml)	Std Dev	% CV	% Accuracy	*N*
S8	0.313	0.307	0.0236	7.7	98.2	2 of 2
S7	0.625	0.647	0.0112	1.7	103.6	2 of 2
S6	1.250	1.29	0.0147	1.1	102.8	2 of 2
S5	2.50	2.42	0.106	4.4	97.0	2 of 2
S4	5.00	4.74	0.180	3.8	94.9	2 of 2
S3	10.0	10.4	0.260	2.5	104.2	2 of 2
S2	20.0	19.9	1.61	8.1	99.4	2 of 2
S1	40.0	40.0	1.96	4.9	100.0	2 of 2

Table 60. Summary of Cycloserine intra-validation quality controls - validation 2

Sample ID	Nominal Conc. (µg/ml)	Mean Observed Conc. (µg/ml)	Std Dev	% CV	% Accuracy	*N*
QC LLOQ	0.313	0.317	0.0451	14.2	101.3	6 of 6
QC L	0.783	0.819	0.0632	7.7	104.6	5 of 6
QC M	16.0	16.3	1.49	9.2	101.6	6 of 6
QC H	32.0	34.6	2.63	7.6	108.1	6 of 6

Table 61. Cycloserine calibration standards accuracy and precision – validation 3

Sample ID	Nominal Conc. (µg/ml)	Mean Observed Conc. (µg/ml)	Std Dev	% CV	% Accuracy	*N*
S8	0.313	0.324	0.0376	11.6	103.6	2 of 2
S7	0.625	0.610	N/A	N/A	97.6	1 of 2
S6	1.25	1.24	0.0453	3.6	99.6	2 of 2
S5	2.50	2.44	0.0562	2.3	97.8	2 of 2
S4	5.00	4.89	0.0735	1.5	97.9	2 of 2
S3	10.0	10.6	N/A	N/A	106.2	1 of 2
S2	20.0	19.8	0.826	4.2	99.1	2 of 2
S1	40.0	40.0	0.496	1.2	100.1	2 of 2

Table 62. Summary of Cycloserine intra-validation quality controls - validation 3

Sample ID	Nominal Conc. (µg/ml)	Mean Observed Conc. (µg/ml)	Std Dev	% CV	% Accuracy	*N*
QC LLOQ	0.313	0.316	0.0147	4.7	100.9	6 of 6
QC L	0.783	0.771	0.0660	8.6	98.4	6 of 6
QC M	16.0	14.4	1.23	8.5	90.3	6 of 6
QC H	32.0	29.2	1.69	5.8	91.3	6 of 6

5.4.3.3.Summary of the combined calibration standard and quality control results

The overall accuracy and precision of the assay procedure is assessed by calculating the accuracy and precision statistics over the within- and between-batch validation batches (3 in total). Accuracy is expressed as the concentration of the analyte found as a percentage of the nominal concentration (% Accuracy), while precision is expressed as the coefficient of variation (CV %). The combined regression, calibration standards and quality control results (all 3 validations) of cycloserine are summarised in Tables 63 - 65.

Table 63. Overall summary of calibration curve parameters- validation 1-3

Validation Batch	Quadratic Calibration Curve Parameters			
	a	B	C	r
1	2.79E+03	6.88E+04	-316	0.9978
2	4.75E+03	1.78E+05	-1.05E+03	0.9991
3	1.08E+04	1.50E+05	-992	0.9996

Table 64. Overall summary of calibration standard accuracy and precision- validation 1-3

Validation Batch	Sample ID / Nominal Conc. / Replicates	STD 1 – ULOQ 40.0 (µg/ml)	STD 2 20.0 (µg/ml)	STD 3 10.0 (µg/ml)	STD 4 5.00 (µg/ml)	STD 5 2.50 (µg/ml)	STD 6 1.25 (µg/ml)	STD 7 0.625 (µg/ml)	STD 8 - LLOQ 0.313 (µg/ml)
	Replicates	Observed Conc.							
1	1	40.3	18.5	8.76	4.81	2.68	1.16	0.557	0.300
	2	39.5	22.2	10.4	5.41	2.48	1.36	0.657	0.323
2	1	38.6	18.7	10.6	4.62	2.35	1.27	0.655	0.291
	2	41.4	21.0	10.2	4.87	2.50	1.30	0.639	0.324
3	1	40.4	20.4	***[24.6]***	4.95	2.41	1.28	0.610	0.298
	2	39.7	19.2	10.6	4.84	2.48	1.21	***[1.13]***	0.351
	n	6	6	5	6	6	6	5	6
	Average	40.0	20.0	10.1	4.92	2.48	1.26	0.624	0.315
	STDEV	0.950	1.45	0.774	0.265	0.111	0.0700	0.0417	0.0225
	% CV	2.4	7.3	7.7	5.4	4.5	5.5	6.7	7.2
	% Accuracy	100.0	100.0	101.1	98.3	99.3	101.1	99.8	100.5

[ ] = Outlier with MNR-ESD Outlier Test, excluded from regression.

Table 65. Overall quality control accuracy and precision estimation

Validation Batch	Sample ID Nominal Conc. / Replicates	QC - DIL 64.0 (µg/ml)	QC - High 32.0 (µg/ml)	QC – Med 16.0 (µg/ml)	QC - Low 0.783 (µg/ml)	QC - LLOQ 0.313 (µg/ml)
		Observed Conc.				
Validation 1	1		31.9	18.8	0.740	0.257
	2		30.1	15.8	0.726	0.337
	3		34.2	15.6	0.890	0.257
	4		34.9	15.2	0.810	0.342
	5		31.9	17.0	0.757	0.292
	6		41.6	17.3	0.861	0.274
Validation 2	1		31.1	14.9	***[0.491]***	0.284
	2		32.9	16.9	0.846	0.304
	3		35.6	15.9	0.830	0.310
	4		38.4	18.2	0.785	0.396
	5		36.2	17.3	0.734	0.271
	6		33.4	14.3	0.901	0.338
Validation 3	1	61.2	*29.6*	13.6	0.749	0.336
	2	60.7	28.6	15.1	0.777	0.298
	3	57.5	27.3	14.1	0.718	0.305
	4	58.7	28.7	12.9	0.724	0.315
	5	60.0	28.8	14.6	0.898	0.331
	6	60.9	32.3	16.4	0.758	0.309
	n	6	18	18	17	18
	Average	59.8	32.6	15.8	0.794	0.293
	STDEV	1.45	3.74	1.61	0.0648	0.0382
	% CV	2.4	11.5	10.2	8.2	13.0
	% Accuracy	93.5	102.0	98.6	101.4	93.7

[ ] = Outlier with MNR-ESD Outlier Test, excluded from regression.

5.4.4. Calibration Range

Results from the validation assays above indicate a valid calibration range of 0.313–40.0 µg/ml for cycloserine. The LLOQ was set at the concentration of the lowest validated standard for cycloserine, 0.313 µg/ml.

5.4.5. Quantification Method

The results show that the method provides sufficient accuracy and precision over the entire range based on analyte peak area with a quadratic calibration curve (weighted by 1/x concentration).

5.4.6. Stability assessment

Various stability experiments were performed to show that all the necessary precautions were taken to ensure that the analyte concentrations were not affected by the assay procedure or associated conditions. The results demonstrate that the assays of study samples could be completed within the period for which the analyte is shown to be stable.

5.4.6.1.Stock solution stability and accuracy

Stock solutions SS1-Cycloserine (prepared on the 17 Nov 2014) were prepared in equal parts water and methanol and kept at approximately -80°C. Test and control samples of these stock solutions were left at room temperature, ~ 4°C and ~ -20°C for 18 hours, respectively. Reference stock solutions, SS2-Cycloserine (prepared on the 27 Nov 2014) were prepared after the storage period of the test and control samples. The reference, test and control samples were diluted with injection solvent to a concentration of 4.00 µg/ml for the analyte and were analysed according to the method procedure. Peak areas of the test and control sample assays were compared to the reference assays and are presented in Table 66 and 67, for the analyte and derivative respectively. Peak areas of the initial stock solution (kept at approximately -80°C) and reference analyte stock solutions were compared for stability at ~ -80°C and accuracy and are presented in Table 68.

Table 66. Stock solution stability of Cycloserine

	Reference (~ -80°C)	Test (Room temp)	Control (~ 4°C)	Control (~ -20°C)
Peak area 1	4070000	3890000	3950000	4140000
Peak area 2	3710000	3690000	3990000	4550000
Peak Area 3	4110000	4010000	4140000	4840000
Peak Area 4	4340000	4020000	4550000	3780000
Peak Area 5	4130000	3580000	4200000	4590000
Peak Area 6	4190000	3620000	4300000	4870000
Average	4091667	3801667	4188333	4461667
STDEV	209420	196715	219947	424802
% CV	5.1	5.2	5.3	9.5
% Difference		-7.1	2.4	9.0

Table 67. Stock solution stability of Cycloserine derivative

	Reference (~ -80°C)	Test (Room temp)	Control (~ 4ºC)	Control (~ -20ºC)
Peak area 1	27600000	26400000	27200000	26200000
Peak area 2	25600000	25400000	26900000	24900000
Peak Area 3	23000000	26300000	25700000	25900000
Peak Area 4	23800000	26200000	26300000	25700000
Peak Area 5	23700000	26500000	24300000	25500000
Peak Area 6	27000000	26000000	25900000	25500000
Average	25116667	26133333	26050000	25616667
STDEV	1906218	398330	1031019	440076
% CV	7.6	1.5	4.0	1.7
% Difference		4.0	3.7	2.0

Table 68. Stock solution accuracy of Cycloserine

	Reference (SS1)	Test (SS2)
Peak area 1	11000000	10400000
Peak area 2	12800000	10700000
Peak Area 3	11400000	10400000
Peak Area 4	11300000	11200000
Peak Area 5	9470000	11600000
Peak Area 6	12500000	11200000
Average	11411667	10916667
STDEV	1188620	491596
% CV	10.4	4.5
% Difference		-4.3

Acceptance Criteria: A high CV (%) (higher than 15%) of the measured values and a difference of more than 15% from the reference solution could indicate instability in the stock/working solution. For accuracy, the % difference between two stock solutions should not be more than 5%.

Conclusion: From the peak areas tabulated above it can be concluded that cycloserine stock solutions prepared in methanol and water are stable for 18 hours stored at room temperature, ~ 4 ºC and ~ -20°C. The initial stock solution kept at ~ -80 ºC compared well with the reference, and was also observed to be stable for 10 days, with a % difference of -0.6.

5.4.6.2. Storage stability in matrix

Samples for the long-term matrix stability assessment of cycloserine in human plasma were prepared in-house on 09 Dec 2014 and was kept at ~ -80°C until 23 Mar 2016 when they were extracted and analysed, as represented in Table 69. The assessment should cover, at a minimum, the length of time from when the first sample was drawn during the clinical phase to the date of final sample analysis during the analytical phase of a study.

Table 69. Long term matrix stability of Cycloserine in plasma

	High Concentration		Medium Concentration		Low Concentration	
	Nominal conc. (µg/ml)	Observed conc. (µg/ml)	Nominal conc. (µg/ml)	Observed conc. (µg/ml)	Nominal conc. (µg/ml)	Observed conc. (µg/ml)
Sample 1	32.0	35.2	16.0	18.7	0.313	0.333
Sample 2		34.1		18.7		0.332
Sample 3		35.4		19.6		0.360
Sample 4		35.6		17.1		0.269
Sample 5		31.1		18.3		0.304
Sample 6		31.5		16.1		0.280
	Average	33.8	**Average**	18.1	**Average**	0.313
	STDEV	2.0	**STDEV**	1.27	**STDEV**	0.0349
	% CV	6.0	**% CV**	7.0	**% CV**	11.1
	% Difference	5.7	**% Difference**	13.0	**% Difference**	0.0

Acceptance criteria: A high % CV and a high % Difference (higher than 15%) of the measured values compared to nominal concentrations could indicate matrix instability.

Conclusion: The % CV and % Difference for cycloserine were within 15% and therefore the long-term matrix stability for cycloserine in plasma is indicated for at least 17 months.

5.4.6.3.“Fresh” vs. “Frozen” stability

In order to qualify the “fresh” vs. “frozen” effect, a fresh set of calibration standards was prepared in plasma (14 Dec 2014) and prior to aliquoting and freezing, tested against stored QC’s (09 Dec 2014). The fresh curve was used to quantify the QC’s and the data is represented in Tables 70 and 71.

Table 70. Calibration Standards Accuracy and Precision – "Fresh" vs. "Frozen" stability of Cycloserine

Sample ID	Nominal Conc. (µg/ml)	Mean Observed Conc. (µg/ml)	Std Dev	% CV	% Accuracy	*N*
S8	0.313	0.289	0.0142	4.9	92.2	2 of 2
S7	0.625	0.623	0.0351	5.6	99.7	2 of 2
S6	1.25	1.29	0.0612	4.8	103.1	2 of 2
S5	2.50	2.44	0.00747	0.3	97.7	2 of 2
S4	5.00	5.54	0.0758	1.4	110.7	2 of 2
S3	10.0	10.1	0.0617	0.6	100.5	2 of 2
S2	20.0	18.9	0.0620	0.3	94.7	2 of 2
S1	40.0	40.6	2.46	6.1	101.4	2 of 2

Table 71. Quality control samples accuracy and precision – "Fresh" vs. "Frozen" stability of Cycloserine

Sample ID	Nominal Conc. (µg/ml)	Mean Observed Conc. (µg/ml)	Std Dev	% CV	% Accuracy	*N*
QC L	0.783	0.764	0.0672	8.8	97.6	6 of 6
QC M	16.0	16.2	1.03	6.4	101.5	6 of 6
QC H	32.0	31.7	1.18	3.7	99.1	6 of 6

Acceptance criteria: The normal acceptance criteria for quality controls apply. The accuracy is required to be within 15% (i.e. % Accuracy should be between 85–115%) and the precision is required to be < 15% (i.e. CV (%) should be < 15%).

Conclusion: The CV (%) and % Accuracy for the analyte was shown to be within 15% which indicates that sample freezing does not influence the accuracy and precision of the assay. It also indicates that the analyte is stable in plasma for at least 5 days when stored at ~ -80°C.

5.4.6.4. Freeze-thaw stability

The freeze-thaw stability evaluation should mimic the intended sample handling conditions to be used during sample analysis. In order to ascertain freeze-thaw stability, low and high quality controls were frozen at ~ -80 ºC, and put through six consecutive freeze-thaw cycles. Sample aliquots were prepared and frozen for at least 24 hours prior to starting this experiment. Each cycle consisted of sufficient thawing time at room temperature followed by 12–24 hours freezing time. These samples were analysed against a valid calibration curve and compared to controls from the batch, analysed at the same concentration. The measured concentrations and calculated differences after six cycles for the two sets of quality control samples are presented in Table 72.

Table 72. Freeze-thaw stability of Cycloserine

	High Concentration		Low Concentration	
	Nominal Concentration (µg/ml)	Observed F/T High (µg/ml)	Nominal Concentration (µg/ml)	Observed F/T Low (µg/ml)
Sample 1	32.0	36.7	0.783	0.746
Sample 2		34.4		0.758
Sample 3		30.5		0.725
Sample 4		29.9		0.763
Sample 5		27.6		0.734
Sample 6		29.0		0.796
	Average	31.4	**Average**	0.754
	STDEV	3.47	**STDEV**	0.0252
	% CV	11.1	**% CV**	3.3
	% Difference	-2.0	**% Difference**	-3.7

Acceptance criteria: A high CV (%) and high % Difference (higher than 15 %) between the test and control values could indicate freeze-thaw instability.

Conclusion: The CV (%) and % Difference for the analyte reported were within 15% which indicates that the analyte is stable in plasma for at least six freeze-thaw cycles.

5.4.6.5.Benchtop stability

In order to ascertain benchtop stability, low and high quality control samples were frozen at ~ - 80ºC, and subsequently left on the bench for approximately 18 hours (maximum anticipated time that future study samples will be left thawed until extracted). Samples were removed on 12 December 2014 at 16H00 and extracted on 13 December 2014 at 10H00. These samples were analysed against a valid calibration curve. The measured concentrations and calculated accuracies for the two sets of quality control samples are presented in Table 73.

Table 73. On bench stability of Cycloserine

	High Concentration		Low Concentration	
	Nominal Concentration (µg/ml)	**Observed BT High (µg/ml)**	**Nominal Concentration (µg/ml)**	**Observed BT Low (µg/ml)**
Sample 1	**32.0**	30.0	**0.783**	0.809
Sample 2		30.2		0.703
Sample 3		32.2		0.742
Sample 4		31.3		0.760
Sample 5		31.0		0.787
Sample 6		32.7		0.854
	Average	31.2	**Average**	0.776
	STDEV	1.07	**STDEV**	0.0530
	% CV	3.4	**% CV**	6.8
	% Difference	-2.4	**% Difference**	-0.9

**Note: Observed QC Mean conc. = Mean found conc. from Validation 3*

Acceptance criteria: A high CV (%) and a high % Difference (higher than 15%) between the test and control samples could indicate on-bench instability.

Conclusion: The CV (%) and % Difference for Cycloserine were reported to be within 15%. On-bench stability for Cycloserine in plasma is indicated for at least 18 hours.

5.4.6.6.Reinjection Reproducibility

Reinjection reproducibility is evaluated to determine if an analytical run can be reanalysed by reinjection in the case of instrument interruptions. Following the injection of the first validation run (Validation 1), the extracted samples (96-well plate) should remain in the autosampler at the method defined temperature. Reinjection of the extracts follows the injection of the second validation run (Validation 2). This demonstrates reinjection reproducibility, as presented in Tables 74 and 75.

Table 74. Calibration Standards Accuracy and Precision – Validation 1 Reinjection

Sample ID	Nominal Conc. (µg/ml)	Mean Observed Conc. (µg/ml)	Std Dev	% CV	% Accuracy	*N*
S8	0.313	0.295	0.0348	11.8	94.2	2 of 2
S7	0.625	0.579	N/A	N/A	92.6	1 of 2
S6	1.25	1.27	0.227	17.9	101.3	2 of 2
S5	2.50	2.69	0.197	7.3	107.7	2 of 2
S4	5.00	5.12	0.357	7.0	102.4	2 of 2
S3	10.0	10.1	0.916	9.1	100.9	2 of 2
S2	20.0	18.5	N/A	N/A	92.6	1 of 2
S1	40.0	40.4	0.0727	0.2	101.0	2 of 2

Table 75. Quality control samples Accuracy and Precision – Validation 1 Reinjection

Sample ID	Nominal Conc. (µg/ml)	Mean Observed Conc. (µg/ml)	Std Dev	% CV	% Accuracy	*N*
QC LLOQ	0.313	0.305	0.0210	6.9	97.4	6 of 6
QC L	0.783	0.815	0.0758	9.3	104.0	6 of 6
QC M	16.0	17.4	1.14	6.6	108.7	6 of 6
QC H	32.0	35.7	4.31	12.1	111.7	6 of 6

Acceptance criteria: The normal acceptance criteria for quality controls apply. The accuracy is required to be within 15% (i.e. % Accuracy should be between 85–115%) and the precision is required to be < 15% (i.e. CV (%) should be < 15%).

Conclusion: The CV (%) and % Accuracy for the analyte are reported to be within 15 % which indicates that a batch may be reinjected within 29 hours.

5.4.6.7.Autosampler stability

In order to assess autosampler stability, the first validation run (day 1) was reinjected after the second validation run (day 2). To assess autosampler (or processed sample) stability, the high and low QC peak areas from the first validation run and the reinjected run was compared. This provides an estimation of autosampler stability over at least 24 hours.

This could not be repeated for a third validation run (day 3) as the reinjected quality controls samples evaporated and not enough sample was left for a third injection. The data are presented in Tables 76 and 77.

Table 76. Autosampler stability for extracted samples -high concentration

High Concentration	
Validation 1	**Peak area**
Injection 1	1880000
Injection 2	1790000
Injection 3	1990000
Injection 4	2020000
Injection 5	1880000
Injection 6	2320000
Average	1980000
STDEV	186226
% CV	9.4

Re injected Validation 1	Peak area
Injection 7	1580000
Injection 8	1640000
Injection 9	1630000
Injection 10	1800000
Injection 11	1750000
Injection 12	2050000
Average	1741667
STDEV	171746
% CV	9.9
% Difference after Re injection	-12.0

Table 77. Autosampler stability for extracted samples - low concentration

Low Concentration	
Validation 1	**Peak area**
Injection 1	53600
Injection 2	52600
Injection 3	63800
Injection 4	58300
Injection 5	54800
Injection 6	61800
Average	57483
STDEV	4590
% CV	8.0
Re injected Validation 1	**Peak area**
Injection 7	44900
Injection 8	46200
Injection 9	54000
Injection 10	55000
Injection 11	50600
Injection 12	53700
Average	50733
STDEV	4296
% CV	8.5
% Difference after re injection	-11.7

Acceptance criteria: A high CV (%) and a high % Difference (higher than 15%) between the test and control samples could indicate autosampler instability.

Conclusion: The CV (%) and % Difference were found to be within 15%. Autosampler stability for the analyte is indicated for at least 29 hours.

5.4.6.8. Whole blood stability on ice

Stability under the appropriate conditions is assessed in order to estimate the maximum amount of time that the whole blood sample may be left on ice before being centrifuged to obtain plasma (i.e. to ensure stability during sample handling). Stability in whole blood was tested after 120, 60 and 30 minutes, the results are presented in Tables 78–80.

Table 78. Whole blood stability of Cycloserine after 2 hours

	High Concentration		Low Concentration	
	Storage: 0 min Peak Area	**Storage: 120 min Peak Area**	**Storage: 0 min Peak Area**	**Storage: 120 min Peak Area**
Sample 1	1110000	1080000	34000	23200
Sample 2	1160000	1050000	32400	25800
Sample 3	1210000	1040000	31400	25000
Sample 4	1190000	1040000	30900	25500
Sample 5	1210000	1010000	29700	26300
Sample 6	1150000	990000	33000	29200
Average	1171667	1035000	31900	25833
STDEV	39200	31464	1547	1964
% CV	3.3	3.0	4.8	7.6
% Difference		-11.7		-19.0

Table 79. Whole blood stability of Cycloserine after 1 hour

	High Concentration		Low Concentration	
	Storage: 0 min Peak Area	**Storage: 60 min Peak Area**	**Storage: 0 min Peak Area**	**Storage:60 min Peak Area**
Sample 1	1090000	1020000	32900	23900
Sample 2	1050000	1030000	33100	27700
Sample 3	1150000	1020000	34500	24600
Sample 4	1190000	999000	36500	23600
Sample 5	1190000	1120000	32300	27400
Sample 6	1150000	1120000	33300	23300
Average	1136667	1051500	33767	25083
STDEV	56095	54014	1521	1961
% CV	4.9	5.1	4.5	7.8
% Difference		-7.5		-25.7

Table 80. Whole blood stability of Cycloserine after 30 minutes

	High Concentration		Low Concentration	
	Storage: 0 min Peak Area	**Storage: 30 min Peak Area**	**Storage: 0 min Peak Area**	**Storage: 30 min Peak Area**
Sample 1	1090000	1040000	32900	33000
Sample 2	1050000	1090000	33100	31500
Sample 3	1150000	1060000	34500	36600
Sample 4	1190000	1080000	36500	34100
Sample 5	1190000	1070000	32300	36900
Sample 6	1150000	1060000	33300	36300
Average	1136667	1066667	33767	34733
STDEV	56095	17512	1521	2213
% CV	4.9	1.6	4.5	6.4
% Difference		-6.2		2.9

Acceptance criteria: Peak areas of the test samples (120 min, 60 min and 30 min) were compared to the peak areas of the reference samples (0 min). A high % Difference (> 15%) between the sets could indicate whole blood instability.

Conclusion: The observed % difference of the 60 and 120 minute samples did not fall within the accepted limits, indicating that Cycloserine is stable in whole blood on ice for only 30 minutes.

5.4.7. Specificity

The very high specificity of the LC-MS/MS assay procedure precludes the detection of any compounds that do not possess the capability to produce the specific parent ion followed by formation of the specific product ion produced and monitored in the mass spectrometer. A representative chromatogram of STD 1 is presented in Figure 30.

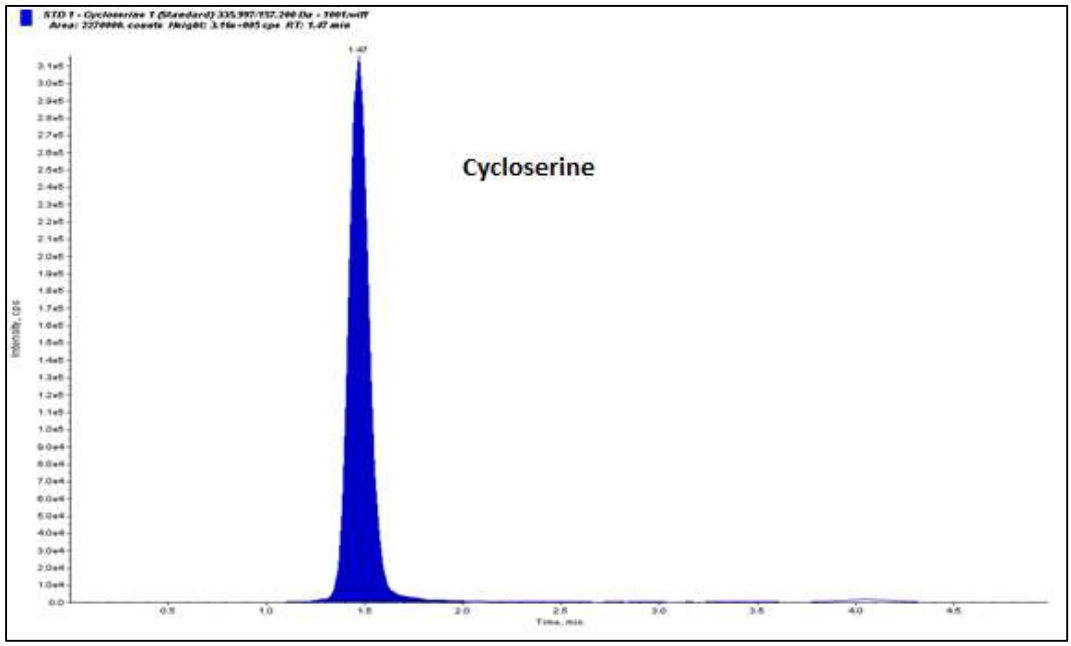

Figure 30. Representative chromatogram of STD 1

5.4.8. Carry-over

A blank sample (without analyte) was positioned in the injection sequence immediately after the highest calibration standard in order to assess possible carry-over effects. A chromatogram of a blank sample is presented in Figure 31.

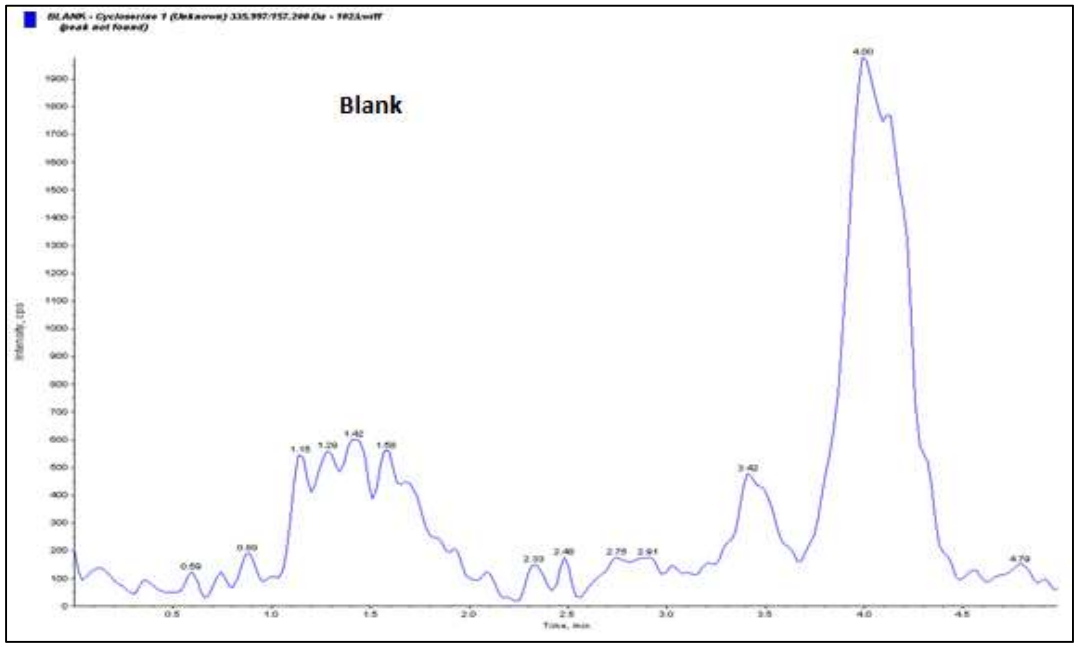

Figure 31. Chromatogram of a blank plasma sample

Acceptance criteria:

Blank assessing carry-over: A peak that is observed for the analyte should not be > 20% of the area of the peak obtained at the LLOQ.

Conclusion: No carry over peaks were observed.

5.4.9. Sensitivity

The LLOQ of this method is 0.313 µg /ml and a representative chromatogram presented in Figure 32 and the signal -to-noise shown in Figure 33.

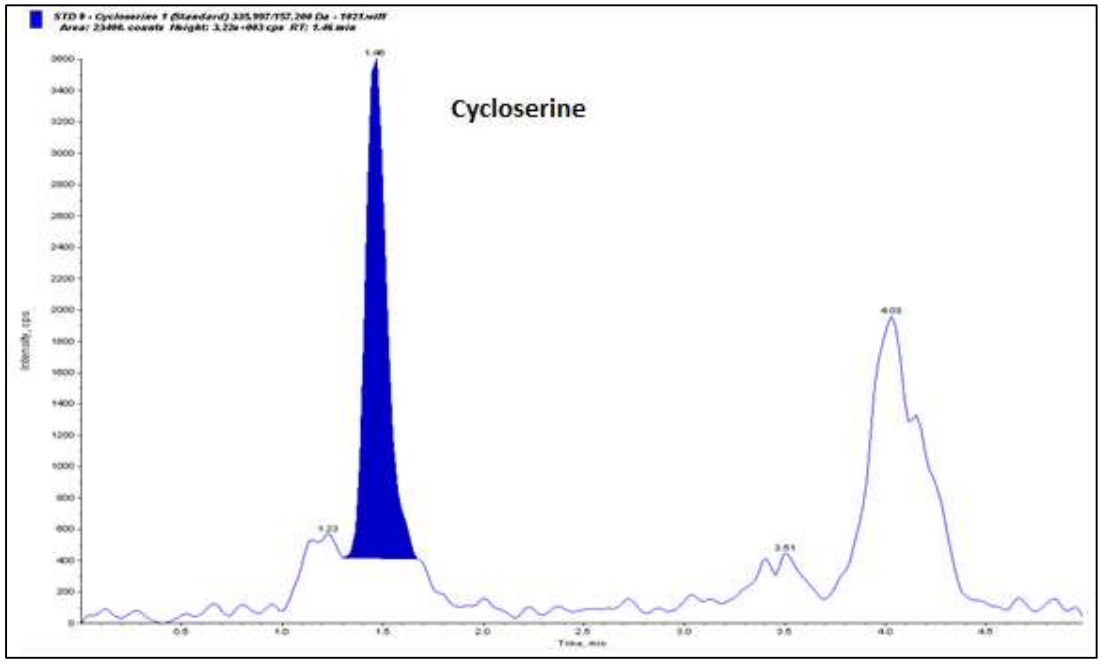

Figure 32. Chromatogram of a lower limit of quantification (LLOQ) sample

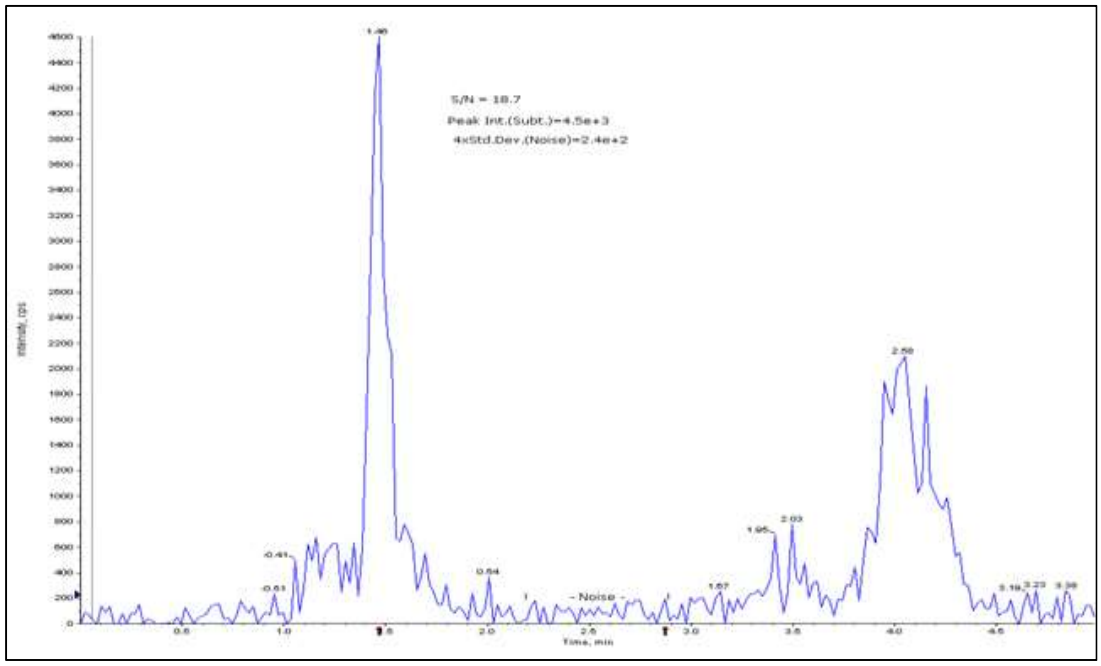

Figure 33. Chromatogram of the S/N ratio of a lower limit of quantification (LLOQ) sample

Acceptance criteria: The mean analyte signal/noise response at LLOQ should be at least > 5 times the response compared to blank response at the retention time of interest.

Conclusion: The raw LLOQ sample chromatograms showed acceptable intensities for the analyte with an average signal/noise ratio of 18:1 for cycloserine (*N=2*).

5.4.10. Recovery

The extraction recovery pertains to the extraction efficiency of the analytical process within the limits of variability. It is determined by comparing the analytical response of blank matrix spiked with the analyte and extracted with the response of the blank matrix first extracted and then spiked with analyte (theoretical, represents 100% recovery).

a. Extracted (test) samples: A minimum of six QCs at each concentration level (low, medium and high) were extracted as per the analytical method.

b. Theoretical samples: Samples were spiked at each concentration level (relative to the final concentration of the corresponding extracted QC's level) in six-fold using extracted blank matrix.

The analyte peak areas found after extraction compared to the theoretical peak area expressed as a percentage recovery as represented in Table 81.

Table 81. Recovery for Cycloserine

	High Concentration (32.0 µg/ml)		Medium Concentration (16.0 µg/ml)		Low Concentration (0.783 µg/ml)	
	Precipitation: Peak Area	Solution: Peak Area	Precipitation: Peak Area	Solution: Peak Area	Precipitation: Peak Area	Solution: Peak Area
Sample 1	2620000	3250000	1370000	1770000	65100	106000
Sample 2	2610000	3040000	1390000	1770000	84800	115000
Sample 3	2570000	3020000	1630000	1470000	77000	112000
Sample 4	2520000	2950000	1430000	1880000	73600	126000
Sample 5	2540000	3140000	1310000	1790000	84500	119000
Sample 6	2460000	3070000	1400000	1800000	91800	125000
Average	2553333	3078333	1421667	1746667	79467	117167
STDEV	59889	104579	109621	141516	9516	7731
% CV	2.3	3.4	7.7	8.1	12.0	6.6
% Recovery		82.9		81.4		67.8
				Average % Recovery		77.4
				Average % CV		10.7

Acceptance Criteria: The mean recovery of a quantitative drug assay method should be consistent and the precision of the measured recovery expressed as percentage coefficient of variation should not exceed 15% for any particular concentration of the analyte at which it is determined. Recovery reproducibility between concentration levels should not be > 15%.

Conclusion: The mean recovery of cycloserine from plasma over the calibration range is 77.4 % with a CV (%) of 10.7.

5.4.11. Matrix effect

In biological chemical analysis, matrix refers to the components of a sample other than the analyte. Matrix effects are of particular importance when dealing with LC-MS analyses, and may only become evident once novel clinical samples are analysed. The presence of co-extracted matrix background components may influence analyte ionization. Appropriate steps should be taken to minimize the influence of matrix components. The Matuszewski method attempts to quantify the matrix effect across the calibration range of the assay [61].

A minimum of six different blank sources of the appropriate biological matrix and anticoagulant type was extracted. Each individual matrix is spiked at low, medium and high

concentration levels (taking into account any calculations for dilutions in the analytical method). The results are presented in Table 82 and the overall CV (%)'s of the slopes calculated.

Table 82. **Cycloserine peak areas**

	High Conc. 4000 ng/ml Peak Area	Medium Conc. 2000 ng/ml Peak Area	Low Conc. 783 ng/ml Peak Area	Area v Conc. Regression Slope
K_3EDTA 1	1340000	917000	51700	32.8
K_3EDTA 2	1200000	898000	54700	29.1
K_3EDTA 3	1240000	899000	57200	30.1
K_3EDTA 4	1250000	929000	65000	30.1
K_3EDTA 5	1260000	976000	62200	30.4
K_3EDTA 6	1300000	946000	71200	31.2
Average	1265000	927500	60333	30.6
STDEV	48888	29965	7203	1.26
% CV	3.9	3.2	11.9	4.1

Acceptance criteria: The peak areas of the analyte for each level in each matrix source are used to generate regressions for each individual matrix. The slope variability (CV %) for the 6 different matrix sources should not be > 5%.

Conclusion: The slope variability (CV %) for 6 different plasma samples was 4.1% for cycloserine which indicates that matrix effects do not adversely influence the precision of the assay.

5.4.12. Effect of Haemolysis

The presence of haemolysed blood in samples could affect the ionization of the analyte during assay. Evidence should be provided that haemolysis has no effect on analyte quantification. Haemolysis was tested at 2% haemolysed blood in plasma.

The influence of haemolysed blood was assessed by the assay of 6 haemolysed samples at high and low cycloserine concentrations and compared to the assay of 6 normal human plasma samples at the same high and low concentrations.

The areas observed for cycloserine in the haemolysed plasma samples were compared with the areas observed in the normal plasma samples, data represented in Table 83.

Table 83. Effect of 2% haemolysis

	High Concentration		Low Concentration	
	Observed Normal Peak Area Ratio	Observed Haemolysed Peak Area Ratio	Observed Normal Peak Area Ratio	Observed Haemolysed Peak Area Ratio
Sample 1	2540000	2830000	81600	70600
Sample 2	2620000	3050000	[100000]	81200
Sample 3	2790000	2840000	69900	79300
Sample 4	2670000	2970000	63700	75200
Sample 5	2610000	2850000	69000	77500
Sample 6	2870000	2870000	71000	75800
Average	2683333	2901667	71040	76600
STDEV	123558	88638	6536	3684
% CV	4.6	3.1	9.2	4.8
% Difference		8.1		7.8

[] Outlier excluded from the analysis

Acceptance criteria: A high % Difference (higher than 15%) between the peak areas observed in haemolysed samples and normal samples and a high CV % (higher than 15%) indicates that haemolysis has an effect on the assay of the analyte.

Conclusion: The results reported above show that the % Difference are within 15% for cycloserine in 2% haemolysed plasma samples and has no significant effect on analyte quantification.

5.4.13. Matrix anticoagulant effect

To evaluate the influence of the matrix anticoagulant on the analytes ionization and accuracy, a set of quality controls was prepared in a matrix containing lithium heparin as the anti-coagulant, which differed from the K_3EDTA used for the preparation of the initial calibration standards and quality controls and analysed in a validation batch. The results are presented in Table 84.

Table 84. Cycloserine anticoagulant effect

High Concentration		Medium Concentration		Low Concentration	
Nominal Conc. (µg/ml)	**Observed Conc. (µg/ml)**	**Nominal Conc. (µg/ml)**	**Observed Conc. (µg/ml)**	**Nominal Conc. (µg/ml)**	**Observed Conc. (µg/ml)**
32.0	28.1	16.0	14.3	0.783	0.850
	29.4		13.3		0.739
	28.1		14.8		0.778
	28.1		14.5		0.814
	27.1		14.4		0.819
	28.4		13.4		0.835
Average	28.2	**Average**	14.1	**Average**	0.806
STDEV	0.738	**STDEV**	0.618	**STDEV**	0.0407
% CV	2.6	**% CV**	4.4	**% CV**	5.1
% Difference	-11.9	**% Difference**	-11.8	**% Difference**	2.9

Acceptance criteria: The normal acceptance criteria for quality controls apply, the accuracy is required to be within 15% (i.e. % Accuracy should be between 85–115%) and the precision is required to be less than 15 % (i.e. CV (%) should be less than 15%).

Conclusion: The resulted precision and accuracy fall within the accepted values, which indicate that the use of lithium heparin vs. K_3EDTA anticoagulant in the matrix has no significant influence on the precision and accuracy of cycloserine concentration determinations.

5.4.14. Dilutions

To determine if samples originally reported as ALQ may be diluted to within the calibration range with accuracy and precision, six extra high QC samples were prepared at a concentration two times higher than the highest QC (64.0 µg/ml) for cycloserine. These were then diluted 1:4 with blank plasma. The concentration was determined and compared with the nominal concentration to determine the percentage accuracy, data presented in Table 85.

Table 85. Sample dilution

Sample ID	Nominal Conc. (µg/ml)	Mean Observed Conc. (µg/ml)	Std Dev	% CV	% Accuracy	*N*
QC DIL	64.0	59.8	1.46	2.4	93.5	6 of 6

Acceptance criteria: The final mean calculated concentration (incorporating the dilution factor) is determined from the calibration curve and compared to the nominal concentration. The accuracy of the diluted samples is required to be within 15% (i.e. % Accuracy should be between 85–115%) and the precision is required to be less than 15% (i.e. CV (%) should be less than 15%).

Conclusion: The resulted precision and accuracy fall within the accepted values.

5.5. Discussion

5.5.1. Validation data summary

Accuracy and precision for the validation of the method were assessed over three consecutive, independent runs and the calibration curve fits a quadratic (weighted by 1/x concentration) regression over the range 0.313–40.0 µg/ml for cycloserine.

A 1:4 dilution of the QC Dilution sample showed that concentrations of up to 64.0 µg/ml of cycloserine in plasma could be analysed reliably when diluted into the calibration range. The method was specific for cycloserine, with no carry over peaks observed.

Endogenous matrix components were found to have an insignificant effect on the reproducibility of the method when human plasma originating from six different sources was analysed. Also, quantification of cycloserine in plasma was not significantly affected by the presence of haemolysed blood (2 %) and when K3EDTA was used as anti-coagulant instead of lithium heparin.

Cycloserine was found to be stable in human plasma for 17 months at ~ -80°C, for up to 18 hours at room temperature and when subjected to 3 freeze-thaw cycles. Stock solutions of cycloserine are stable in water and methanol for 10 days when stored at ~ -80°C and for 18 hours stored at room temperature, ~4°C and ~ -20°C, respectively. Cycloserine is shown to be stable on-instrument over a period of ~29 hours. Reinjection reproducibility experiments indicate that a batch may be re-injected within 29 hours. Cycloserine is stable in whole blood (on ice) for up to 30 minutes.

In summary, the method is suited for the analysis of cycloserine in plasma using a derivatization procedure with dansyl chloride.

5.6. Clinical application of the method.

This method was successfully use for the assay of samples in a clinical study.

5.6.1.1. Summary of study protocol

TITLE: PHARMACOKINETICS AND TOXICITY OF SECONDLINE ANTITUBERCULOSIS DRUGS IN HIV-INFECTED AND UNINFECTED CHILDREN

NIH R01: 069169-01

The project aims to improve the health of children with drug-resistant tuberculosis (DR-TB) in need of second-line anti-TB drugs, through examining the pharmacokinetics (PK), safety profile and toxicity of commonly used second-line anti-TB drugs in children, with and without HIV co-infection. The specific aims are:

1) to compare the PK of second-line anti-TB drugs in children (≤ 15 years) by age;

2) to compare the plasma concentrations of anti-retroviral (ARV) drugs in HIV-infected children (≤ 15 years) on second-line anti-TB drugs to those not on anti-TB therapy; and

3) to characterize the tolerability and toxicity of second-line anti-TB drugs in HIV-infected and uninfected children.

Childhood TB represents 15–20% of the disease burden in settings where TB and HIV infection is prevalent. MDR-TB; i.e. TB resistance to both rifampin and isoniazid, is an emerging epidemic, with an estimated 489 000 global cases annually [13]. MDR-TB patients often experience prolonged diagnostic delay, resulting in transmission of these strains especially to young children, with a sharp increase in the numbers of children on treatment and prophylaxis for MDR-TB. This hospital-based study was implemented in Cape Town, Western Cape Province, South Africa at 2 referral hospitals – Brooklyn Hospital for Chest Diseases and Tygerberg Children's Hospital. The prevalence of MDR-TB amongst children with culture-confirmed TB was 8.6% and the prevalence of HIV infection in children with TB was 25-30% during 2009. The most frequently used second-line anti-TB drugs in children in this setting was ethionamide, fluoroquinolones, amikacin and terizidone.

Capreomycin, linezolid and PAS were typically used for treatment of extensively drug-resistant TB. Although second-line anti-TB drugs are routinely given and recommended in children, there is limited information available to inform the accurate dosing in young HIV-infected children, who may have altered drug metabolism and unique drug-drug interactions. The literature provides limited data on toxicity of these anti-TB drugs in children, who are typically treated for 18–24 months for DR-TB.

This study provides a longitudinal, hospital-based, observational PK observation of children aged 0–15 years who received routine chemotherapy or chemoprophylaxis for the treatment or prevention of DR-TB. 318 children were enrolled for observation consecutively over 3.5 years, undergoing intensive PK sampling of second-line anti-TB drugs at baseline. The subjects were undertaking routine treatment for DR disease until treatment completion for clinical outcomes, including TB treatment response and drug adverse effects.

Approximately 30% of the sample were co-infected with HIV. An equal number of HIV-infected children with and without anti-TB therapy (concurrent controls; 42 on efavirenz and 22 on lopinavir) were enrolled, based on the required age strata to allow for comparison of the effect of second-line TB drugs on ARV levels in HIV-infected children with and without TB treatment. HIV-infected children had ARV PK sampling done at baseline. Enrolment was balanced by HIV status and age to ensure adequate numbers of children 0–2 years of age and adequate numbers of HIV-infected children.

The TB medication regimen was based on local and international recommendations for routine care. The PAS local and international dose recommendations for children with TB is 150 mg/kg body weight up to a maximum of 1200 mg/day. This was therefore, not an interventional study. No new medications or new dosages were assessed.

5.6.1.2. Ethical approval for the study.

The study was formally approved by the Faculty of Health Science Research Ethics Committee, see Appendix 2 for approval letter.

5.6.1.3.Accuracy and Precision

This method was accurate and precise, showing a coefficient of variation and bias within 15% at all levels of quality controls used in each the 12 batches of unknown sample runs, data represented in Table 86.

Table 86. Summary of Quality Control data from 12 sample run batches

	High (32.0 µg/ml)	Medium (16.0 µg/ml)	Low (0.783 µg/ml)
Mean % Accuracy	104.4	99.7	98.0
STDEV	11.20	7.03	8.63
RSD (%)	10.8	7.0	8.8
N	24/24	*23/24	24/24

** = One outlier with MNR-ESD Outlier Test, excluded from table.*

The mean % accuracy for the low, medium and high quality control concentrations were 98.0, 99.7 and 104.4 with a precision CV % of 8.8, 7.0 and 10.8, respectively.

5.6.1.4. Suitability

The calibration range of 0.313–40 µg/ml was appropriate for the quantification of cycloserine samples generated in this study. Only 0.15% of the total number of sample (657) was below the limit of quantification and 6.8% of the sample was ALQ of the calibration range. All ALQ samples was diluted into the calibration range, none of the diluted samples was above the validated dilution range of 64 µg/ml.

The method was sensitive and LLOQ of this method, 0.313 µg/ml, enabled the determination of cycloserine concentrations over the total drug profile for at least 8 hours. With a half-life of 10 hours it was possible to follow the drug elimination for approximately one half-life in the 8 hour drug profile.

5.6.1.5. Drug levels measured in patient samples.

The average plasma concentration (standard deviation) versus time profiles is shown in Figure 34 for 109 children dosed with terizidone at 10–20 mg/kg/day. The Cmax varied between subjects with 14.3 µg/ml as the lowest and 54.6 µg/ml as the highest Cmax concentration and an average Cmax of 26.8 µg/ml at an average Tmax at 2 hours. With a MIC for terizidone / cycloserine of 5–20 µg/ml this MIC was achieved in all of the subjects.

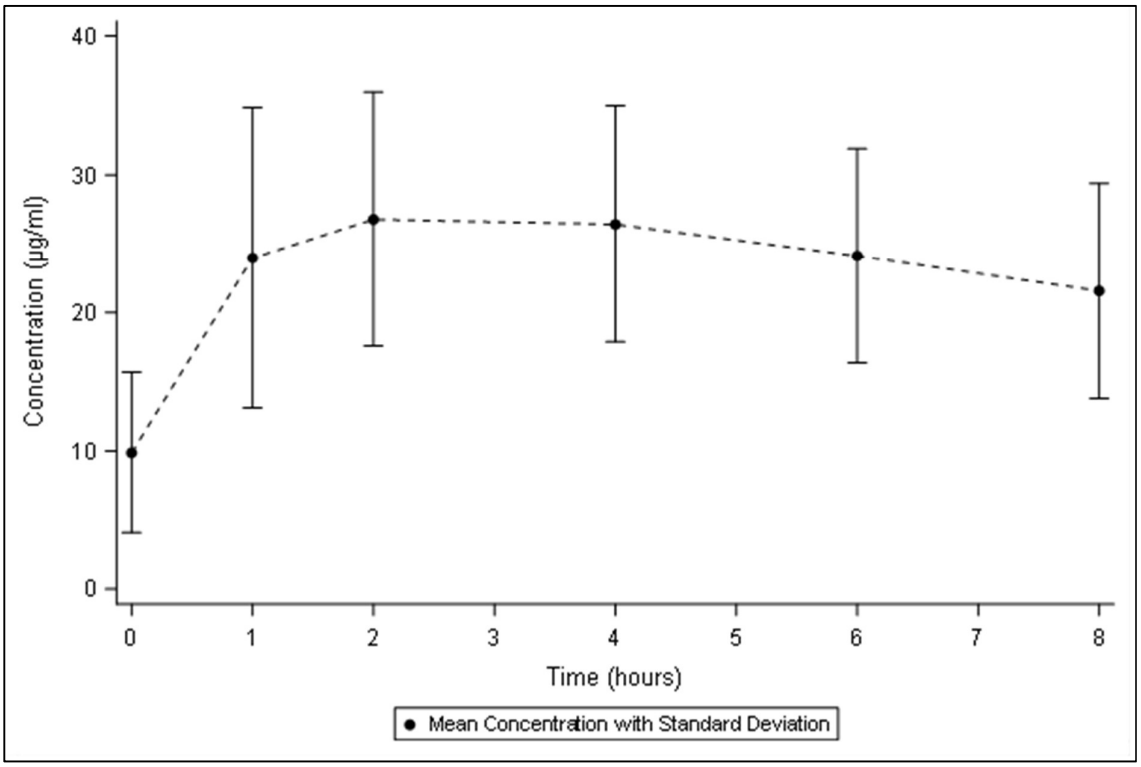

Figure 34. Average concentration with standard deviations vs time profile in children after receiving a terizidone dose of 10–20 mg/kg/day.

5.7. Conclusion

The developed LC–MS/MS method was successfully validated and employed for quantitative determination of cycloserine concentrations in a clinical study following oral administration of 10–20 mg/kg/day terizidone in children with DR-TB in need of second-line anti-TB drugs. The proposed method is affordable, reliable, simple, selective, rugged and reproducible. The method was developed with good peak shape and low base line noise. The mobile phase was optimized in simple isocratic mode and one of the major advantages of this method is that only 20 µl of plasma is needed for analysis, which greatly facilitates the collection of blood samples in children. The method produced reliable data that contributed to the pharmacokinetics and understanding of terizidone as a second line anti-TB drug.

6. APPENDIX 1

Development, validation and application of a LC-MS/MS method to accurately quantify *para*-aminosalicylic acid in 20 µl human plasma

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Abstract

A selective and sensitive liquid chromatography-tandem mass spectrometry method (LC-MS/MS) was developed and validated for the quantification of *para*-aminosalicylic acid (PAS) in 20 µl human plasma over the range of 0.391 to a 100 µg/ml. Sample extraction included protein precipitation with 200 µl of methanol containing rilmenidine as the internal standard. The mean recovery was 100.3% across low, medium and high test concentrations. The retention times for PAS and rilmenidine were 2.4 and 1.6 minutes respectively. PAS was shown to be stable in methanol for two days at approximately -80°C, and for 24 hours at ~4°C and ~-20°C. PAS was stable for up to 21 hours at room temperature and when subjected to three freeze-thaw cycles. Extracted samples were stable on instrument over a period of 56 hours. The overall accuracy of the validation batches ranged between 93.3 and 108.5% with a percentage coefficient of variation below 5.1% for high, medium and low quality controls and 13.4% for the lower limit of quantification. The assay was robust and performed well when applied to paediatric clinical samples generated during a clinical multidrug-resistant tuberculosis research study in South Africa.

Keywords: Tuberculosis, *Para*-aminosalicylic acid, LC-MS/MS.

1. Introduction

Para-aminosalicylic acid (PAS) is a second-line drug used to treat multidrug-resistant tuberculosis (MDR-TB) and is classified as a Group D drug for MDR-TB (i.e. an "add-on agent") by the World Health Organisation[1]. It was the first drug discovered to be active against tuberculosis (TB) and was introduced for clinical use in 1946[2, 3], but its popularity decreased after the introduction of the more potent drugs: rifampicin and pyrazinamide[4]. The emergence of MDR-TB has raised the significance of PAS in combination therapies. PAS both delays resistance

against streptomycin and isoniazid[5] and has a relatively well-tolerated adverse effect profile compared to other second-line drugs[1]. Effective as a bacteriostatic prodrug, PAS impacts the mycobacterial folate pathway by targeting dihydropteroate synthase (DHPS) and dihydrofolate synthase (DHFS), generating a hydroxyl dihydrofolate antimetabolite, which inhibits DHFR activity[6]. Clinical studies have demonstrated bactericidal effects at higher concentrations[7].

Pharmacokinetic and pharmacodynamic studies to optimize the dosing of PAS in combination MDR-TB therapies are essential. PAS has been shown to lower plasma rifampicin concentrations by half following oral administration[8]. Children account for approximately 25 000-32 000 cases annually[9,10]. Currently, treatment guidelines for children with MDR-TB are extrapolated from adult studies, despite children having different absorption and clearance profiles compared to adults[11]. Pharmacokinetic studies of second-line anti-tuberculosis drugs, including PAS, in children with MDR-TB are important as current data is limited to a few studies[12].

Published bioanalytical methods to determine PAS concentrations require between 200 and 1000 µl plasma[13, 14], which is not optimal for therapeutic drug monitoring in young children where blood volumes are a major consideration [15].

This paper presents a sensitive and selective bioanalytical method capable of measuring PAS over the range of 0.391 to a 100 µg/ml from just 20 µl of plasma in order to study the pharmacokinetics of PAS, used in combination with other second-line TB drugs, in South African children.

2. Materials and methods

2.1. Materials and chemicals

Methanol (Burdick and Jackson, LC-MS) was purchased from Honeywell International Inc. (Muskegon, MI, USA), formic acid was purchased from Merck (Darmstadt, Germany) and all water used, purified by a Milli-Q reverse osmosis system and a Synergy S Kit Millipore Water Purification System from EMD Millipore (Billerica, MA, USA). PAS and rilmenidine reference standards were supplied by Toronto Research Chemicals (TRC, Ontario, Canada) and Shenzen Haorui Industrial Dev Co., Ltd. (Shenzhen, China), respectively. Drug free plasma was obtained from Western Province Blood Transfusion Services, South Africa. A Phenomenex Synergi Hydro-RP 4µm, 150 x 2.0 mm analytical column (Phenomenex, USA) was used for the separation of PAS and the internal standard.

2.2. Instrumentation

Liquid chromatography-tandem mass spectrometry method (LC-MS/MS) analysis was performed using an Agilent 1200 series binary pump (Agilent, CA, USA) interfaced with an AB Sciex API 3000 mass spectrometer (AB Sciex, Ontario, Canada) with an electrospray ionization (ESI) source. An HTC PAL autosampler (CTC Analytics AG, Zwingen, Switzerland) was used to inject samples.

2.3. Preparation of calibration standards and quality controls

Calibration standards and quality controls were prepared volumetrically in blank human plasma (anticoagulant K3EDTA) at room temperature. Two PAS stock solutions were prepared in methanol at 5000 µg/ml each and the first spiked into 4.90 ml blank plasma for the highest calibration standard of 100 µg/ml. This standard was serially diluted with normal blank plasma to produce a total of 9 calibration standards (50.0, 25.0, 12.5, 6.25, 3.13, 1.56, 0.781, 0.391 µg/ml). Five quality control samples were prepared using the same methodology; namely a dilution, high, medium, low and lower limit of quantification (LLOQ) control at concentrations: 160, 80.0, 40.0, 0.781 and 0.391 µg/ml, respectively. The samples were aliquoted into polypropylene tubes and stored at approximately -80°C to allow for duplicate 20 µl extractions from each tube.

2.4. Extraction procedure

A protein precipitation method was used to prepare samples at room temperature. Briefly, 20 µl of the thawed plasma sample was precipitated with 200 µl of a 250 ng/ml internal standard solution in methanol, except for the double blank sample, which was precipitated using pure methanol. Samples were vortexed for 30 seconds then centrifuged for 5 minutes at approximately 16 000 rcf. A hundred and fifty microliters of the supernatant was transferred to a 96 well plate and 50 µl of 0.2% formic acid in water (v/v) was added. Five microliters was injected onto the LC-MS/MS for quantification.

2.5. Liquid Chromatography

A Synergi Hydro-RP 4µm, 150 x 2.0 mm analytical column was used for chromatographic separation. An isocratic mobile phase containing 0.2% formic acid in water (v/v) and methanol (60:40, v/v) was used at a flow rate of 300 µl/min for 5 minutes. The retention times were between 1.60 and 1.70 minutes for rilmenidine and 2.20 and 2.40 minutes for PAS (shown in Figure 1).

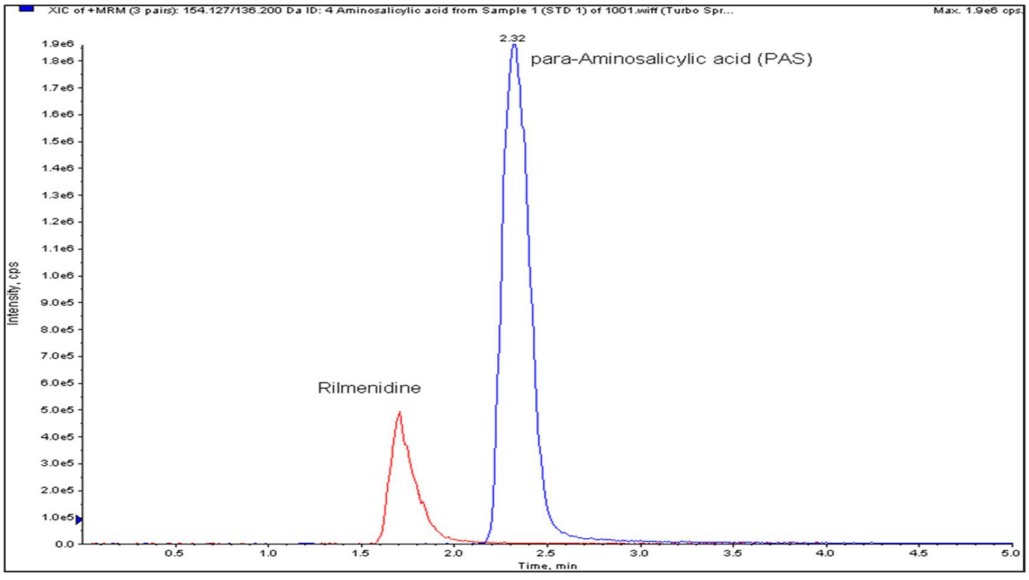


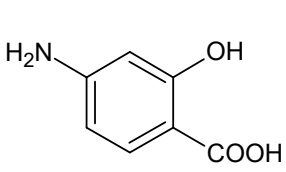
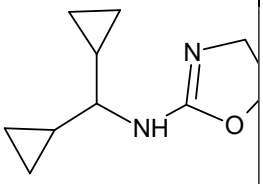
Fig. 1. Chromatogram of PAS (*para*-aminosalicylic acid) and internal standard (rilmenidine) at the highest calibration standard concentration.

2.6. Mass Spectrometry

ESI was performed in the positive ion mode with zero air used as the nebulizing and turbo spray gas. Nitrogen was

used as curtain gas, with the optimum values set at 6, 8 and 6 (arbitrary values) for nebulizing, turbo spray and curtain gas, respectively. A heated nebulizer temperature of 500°C and ion spray voltage of 5000 V was used. The instrument response was optimized for PAS and the internal standard rilmenidine, by infusing a solution of the compounds dissolved in mobile phase at a constant flow rate. The instrument was operated at unit resolution in the multiple reaction monitoring (MRM) mode with their respective transitions shown in Table 1. The pause and dwell times were set to 5 milliseconds and 150 milliseconds, respectively, and the collision gas (N_2) was set to medium (arbitrary value). The instrument was interfaced with a computer running Analyst software (version 1.4.2).

Table 1. Summary of the multiple reaction monitoring transitions for PAS (*para*-aminosalicylic acid) and internal standard rilmenidine

	PAS	Rilmenidine
Chemical Structure		
Protonated molecular ion mass (m/z) $[M+H]^+$	154.127	181.156
Product ion mass (m/z) Quantifier	80.151	95.1
Product ion mass (m/z) Qualifier	136.2	67.0

m/z = mass-to-charge ratio

2.7. Method Validation

2.7.1. Calibration standards, quality controls and dilution quality controls

Intra- and inter-batch accuracy and precision were determined by validating the calibration curve over a period of three days. High, medium, low and LLOQ quality control samples were analyzed in six-fold while the calibration standards were analyzed in duplicate over the range 0.391 to 100 µg/ml. Calibration curves were constructed using a weighted quadratic regression (1/concentration) of the peak area ratio of PAS to the internal standard versus nominal concentration. Accuracy and precision of the dilution procedure was evaluated by diluting and extracting six dilution quality control samples prepared at a PAS concentration double that of the highest quality control sample, namely 160 µg/ml. The dilution quality control samples were diluted 1:1 and 1:4 with blank plasma and their respective concentrations determined and compared with the nominal concentration.

2.7.2. Recovery

Recovery was evaluated at high, medium and low concentrations. Blank plasma was extracted as described for reference samples and spiked with PAS to generate matrix containing reference samples in six-fold at high, medium and low concentrations. The recoveries were calculated by comparing the peak areas of the post-spiked quality controls representing 100% theoretical recovery with those of the reference samples, also performed in six-fold.

2.7.3. Stock solution stability

Stock solution stability of PAS and rilmenidine in methanol was evaluated over 24 hours at room temperature, ~4°C, ~-20°C and over 48 hours at ~-80°C. Reference stock solutions were prepared fresh. Reference and test samples were diluted with injection volume to 100 µg/ml and 0.250 µg/ml for PAS and rilmenidine, respectively, and the peak areas of six separate samples compared for each.

2.7.4. Freeze-thaw stability

Freeze-thaw stability was evaluated at high and low concentrations, with quality control samples frozen at ~-80ºC, and put through three consecutive freeze and thaw cycles. Samples were frozen for at least 24 hours prior to starting this experiment. Each cycle consisted of sufficient thawing time at room temperature followed by 12–24 hours freezing time. These samples were analyzed against a fresh calibration curve and compared to the observed mean of the quality controls analyzed within the batch.

2.7.5. Benchtop and on-instrument stability, and reinjection reproducibility

To evaluate benchtop stability, low and high quality control samples were frozen at ~-80ºC, and subsequently left on the bench at room temperature to thaw for approximately 21 hours. Samples were analyzed against a fresh calibration curve. Reinjection reproducibility and on-instrument stability were determined by reinjecting the first validation batch after being kept in the autosampler at ~10°C over the course of 56 hours.

2.7.6. Whole blood stability, matrix effects, anticoagulant effect and haemolysis effect

To replicate typical clinical sampling conditions, whole blood stability was evaluated over 2 hours on ice. Whole blood was spiked, as previously described for plasma samples, at concentrations similar to high and low quality control concentrations. Six reference samples were spiked and immediately centrifuged and plasma stored, while six test samples were left on ice for 2 hours before centrifuging and storing plasma. Both sets were stored at ~-80°C before analysis.

Matrix effect was determined using the Matuszewski method[16]. Six different blank sources of plasma were extracted and spiked with PAS at low, medium and high concentration levels and a single concentration of internal standard, rilmenidine. The peak area ratios of PAS and the internal standard for each level in each matrix source were used to generate and compare regressions for each individual matrix. To evaluate the influence of matrix anticoagulant effects on analyte and internal standard ionization, high, medium and low quality controls were prepared in plasma containing lithium heparin as an anticoagulant and compared to the nominal concentration of the corresponding quality controls, when analyzed against the calibration curve prepared in K3EDTA.

The influence of haemolysed blood on ionization was evaluated by assaying 2% haemolysed samples at high and low

concentrations and comparing peak area ratios to six reference high and low quality controls.

2.7.7. Long term matrix stability

The long-term matrix stability in plasma was assessed by comparing test samples, prepared at high and low concentrations and kept for a period of time at approximately -80°C, to freshly prepared samples.

3. Results and Discussion

The LC-MS/MS method to determine PAS concentrations was successfully validated in accordance with the bioanalytical validation guidelines of the US Food and Drug Administration [17,18], European Medicines Agency[19,20], and White Paper publications[21–28]. The overall accuracy and precision of the assay procedure, as calculated by the accuracy and precision over the three validation batches, are shown in Table 2. A range of 93.3 to 108.5% for quality control concentrations (N = 18, with one outlier observed for the low-quality control), is well within international accepted guidelines of 85–115% accuracy with a coefficient of variance (CV) below 15 and 20% for the LLOQ. Dilutions of 1:1 and 1:4 were successfully validated for concentrations of up to 160 µg/ml. The calibration curve fits a quadratic regression using peak area ratio of analyte to internal standard with 1/concentration weighting and showed high reproducibility with a mean r^2 of 0.9995 over the three validation batches.

Table 2. Summary of validation quality control results

Sample ID	Nominal Concentration (µg/ml)	Mean Observed Concentration (µg/ml)	Standard Deviation	% CV	% Accuracy	N
QC 6 - LLOQ	0.391	0.385	0.0516	13.4	98.4	18 of 18
QC 5 - Low	0.781	0.847	0.0266	3.1	108.5	*17 of 18
QC 2 - Med	40.0	37.3	1.918	5.1	93.3	18 of 18
QC 1- High	80.0	76.4	3.11	4.1	95.5	18 of 18
QC DIL 1:1	160	162	11.6	7.1	101.2	6 of 6
QC DIL 1:4	160	156	8.7	5.6	97.3	6 of 6

* = Outlier with MNR-ESD Outlier Test

The LLOQ for this method, when injecting 5 µl onto the column, was validated at 0.391 µg/ml. A representative raw chromatogram is shown in Figure 2. The signal to noise ratio at LLOQ was well above the minimum international accepted criteria of greater than 5. Increasing injection volume could improve the sensitivity of this method, but this LLOQ was found to be adequate for this study. No interfering or late peaks were observed showing a highly specific LC-MS/MS method.

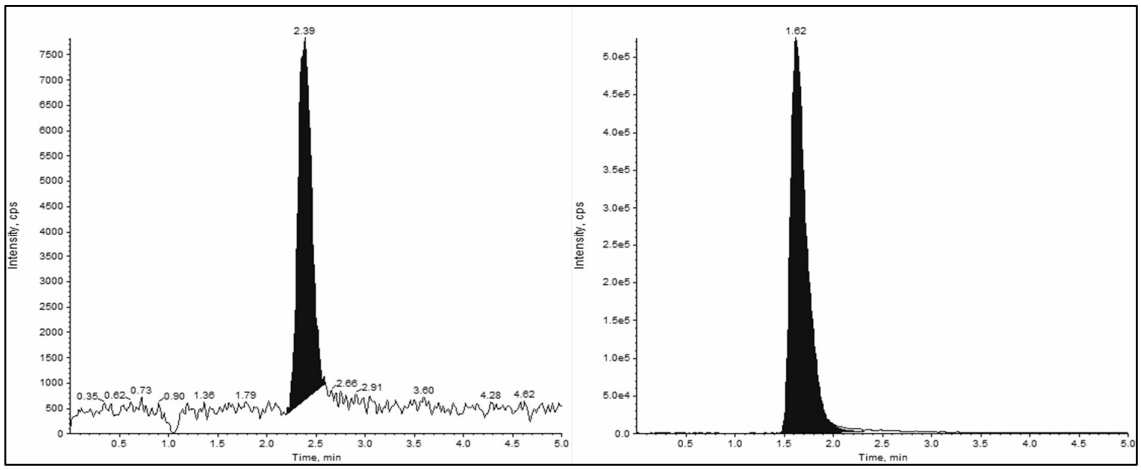


Fig. 2. Chromatogram of PAS (*para*-aminosalicylic acid) calibration standard at LLOQ (0.391 µg/ml).

The protein precipitation method used showed satisfactory extraction efficiency, with a mean recovery of 100.3% and a reproducibility CV of 3.3% for test controls of 100, 50.0 and 0.391 µg/ml ($N = 6$).

Stock solutions were shown to be stable under experimental conditions over a 24-hour period and had a percentage difference to fresh reference stocks of 1.6% at room temperature (CV% = 5.3, $N = 6$), 2.4% at ~4°C (CV% = 7.9, $N = 6$) and 0.03% at ~-20°C (CV% = 1.9, $N = 6$). The initial stocks kept at ~-80°C (CV% = 2.6, $N = 6$) were shown to be stable over 48 hours with a percentage difference to the freshly prepared reference stocks (CV% = 5.9, $N = 6$) of 0.9%.

Freezing calibration samples were found to have no significant impact on stability after eight days at ~-80°C when compared to a freshly prepared calibration curve with high, medium, low and LLOQ controls showing 92.3 - 109.3% accuracy and a highest observed CV of 6.9%. Freeze thaw stability was performed over three cycles at high and low concentrations and analyzed against a valid calibration curve. The samples were within 2.5% of the nominal concentration (CV% = 5.1, $N = 6$) showing acceptable freeze-thaw stability over 3 cycles when stored at ~-80°C.

Plasma samples were shown to be stable on bench over 21 hours at room temperature with a percentage difference of 6.9% and 0.9% for high (CV% = 5.0, $N = 6$) and low (CV% = 7.9, $N = 6$) quality controls respectively.

Reinjection reproducibility was determined by reinjecting the first validation run after both the second and third validation runs and showed satisfactory accuracy and precision, which indicated that a batch may be reinjected within 56 hours and quantified using the standards contained within the batch.

On-instrument stability for the analyte was indicated for at least 56 hours using high (CV% = 7.9, $N = 6$) and low (CV% = 7.9, $N = 6$) quality controls stored at an autosampler temperature of ~10°C.

PAS was shown to be stable for at least 2 hours on ice in whole blood for high (CV% = 5.2, *N* = 6) and low (CV% = 9.1, *N* = 6) quality controls with a difference of -2.2% and 0.9%, respectively.

No significant matrix, matrix anticoagulant or haemolysis effects were observed. Six plasma sources were used to assess matrix effect and slope variability was found to be 4.1%. Precision and accuracy of high, medium and low lithium heparin quality controls compared to a valid calibration curve prepared in K3EDTA had no significant influence on the precision and accuracy of the analyte concentration and was found to be within the allowed ranges for accuracy and precision. High (CV% = 6.7, N = 6) and low (CV% = 8.1, N = 6) quality control levels were used to assess effect of haemolysis in 2% haemolyzed samples and had a percentage difference of -6.6 and -11.1%, respectively. PAS was stable in plasma for 21 months kept at approximately -80°C, at high (CV% = 5.9, Difference% = -9.6%) and low (CV% = 3.9, Difference% = 0.2%) concentrations.

Small carry-over peaks were observed in the double blank samples positioned immediately after the highest calibration standard, but the peak areas were less than 20% of the LLOQ peak areas of the analyte and less than 5% of the internal standard peak areas, shown in Figure 3.

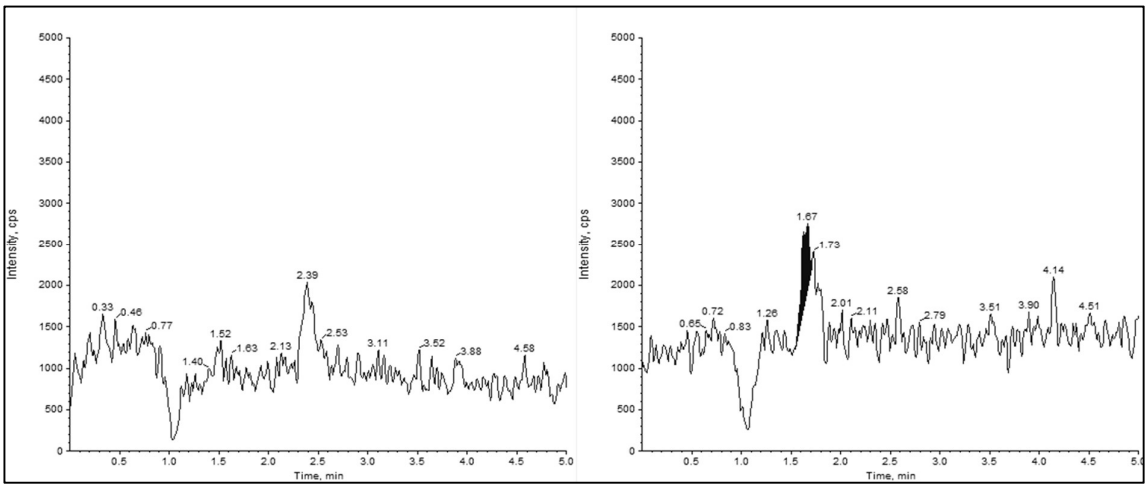


Fig. 3. Chromatogram showing minimal PAS (*para*-aminosalicylic acid) and rilmenidine carryover in the double blank sample after injecting the highest calibration standard.

This method was successfully used for the analysis of samples from a clinical study in children with MDR-TB, with and without HIV co-infection (NIH R01: 069169-01). All TB medications were dosed according to local and international recommendations for routine TB care in children. For PAS, this dose was 150 mg/kg body weight up to a maximum of 12 000 mg/day. This method proved to be accurate and precise, showing a coefficient of variation and bias below 7% for all quality control concentrations, high (CV% = 6.4, N = 10), medium (CV% = 5.6, N = 10) and low (CV% = 6.6, N = 10). A mean concentration versus time profile from 28 children is presented in Figure 4. Full clinical results will be reported elsewhere.

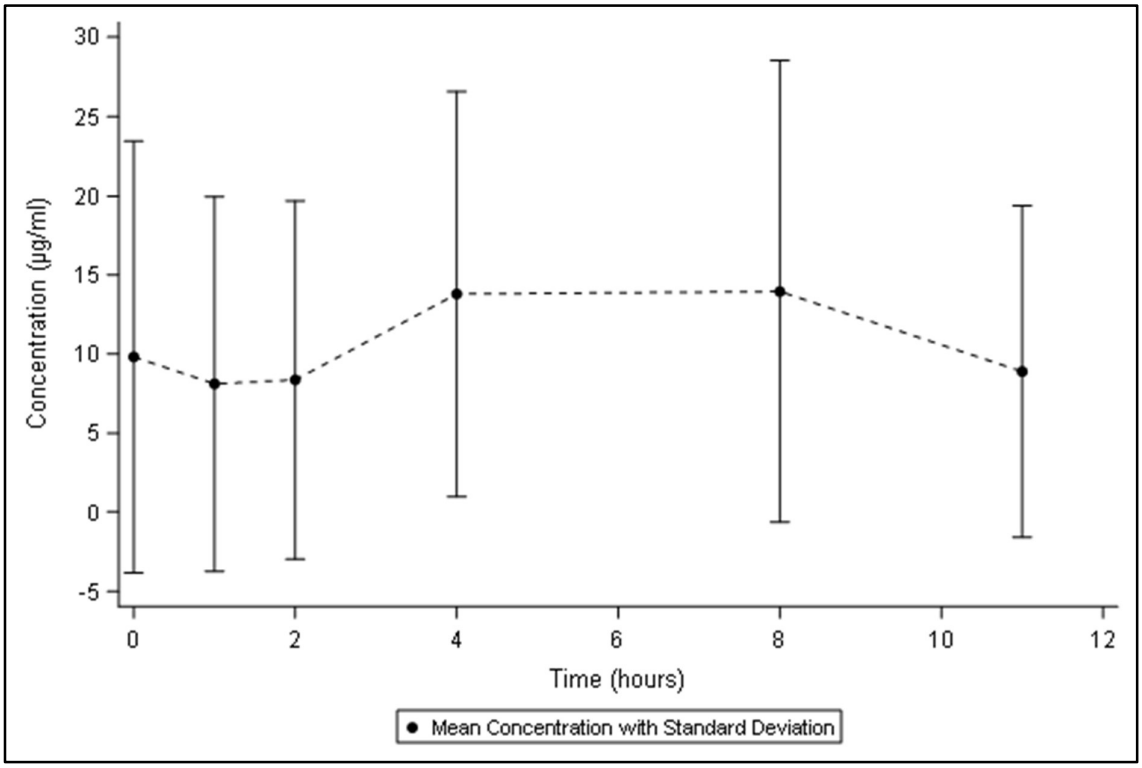


Fig. 4. Mean concentration vs. time profile from 28 children receiving a 150 mg/kg body weight dose of PAS (*para*-aminosalicylic acid).

4. Conclusions

A robust method to quantify PAS in 20 µl of plasma by LC-MS/MS using a simple protein precipitation extraction was successfully validated and applied to clinical samples. PAS was shown to be stable across various sample handling conditions and the extraction of 20 µl plasma showed high recovery, making this method ideally suited for high-throughput sample analysis and appropriate for monitoring PAS in adult and pediatric pharmacokinetic studies.

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The authors declare that there are no conflicts of interests.

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7.APPENDIX 2

UNIVERSITY OF CAPE TOWN

Faculty of Health Sciences
Faculty of Health Sciences Research Ethics Committee
Room E52-24 Groote Schuur Hospital Old Main Building
Observatory 7925
Telephone [021] 406 6338 • **Facsimile** [021] 406 6411
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28 September 2011

HREC REF: 397/2011

A/Prof H McIlleron
Pharmacology
K-floor
OMB

Dear A/Prof McIlleron

PROJECT TITLE: PHARMACOKINETICS AND TOXICITY OF SECOND-LINE ANTI-TUBERCULOSIS DRUGS IN HIV-INFECTED AND UNINFECTED CHILDREN (NIH R01; in response to RFA - Invesitgator -initiated)

Thank you for addressing the comments raised by the committee.

It is a pleasure to inform you that the Ethics Committee has **formally approved** the above-mentioned study.

Approval is granted for one year till the 30 September 2012.

Please submit a progress form, using the standardised Annual Report Form (FHS016), if the study continues beyond the approval period. Please submit a Standard Closure form (FHS010)if the study is completed within the approval period.

Please note that the ongoing ethical conduct of the study remains the responsibility of the principal investigator.

Please quote the REC. REF in all your correspondence.

Yours sincerely

Cedey Mendy

pp **PROFESSOR M BLOCKMAN**
CHAIRPERSON, HSF HUMAN ETHICS

Federal Wide Assurance Number: FWA00001637.
Institutional Review Board (IRB) number: IRB00001938

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