LC-MS/MS METHOD DEVELOPMENT AND VALIDATION FOR SIMULTANEOUS QUANTIFICATION OF FIRST-LINE HIV DRUGS AND SECOND-LINE TB DRUGS IN RAT PLASMA

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213511436

2018

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2018

This thesis is submitted to the School of Health Sciences, College of Health Sciences, University of KwaZulu-Natal, for the degree of Master of Medical Science in Pharmaceutical Chemistry.

This is the thesis in which the chapters are written as a set of discrete research publications that have followed the Journal of Pharmaceutical and Biomedical Analysis format with an overall introduction and final summary. Typically, these chapters will have been published in internationally recognized, peer-reviewed journals.

This is to certify that the contents of this thesis are the original research work of Ms. Thembeka Hlengiwe Malinga carried out under our supervision at the Catalysis and Peptide Research Unit, Westville campus, University of KwaZulu-Natal, Durban, South Africa.

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ABSTRACT

Tuberculosis (TB) and human immunodeficiency virus (HIV) co-infection continues to be a major public health concern, worldwide. HIV infection has increased the TB incidence over the past twenty years, making it hard to eliminate TB. At the same time, TB continues to be responsible for approximately 30% of deaths among HIV-infected individuals. Emtricitabine (FTC), efavirenz (EFV), and tenofovir (TFV) are constituents of a one-day-pill, Atripla™, which was approved in 2006 by the Food and Drug Administration (FDA). Atripla™ is a triple combination anti-HIV drug that provides an efficient dosing plan. Streptomycin (STR), kanamycin (KAN), and ofloxacin (OFL) are second-line anti-TB drugs used to treat multidrug-resistant/ extensively drug-resistant tuberculosis (MDR/XDR-TB). The worldwide increase in the prevalence of anti-TB drugs resistance is of concern to researchers since it remains one of the most significant threats to the community. Co-prescription of anti-HIV and anti-TB drugs poses a challenge of drug-drug interactions, which causes adverse effects. Therapeutic drug monitoring (TDM) seems to be the tool for a solution to these problems since it personalizes doses thus reducing drug toxicity. LC-MS/MS methods with short run times are required to produce effective TDM studies. Therefore, this study aimed to evaluate the new Ascentis Express column technologies [pentafluorophenylpropyl (F5), octadecyl (C18), biphenyl, and reversed phase amide (RP-Amide)] and their applicability to the simultaneous quantification of current first-line anti-HIV treatment Atripla. It also aimed to develop, optimize and validate a liquid-chromatography tandem mass spectrometry (LC-MS/MS) methods for the simultaneous quantification of anti-HIV drugs (FTC, EFV, and TFV) and second-line anti-TB drugs (STR, KAN, and OFL) in rat plasma for the usage of TDM. The currently used HPLC columns have longer run times making them impractical in a point of care environment since the number of patients and diseases is globally increasing. There are also no or very few studies regarding the LC-MS/MS method of simultaneous HIV and TB drugs for HIV positive TB patients. The biphenyl column showed consistency and optimum performance with regard to the number of theoretical plates, resolution and peak asymmetry factor. It showed good separation and overall effectiveness. However, this does not rule out other columns for other purposes intended to be accomplished. The LC-MS/MS method developed for the simultaneous quantification of anti-HIV drugs and second-line anti-TB drugs was short to eleven minutes and met all the recommendations by the European Medicines Agency (EMA) guidelines for bioanalytical method validation. The new HPLC column matrices (F5, C18, biphenyl, and RP-Amide) offer various benefits such as the potential of saving solvents and short runtimes, essential in TDM studies. Therefore, the usage of the new HPLC column technologies

will be beneficial in a point of care environment in terms of saving time and money. The LC-MS/MS method validated in this study can be used in clinical trials and in the simultaneous determination of the effective plasma concentrations of anti-TB and anti-HIV drugs, making it a strong candidate for TDM in a point of care setting.

DECLARATIONS

DECLARATION 1- PLAGIARISM

I, **Thembeka Hlengiwe Malinga** declare that

1. The research reported in this thesis, except where otherwise indicated, is my original work.
2. This thesis has not been submitted for any degree or examination at any other university.
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4. This thesis does not contain other person's writing, unless specifically acknowledged as being sourced from other researchers. Where other written sources have been quoted, then:
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Signed 

DECLARATION 2 - PUBLICATION

Detail of contribution to publication that form part and/or include research presented in this thesis (include publication in preparation, submitted and give details of the contributions of the first author to the experimental work)

LIST OF MANUSCRIPTS

1. **Performance comparison of fused core particle columns for the quantitation of ARVs in Atripla**

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Contribution:

Thembeka H. Malinga and *Xylia Q. Peters* - both contributed to the experimental procedure, study design, and writing of this study and publication.

Sphamandla Ntshangase, Sipho Mdanda, Annapurna Pamreddy, and Maya M. Makatini - provided technical assistance and support for this study.

Hendrik G. Kruger, Tricia Naicker, Thavendran Govender, and Sooraj Baijnath - supervised this study.

(Submitted to the Journal of Pharmaceutical and Biomedical Analysis, manuscript ID is **JPBA_2017_2611**)

2. LC-MS/MS method development and validation for simultaneous quantification of first-line HIV drugs and second-line TB drugs in rat plasma

Thembeka H. Malinga[1], Sphamandla Ntshangase[1], Sipho Mdanda[1], Annapurna Pamreddy[1], Hendrik G. Kruger[1], Thavendran Govender[1], Sooraj Baijnath[1]*

Contribution:

Thembeka H. Malinga - contributed to the experimental procedure, study design, and writing of this study and publication.

Sphamandla Ntshangase, Sipho Mdanda and Annapurna Pamreddy - provided technical assistance and support for this study.

Hendrik G. Kruger, Tricia Naicker, Thavendran Govender, and Sooraj Baijnath - supervised this study.

(**To be submitted**)

ACKNOWLEDGEMENTS

I would like to express sincere words of gratitude to:

- My supervisors, Prof Thavendran Govender, Dr Sooraj Baijnath, Prof Tricia Naicker and Prof Gert Kruger for their remarkable guidance, support and motivation throughout my studies. They have introduced me in medicinal chemistry perspective and understanding.
- My mentors Sphamandla Ntshangase, Sipho Mdanda and Dr. Annapurna Pamreddy for eagerly supporting me during my studies.
- The CPRU group 2017/2018 for their professionalism, eagerness to assist and each having ability to be a teamer player.
- I thank my family, my husband and my friend for their continuous support throughout the course of this degree.
- Biomedical Resource Unit, UKZN Westville, for use of their facilities for all animal studies.
- National Research Foundation (NRF, SA) and the University of KwaZulu-Natal (College of Health Sciences), for financial support.

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LIST OF ABBREVIATIONS

ADV-DP	Adefovir dipivoxil
AIDS	Acquired Immune Deficiency Syndrome
APCI	Atmospheric Pressure Chemical Ionization
APPI	Atmospheric Pressure Photo-Ionization
ART	Antiretroviral therapy
ARVs	Antiretrovirals
As	Asymmetry factor
ATV	Atazanavir
CD4	Cluster of Differentiation 4
CID	Collision-Induced Dissociation
C_{max}	Maximum Plasma Concentration
C_{min}	Minimum Plasma Concentration
CNS	Central Nervous System
d4T	Stavudine
DNA	Deoxyribonucleic acid
DTG	Dolutegravir
EFV	Efavirenz
EIC	Extracted ion chromatogram
EMA	European Medicines Agency

ENF	Enfuvirtide
EPG	Embedded Polar Group
ESI	Electrospray Ionization
ETR	Etravirine
F5	Pentafluorophenyl
FDA	Food and Drug Administration (USA)
FTC	Emtricitabine
GC-MS	Gas Chromatography Mass Spectrometry
HAART	Highly Active-Antiretroviral Therapy
HIV	Human Immune Virus
HPLC	High-Performance Liquid Chromatography
HQC	High quality control
IM	Intramuscular
INH	Isoniazid
InSTs	Integrase inhibitors
IS	Internal standard
KAN	Kanamycin
LC-MS	Liquid Chromatography Mass Spectrometry
LLOQ	Lower limit of quantification
LOD	Limit of detection

LPV	Lopinavir
LQC	Lower quality control
LTBI	Latent Tuberculosis Infection
M.tb	Mycobacterium tuberculosis
m/z	Mass to charge
MDR-TB	Multidrug-resistant Tuberculosis
MQC	Middle quality control
MRC	Maraviroc
MRM	Multiple-Reaction Monitoring
MS	Mass Spectrometry
N	Number of theoretical plates
NNRTIs	Non-nucleoside reverse transcriptase inhibitors
NRTIs	Nucleoside reverse transcriptase inhibitors
NVP	Nevarapine
OFL	Ofloxacin
PIs	Protease inhibitors
QC	Quality control
QqTOF	Quadrupole Time-of-Flight
R^2	Colleration coefficient
RGV	Raltegravir

RMP	Rifampicin
RNA	Ribonucleic acid
RP	Reversed phase
RP-Amide	Reversed-Phase Amide
Rs	Resolution
RSD	Relative standard deviation
RT	Reverse transcriptase
RTV	Ritonavir
S/N	Signal to noise ratio
STR	Streptomycin
TB	Tuberculosis
TDM	Therapeutic Drug Monitoring
TFV	Tenofovir
TFV-DP	Tenofovir disoproxil fumarate
TIC	Total ion chromatogram
T_{max}	Time at Maximum Plasma Concentration
TOF-MS	Time of flight-mass spectrometry
WHO	World Health Organization
XDR-TB	Extensively drug-resistant Tuberculosis
ZDV	Zidovudine

CHAPTER 1

1. Introduction

1.1 Human immunodeficiency viral infection

The historical evolution of the Human Immunodeficiency Virus (HIV) and Acquired Immune Deficiency Syndrome (AIDS) is very noteworthy. The first human HIV infection is likely to have occurred around 1933 in Africa due to cross-species transmission of the disease [1-3]. From Africa, it was presented in Haiti around 1969 and thereafter, in New York City (America) around 1972 [1-3]. When the first case of AIDS was recognized in 1981, HIV had been present in the United States for ten years [1-4].

In 2016, the approximate number of HIV positive individuals was 36 700 000 and 7 100 000, globally and in South Africa, respectively [5]. As of late, highly active antiretroviral therapy (HAART) has become an essential treatment strategy for patients suffering from HIV infection [6]. With the widespread utilization of antiretroviral drugs, the survival rate of HIV positive patients has significantly improved. Antiretroviral drugs ensure the patient's health is improved by increasing CD4 T-cells, fighting opportunistic infections, reducing time spent in hospital and regaining of body weight [6].

The virus's main objective, when it invades a human host is to multiply and replicate itself as many times as possible [7-9]. Its life cycle has six stages (**Figure 1**) [9]. The HI virus firstly attaches and enters a human cell [9-13]. When it enters the cell, its genetic code is in the form of ribonucleic acid (RNA). However, when it enters the host cell, RNA is transformed to deoxyribonucleic acid (DNA), so it could incorporate itself into the genetic machinery of the human cell using the enzyme called reverse transcriptase (RT) [9-13]. RT enzyme reads and transcribes the viral RNA sequence to complementary DNA [14-16]. The newly formed viral DNA then integrates with that of the human cell and triggers it to produce nucleotide sequences of the virus which is then referred to as the provirus. [9-13]. In addition to producing its nucleotides, it also produces long proteins which are cut into shorter proteins by a viral protease enzyme [9-13]. Some of these short proteins are used to form more viral enzymes, while the others are transformed to virus structural elements. The mature HIV virion then buds off the cell to infect other cells in the body [9-13]. Infected individuals are introduced to HIV treatment that will reduce rapid viral replication [9-13].

1.2 Antiretroviral therapy (ART)

The purpose of ART is to decrease and maintain the viral load in plasma at the lowest levels possible [17]. This antiretroviral therapy brings about the gradual recovery of the immune system in many patients, greatly reducing the risk of disease progression and death. Adherence to antiretroviral treatment is critical to guarantee viral control, and reduction [17].

There are currently five classes of antiretroviral therapy (ART) available in South Africa, namely; nucleoside and nucleotide reverse transcriptase inhibitors (NRTIs), [18-20] non-nucleoside reverse transcriptase inhibitors (NNRTIs), [18-20] protease inhibitors (PIs), [21-23] integrase inhibitors (InSTIs) [24-26] and fusion inhibitors [27, 28]. Each of these classes inhibits a specific stage in the HIV replication cycle (**Table 1, Figure 1**).

Table 1: Mechanism of action for ART classes

ART class	Drug	Mechanism of action	Reference
NRTIs	Tenofovir disoproxil fumarate (TFV-DP) Emtricitabine (FTC) Zidovudine (ZDV) Stavudine (d4T)	Inhibit transcription of viral RNA to DNA by mimicking the natural building blocks of DNA	[9, 27, 29]
NNRTIs	Efavirenz (EFV) Nevarapine (NVP) Etravirine (ETR)	Change the conformation of the catalytic site of RT and completely prevent its action	[9, 27, 29]

PIs	Ritonavir (RTV) Lopinavir (LPV) Atazanavir (ATV)	Inhibit the final maturation stages of HIV replication, resulting in the formation of non-infective viral particles	[27, 29]
InSTIs	Raltegravir (RGV) dolutegravir (DTG)	Inhibit the transfer of proviral DNA strands into the host chromosomal DNA	[27, 29]
Fusion Inhibitors	Maraviroc (MRC) enfuvirtide (ENF)	Bind to viral gp120 or gp41 or host cell CD4+ or chemokine (CCR5) receptors in order to prevent fusion of the HIV virus with the target CD4 cell	[27, 29]

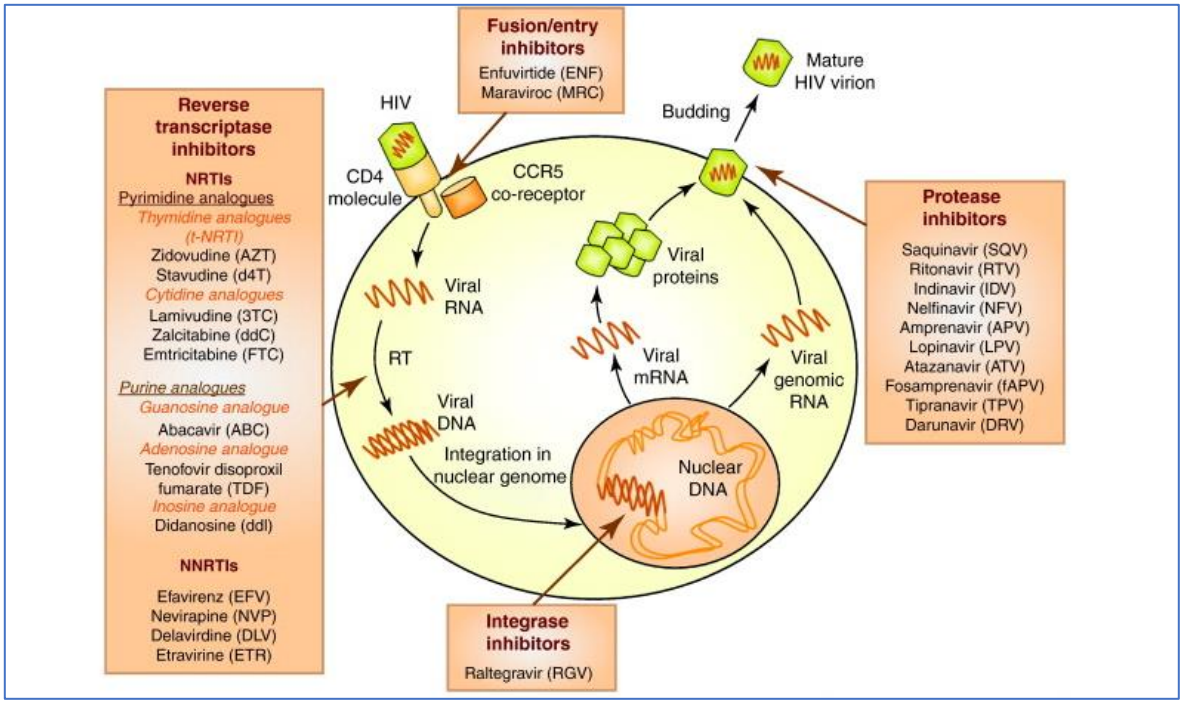

Figure 1: Antiretroviral classes mechanism and site of action (Figure is reused with permission from ref [28]). Copyright © 2011 Elsevier Ltd

South Africa has recently adopted a recommendation by the World Health Organization (WHO), that all HIV positive patients should be on ART regardless of their CD4 count [30]. Anti-HIV drugs are available as a fixed-dose combination and were implemented for best patients' benefits. Among the fixed-dose combination drugs available for HIV treatment, there are triple fixed-dose combinations, which generally consists of two NRTIs and an NNRTI or PI, for example, a one-day-pill, Atripla™, which was approved in July 2006 by the US Food and Drug Administration (FDA). It comprises of two NRTIs (FTC and TFV-DP), and one NNRTI (EFV) (**Figure 2, 3** and **4**) [31]. Of all fixed-dose combination treatments accessible now, Atripla™ presents the most effective dosing plan and the least number of pills [32, 33].

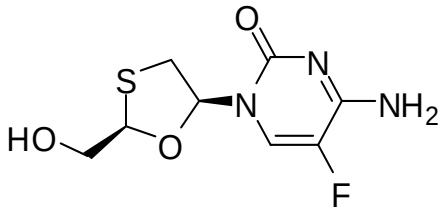

Figure 2: FTC drug structure

FTC (**Figure 2**) also known as Emtriva or 5-fluro-1-(2*R*, 5*S*)-[2-9hydroxymethyl]-1,3-oxathiolan-5-yl). It is classified as an NRTI that works against hepatitis B virus and HIV types I and II. FTC produces emtricitabine 5′-triphosphate by phosphorylation carried by cellular enzymes, which competes with deoxycytidine 5′-triphosphate and stops the new string of nucleotides that form the provirus [34].

A study by Molina et al., (2004), found the time at maximum plasma concentration (T_{max}) for FTC to be 1.8 hours with the maximum plasma concentration (C_{max}) of 1.8 mg/L and minimum plasma concentration (C_{min}) was found to be 0.04 mg/L when 200 mg of FTC on prescription was orally dosed once-daily for each patient [35]. In another study, Blum et al., (2007), found a T_{max} of 3.02 hours in plasma with a C_{max} of 1.77 mg/L and the C_{min} was found to be 0.064 mg/L when 200 mg of FTC was orally dosed once-daily for each patient [36].

Another way of determining the effectiveness of a drug is to determine its inhibitory concentration. A study by de Lastours et al., (2011), found FTC to have a concentration at which 50% infection was inhibited (IC_{50}) of 2 ng/mL against wild-type HIV in peripheral blood mononuclear cell [37] Another study, Saravolatz, (2006), found FTC to have an IC_{50} of 0.01 µmol/L in peripheral blood mononuclear cells in vitro activity against HIV [38].

Adverse effects of this drug includes the development of a rash, back pain, skin discoloration particularly on the soles of the feet and palms, abdominal pain, paresthesia, anxiety, pneumonia, urticarial, arthralgia, fever, increased cough, myalgia, dyspepsia, peripheral neuropathy, allergic reactions, and rhinitis, rash, urticaria [39].

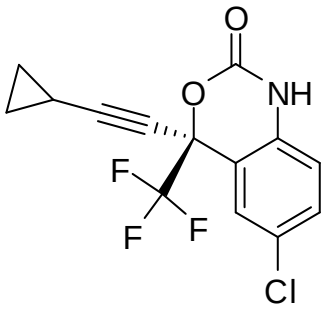

Figure 3: EFV drug structure

EFV (**Figure 3**) also known as Sustiva or (4S)-6-chloro-4-(2-cyclopropylethynyl)-4-(trifluoromethyl)-1H-3,1-benzoxazin-2-one. It belongs to the NNRTI class and is effective against HIV type I [40]. A study by Veldkamp et al., (2001), found the T_{max} of EFV to be 2 hours in plasma

with the C_{max} of 3.63 mg/L and C_{min} was found to be 1.55 mg/L when 600 mg once daily was orally dosed for each patient [41]. Another study by Molina et al., (2004), found the T_{max} of 2.5 hours in plasma with the C_{max} of 2.5 mg/L and C_{min} was found to be 1.0 mg/L when 600 mg of EFV once daily was orally dosed for each patient [35].

A study by Adkins and Noble, (1998), found that EFV demonstrated good inhibitory activity against wild-type HIV-1 replicative spread in primary lymphoid and monocytoid cell cultures with concentration producing 95% inhibition (IC_{95}) of 1.5 to 3.0 nmol/L [40]. Another study by Parkin et al., (2004), found EFV to have an IC_{50} of 1.6 nmol/L against wild-type HIV-1 in human plasma cell culture [42].

Adverse effects of this drug include insomnia, inability to concentrate, dizziness, abnormal dreams, headache, potential teratogenicity, benzodiazepine screening assays, and false-positive cannabinoid [43].

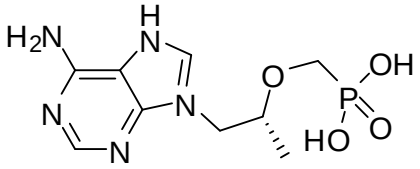

Figure 4: TFV drug structure

TFV-DP also known as Viread or 9-[(*R*)-2-[[bis[[(isopropoxycarbonyl) oxy] methoxy] phosphinyl] methoxy] propyl). TFV-DP is an NRTI under the class of acyclic nucleoside phosphonates and is effective on different drug-resistant HIV type I strains *in vitro*. TFV-DP is a prodrug found as tenofovir (TFV) (**Figure 4**) which is chemically described as; 9-[(*R*)-2-(phosphonomthoxy)-propyl] adenine) in blood plasma and converts intracellularly to its active metabolite by diphosphorylation [34]. A study by Du et al., (2017), found the T_{max} of TFV to be 1.3 ± 0.4 hours in plasma with the C_{max} of 447.1 ± 217.4 ng/mL when 300 mg once daily was orally dosed for each patient [44]. Another study by Blum et al., (2007), found the T_{max} of 2.43 hours in plasma with the C_{max} of 297 ng/mL when 300 mg of TFV, once daily was orally dosed for each patient [36].

A study by de Lastours et al., (2011), found TFV to have an IC_{50} of 10 ng/mL against wild-type HIV in peripheral blood mononuclear cells [37]. Another study by Grayson et al., (2010), reported TFV to have IC_{50} of 0.007 and 0.005 µM in MT-2 cells and peripheral blood mononuclear cells, respectively [45].

Adverse effects of this drug include vomiting, renal failure, asthenia, nausea, renal toxicity, headache, proteinuria, diarrhea, flatulence, and decreased bone mineral density[43].

Most of the adverse impacts of antiretroviral drugs come from drug-drug interactions [46]. The study on discontinuation of Atripla™ as first-line therapy in HIV-1 infected individuals that was conducted by Scourfield et al., (2012), found that Atripla™ is an effectual first-line antiretroviral treatment, yet roughly one of every five of all people starting Atripla™ should change treatment, usually due to side effects such as spontaneous abortions, rash, allergic reactions, diarrhoea, nausea, vomiting, mild and transient headaches, central nervous system (CNS) toxicity and dizziness [39, 47, 48].

Patients are always sceptical and have queries regarding drug toxicity, drug-to-drug interactions and drug resistance. A few investigations have shown a connection between efficacy and/or toxicity and plasma drug concentrations. Thus, therapeutic drug monitoring (TDM) of antiretroviral drugs is critical in clinical maintenance, in selecting the best dosage regimen, personalized to each patient to lessen the danger of virological failure from low plasma drug concentration and to prevent the toxicity associated with high plasma concentrations [31]. In scientific research, it is broadly acknowledged that an adherence level of no less than 90% is important to control the infection adequately in order to evade the danger of mutations and to avoid the occurrence of drug-resistant strains and drug failure [17].

1.3 Tuberculosis (TB)

South Africa is one of the countries experiencing a higher burden of TB, with the WHO estimating the prevalence of about 438,000 of people with active TB in 2016 [5]. Tuberculosis (TB) is caused by a bacterium called *Mycobacterium tuberculosis* (*M.tb*) which is an acid-fast and alcohol bacillus. It belongs to a group of microorganisms called *M.tb* complex [49]. Tuberculosis is an ancient human disease which primarily affects the lungs, resulting in pulmonary diseases. Extrapulmonary TB affects different organ systems, particularly, the central nervous system, the respiratory system, the skin, the gastrointestinal system, the liver, the musculoskeletal system, the lymph reticular system, and the reproductive system. The symptoms of this disease include a bad cough that lasts 3 weeks or longer, fever, fatigue, weight loss, coughing up mucus or blood, hemoptysis, loss of appetite, night sweats and sputum expectoration [50, 51]. TB is still responsible for a large burden of mortality and morbidity globally [49]. In 2016, there were approximately 10.4 million new (incidents) TB cases around the world, 90% of which were adults [5].

1.4 HIV-TB co-morbidity

Tuberculosis is still a major cause of death in developing nations particularly among people with a repressed immune system. [52]. HIV alters the clinical form of TB from a gradually advancing disease to one with a higher death rate [53, 54]. In 2016, WHO approximated that amongst people with latent TB infection (LTBI), individuals who are HIV-positive have a 26-fold-higher danger of the development of active TB when compared to those who are HIV-negative [53]. The HI Virus attacks the body's immune system by lessening the number of CD4-T cells, which are important in achieving a regulated active immune response to pathogens [52, 55]. This then brings about the loss of the body's capacity to stop the spread of the *M.tb* from localized granulomas leading to the spread of infection. TB can bring about the depletion of the $CD4^+$ cells and increment of the viral load thus speeding up the advancement of HIV disease to AIDS [53, 54].

TB accounted for 22% of worldwide mortality in people with HIV/AIDS in 2016 [5]. In 2008, South Africa accounted for 24% of HIV and TB incidence in the world while having just 1% of the planet's population [53]. This demonstrates that HIV is still the largest risk factor for TB around the world, and in locations where HIV incidence is high, the TB-HIV co-morbidity is rapidly growing [53]. In 2016, WHO estimated that 59% of TB incidence in South Africa is co-infected with HIV and 88% is on ART (**Figure 5**) [5].

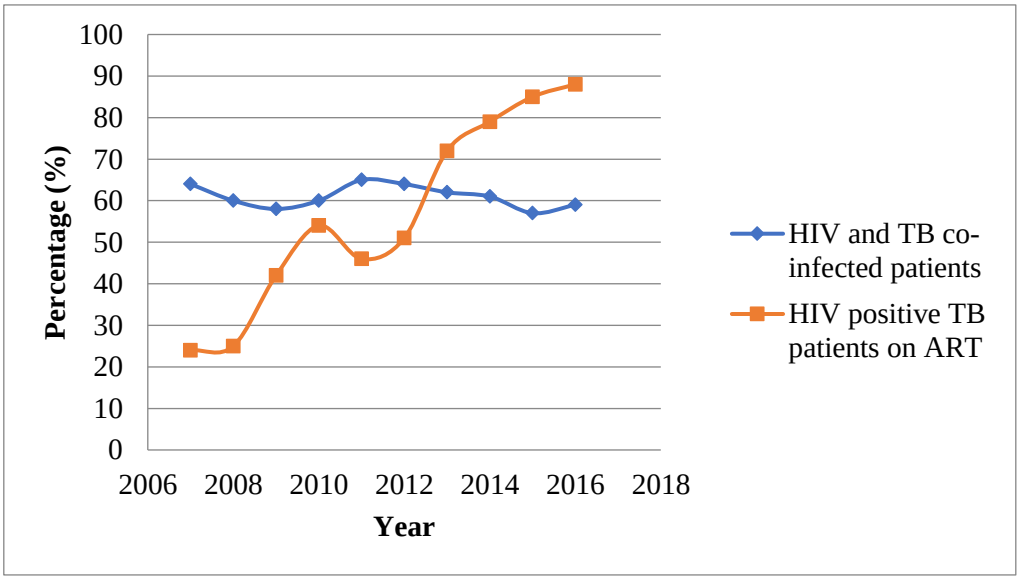

Figure 5: Co-epidemics of TB and HIV over the past ten years in South Africa (Data obtained with permission from ref [5]). Copyright World Health Organization (WHO), 2018

Clinical symptoms of TB in HIV positive individuals vary from classic manifestations of weight loss, prolonged fever, night sweats, productive cough, or haemoptysis, to negligible or nonspecific manifestations [54].

HIV patients co-infected with TB require an integrated co-treatment for these two diseases since TB is an opportunistic disease and it arises after the first couple of months in HIV positive patients that are only on ART [54]. Simultaneous therapy of both HIV and TB, considering the available pharmacokinetic evidence from investigations of these two diseases, individually, has appeared to be possible and proficient in managing the diseases and provides better survival in different clinical backgrounds [54, 56].

1.4.1 Tuberculosis drug resistance

M.tb has natural, unsurprising rates of chromosomally borne mutations that present resistance to antimicrobial drugs. These mutations are not related to each other; thus, resistance to a drug is commonly on a certain drug group targeting a specific stage in TB life cycle. The rise of drug resistance signifies the survival of random previous mutations [57].

In 2016, an estimated number of 490 000 individuals globally developed multidrug-resistant TB (MDR-TB) [5]. South Africa presented 3.4% new cases of individuals who developed MDR-TB in 2016 [5]. The development and increment of multidrug-resistant strains (MDR-TB; described as resistance at least to rifampicin (RMP) and isoniazid (INH) and extensively drug-resistant (XDR-TB); described as MDR in addition to extra resistance to no less than one fluoroquinolone and one second-line injectable drug) of *M.tb* has been disturbing experts around the world [53, 58]. These tuberculosis strains show low cure rates and high death rates because of challenges in their treatment [58]. Additionally, HIV seems by all accounts to be an unassuming danger factor for MDR-TB. In the latest meta-examination, the likelihood of MDR-TB was 1.26-fold higher amongst those who are HIV-positive than amongst those who are HIV-negative [47]. High death rates due to MDR-TB and in a few cases, XDR-TB prevalence, as well as transmission, has been most noticeable in individuals with HIV [53].

Following the resistance of some *M.tb* strains to first-line TB drugs, there was the vital need for the development of second-line drugs for tuberculosis treatment. Second-line TB drugs comprise of fluoroquinolones, aminoglycoside, injectable agents, oral bacteriostatic and anti-TB drugs with less evidence on effectiveness and/or long-term safety in the treatment of MRD-TB/XDR-TB [59]. The

study of the new treatment against TB assumes an essential part in decreasing the occurrence and mortality, important to accomplish worldwide objectives set up by the WHO [58].

1.4.2 Treatment of drug-resistant TB

Discontinuation of the standard TB treatment may lead to drug resistance. Second-line TB drugs, which are expensive and have more side effects will then be required to treat MDR-TB. Ofloxacin (OFL), [60-62] streptomycin (STR) [63-65] and kanamycin (KAN) [66-68] are among the second-line drugs used for MDR-TB treatment [52].

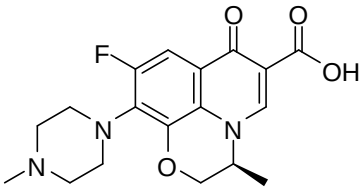

Figure 6: OFL drug structure

OFL (**Figure 6)** is a fluoroquinolone that is used to treat MDR-TB and also works against urethral chlamydia infections, *Streptococcus pneumoniae* pulmonary infections and effectively eradicate staphylococcal infections [69, 70]. A study by Yuk et al., (1991), found the T_{max} of OFL to be 1.74 ± 0.57 hours in plasma with the C_{max} of 3.14 ± 0.53 µg/mL when 400 mg once daily was orally dosed for each volunteer [71]. Another study by Lode et al., (1987), found the T_{max} of 1.9 hours in plasma with the C_{max} of 3.51 ± 0.7 µg/mL when 400 mg of OFL, once daily was orally dosed [72].

Minimum inhibitory concentration (MIC) is important for determining the effectiveness and susceptibility of a drug. A study by Heysell et al., (2015), found 14 OFL-susceptible isolates of *M.tb* with the MICs of ≤ 1µg/mL and nine OFL-resistant isolates with the MICs of ≥ 8 µg/mL on the 96-well Sensititre MYCOTB plate [73]. Another study by Chen et al., (1989), found OFL-susceptible strains of *M.tb* with the MIC of 2 µg/mL when determined in 7H12 broth radiometrically [74].

Adverse effects include nausea, anorexia, vomiting, diarrhoea, skin or hypersensitivity reactions, and central nervous system events [56, 70].

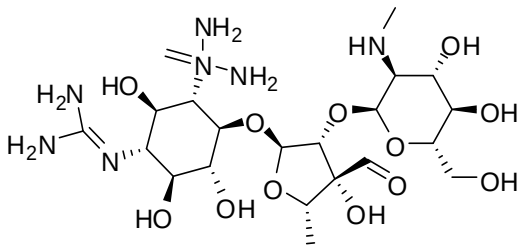

Figure 7: STR drug structure

STR (**Figure 7**) is an aminoglycoside bactericidal originated from *Streptomyces griseus* that is utilized as part of the treatment of TB and sensitive Gram-negative diseases. Individuals with MDR-TB are given ethambutol, STR, and moxifloxacin for a year and a half [57]. A study, Park et al., (2015), found the T_{max} of STR to be 1.0 (0.5–1.5) hours in plasma with the C_{max} of 42.0 ± 10.8 mg/L when 1 g once daily was dosed for each volunteer [75]. In a study, Zhu et al., (2001), where 18 mg/kg of STR was intramuscularly (IM) dosed, T_{max} in plasma was found to be 1.51 (0.58–2.70) hours with the C_{max} of 42.6 (2.90–85.2) mg/L [76]. In nother study, Prasad et al., (1978), where 20 mg/kg of STR was IM dosed, T_{max} was found to be 0.5 hours in plasma with the C_{max} of 12.3 ± 1.79 mg/L [77].

Susceptibility of *M.tb* to a drug is determined by MICs. A study, Franzblau et al., (1998), found 18 strains of *M.tb* susceptible to STR with the MICs of 2 μg/mL in the BACTEC system and was ≤ 1 μg/mL by the microplate-based Alamar Blue assay (MABA). For 11 of the 13 strains resistant to STR, MIC was 6 μg/mL in the BACTEC system and 2 to 8 μg/mL by MABA. For two resistant strains to STR at 6 μg/ml in the BACTEC system, the MICs by MABA was ≥32 μg/ml [78]. Another study, Schönfeld et al., (2012), found five (19.2%) strains of *M.tb* which showed sensitivity to STR with MICs below or equal to 4.0 μg/mL and most resistant strains to STR showed MICs above 20 μg/ml (88.9 %) on Löwenstein–Jensen medium (LJ medium) [79].

Side effects incorporate deafness (no wax on otoscopy), rash, deafness, sterile abscesses can develop at injection spots and injections are aching, hypersensitivity responses are normal and can be serious, dosage ought to be decreased if vertigo, headache, tinnitus, and vomiting take place. Aplastic anaemia, agranulocytosis, thrombocytopenia, haemolytic anaemia, lipoid responses, and agranulocytosis are uncommon side effects [52, 56].

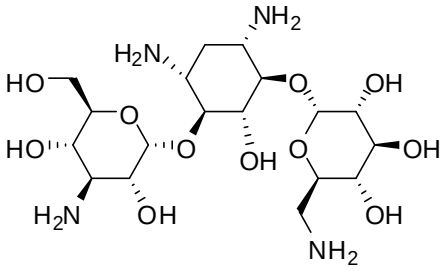

Figure 8: KAN drug structure

KAN (**Figure 8)** is also an aminoglycoside anti-microbial drug that inhibits protein synthesis by binding to the ribosomes of the bacteria [80]. A study, Clarke et.al., (1974), found the T_{max} of two hours in plasma with the C_{max} of 18 mg/L after a 7.5 mg/kg IM single dose of KAN [81]. Another study, Doluisio et.al., (1973), found similar results, T_{max} was one hour in plasma with the C_{max} of 23 mg/L after a 7.5 mg/kg IM dose of KAN in 12 hours [82].

A study, Bastian et al., (2001), found 36 of 37 kanamycin-susceptible isolates of *M.tb* with the MICs of ≤ 2.5 µg/mL while all 35 kanamycin-resistant isolates with the MICs of ≥ 5.0 µg/mL by the MABA method using 7H9-S broth [83]. Another study, Heysell et al., (2015), found 55 KAN-susceptible isolates of *M.tb* with the MICs of ≤ 2.5 µg/mL and 1 KAN-resistant isolate with the MIC of ≥ 20.0 µg/mL on the 96-well Sensititre MYCOTB plate [73].

Adverse effects of this drug incorporate azotaemia, hearing loss, ataxia, nystagmus, proteinuria, eosinophilia, thrush, serum electrolyte abnormalities, stomach pains, tremulousness headache and anxiety [56, 57, 84].

1.5 Therapeutic drug monitoring (TDM)

Therapeutic drug monitoring (TDM) is the measurement of specific drug concentrations at timed intervals in the patients' plasma [85]. The main aims of TDM are to avoid therapeutic failures due to bad compliance or too low dose of a given drug, as well as adverse or toxic effects due to an excessive dose by individualizing therapeutic regimens for ideal patient's advantage [85, 86]. The utilization of TDM in the case of different diseases enables the clinicians to make informed decisions with respect to the dosage and the duration of medication treatment [87]. A small number of patients do not adhere well to treatment or are in danger of drug-drug interactions or have MDR or XDR TB or have

simultaneous disease conditions that further complicate the clinical setting. TDM is especially beneficial to these kinds of patients that have a complicated clinical background [87].

Drug-drug interactions are likely to occur for HIV positive TB patients using at least three drugs for their treatments, combined. The approved drug guidelines only show drug-drug interactions of two drugs, which become inefficient when the patient is treated with three or more drugs [87]. Under these difficult conditions, TDM frequently is the most accessible technique for quantifying these interactions and assigning the patient the correct dosage that they need [87]. Comparisons of concentration/dose ratios in exposed and unexposed patients are performed using TDM data to study drug interactions [88].

The plasma drug concentrations are helpful especially for few compounds that have a narrow interval between the therapeutic dose and the toxic dose. Tests for a substantially more broader scope of compounds, and metabolites might be needed to survey adherence (concordance, consistency) to treatment, side effects, acute poisoning, or drug-drug interactions [89].

1.6 Mass spectrometry

Mass spectrometry (MS) is amongst the most accurate, reliable, and easily accessible methods to identify the atomic and molecular masses of compounds with high precision in a single evaluation [90-92]. MS converts analyte molecules into ions; thereafter the analyte's mass (quantitative) and structure (qualitative) can be determined upon detection [93].

A key component of MS depends on the capacity of electric and magnetic fields to impact the movement of charged particles and atoms, ordinarily in a vacuum relative to their mass and charge [90]. Positive or negative charges are obtained upon ionization of an analyte molecule when it is introduced into the ionization source of the MS. The ions are separated by the detector on the basis of their mass/charge (*m/z*) ratio after they have travelled through the mass analyzer. A computer system records readable signals upon the arrival of the ions at the detector. The signals in a computer system are displayed as spectra based on their *m/z* ratio. Information that can be obtained from mass spectral data includes purity of the sample, structure and molecular weight [93].

Numerous technologies exist for ionization and mass analysis, leading to many various kinds of MS's with various combinations of these two processes. Available ion sources include electrospray ionization source (ESI), atmospheric pressure chemical ionization source (APCI), and atmospheric

pressure photo-ionization (APPI). Mass analyzers available include ion trap, hybrid, time of flight, and quadrupole analyzers [91].

ESI utilizes electrical energy to help with the transmission of ions from a liquid phase to a gas phase before they are subjected to mass spectrometric analysis. Ionic species in the liquid phase would, therefore, be able to be analyzed by ESI-MS with higher sensitivity. The transmission of ions from a liquid into the gas phase by ESI includes three steps: dispersion of a fine spray of charged droplets, solvent evaporation and ion ejection from the highly charged droplets tube [93].

A fine spray of charged droplets is formed at the tip of the capillary after the in-solution samples are pumped at 3 to 5 kV and nebulized. The capillary is normally orthogonal to or off-axis from, the passage to the mass spectrometer with a specific goal to reduce contamination. Use of dry nitrogen and heat quickly evaporate the droplets, and the residual electrical charge on the droplets is transferred to the analytes. Focusing voltages and small apertures are used to transfer the ionized analytes into the high vacuum of the mass spectrometer. Detection of negative or positive ions is accomplished after the ions have gone through the ion source and mass analyzer [91].

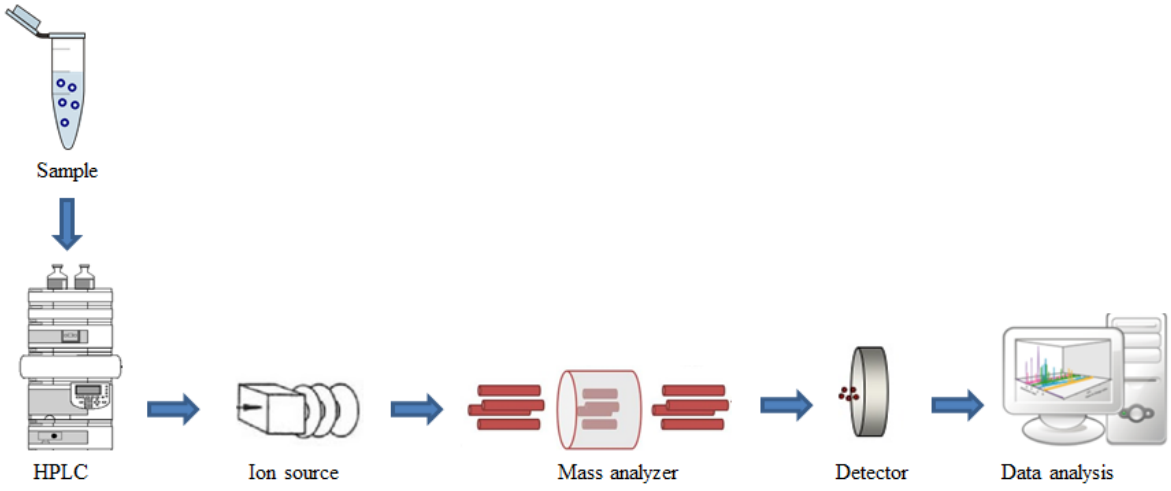

Figure 9: Basic diagram of instrumentation of typical liquid chromatography-mass spectrometry (Information obtained from literature [94])

Under typical circumstances, ESI is viewed as a “soft” ionization source, implying that generally little energy is sent to the analyte, and thus little fragmentation takes place. This is as opposed to other MS ion sources, for instance, the electron impact source ordinarily utilized in gas chromatography mass

spectrometry (GC-MS), which causes extensive fragmentation. It is possible to increase ESI "in-source" fragmentation by increasing voltages inside the source to increase collisions with nitrogen molecules. This has been utilized as part of LC-MS analyses to distinguish compounds with common structural characteristics [91].

In APCI, nebulization occurs at the tip after the liquid is injected through a capillary. Next to the capillary tip, a corona discharge occurs, where gas and solvent molecules are ionized. An analyte is then ionized by charge transfer after it has reacted with the ions formed from gas and solvent molecules. APCI is especially used for thermally stable and small molecules. In APCI, singly-charged ions are dominant, unlike in ESI where multiple charging is dominant. APCI has also been used for fat-soluble vitamins and lipids [91, 95-99].

In APPI, after nebulization, photons are used to excite and ionize molecules. Simultaneous ionization of solvents and ion source gases are minimized by the energy of the photons. In APPI, singly-charged ions are also dominant. APPI is used for neutral compounds, for example, steroids [91, 100, 101].

The components of a liquid chromatography-mass spectrometry (LC-MS) system (**Figure 9)** incorporate the high-performance liquid chromatography (HPLC) system, the mass spectrometer, the autosampler and the ionization source (which connects the MS and the LC). Preferably, these components are all controlled by one PC system. The HPLC system is made up of various components such as the mobile phase reservoir, pump, injector, and column [102]. It ought to be pointed out that to link HPLC with MS; there are a few limitations on the mobile phases and rate of flow that can be utilized. Acetonitrile or methanol and water are utilized as mobile phases in reversed phase HPLC systems linked with MS. There are many mobile phase modifiers, and these should be volatile in many instances. Mobile phase modifiers are additional chemicals to the mobile phase that are fundamentally utilized to enhance the chromatography of the analytes of concern. Common mobile phase modifiers would incorporate acetic acid, formic acid, and ammonium acetate [103].

1.6.1 Tandem mass spectrometry

Two ions with the equal mass-to-charge (*m/z*) ratio cannot be differentiated by the mass analyzer, as would be the case with stereoisomers. In any case, these particles can regularly be differentiated by breaking the ions separated and examining the ion fragments using a second mass analyzer. To achieve this, mass spectrometers can be designed to store, channel and fragment ions [90]. Since each of these tasks may require extra equipment, contemporary multiplexed mass spectrometer systems

are frequently hybrids and incorporate more than one mass analyzer, equipped for multistage operation. This multiplex method is mostly denoted as coupled mass spectrometry, or MS/MS, to depict the two phases of analysis [90].

Liquid chromatography tandem mass spectrometry (LC-MS/MS) quantification has become the gold standard tool due to its capabilities of ion fragmentation, which is essential for identification of analytes in matrices [90, 104-113]. To distinguish analytes of the same mass, ion fragmentation for each analyte is required [90]. Coupled MS enhances identification by means of fragmentation in light of the fact that the coupled mass spectrum may give a different profile of the fragment ions, representing a chemical structure. Ordinarily, MS/MS analysis is achieved by a collision of a chosen ion with inert gas molecules, for example, argon or nitrogen, and the subsequent fragments go through mass analysis. MS/MS is utilized to analyze lipids, peptides, small oligonucleotides and structurally characterize carbohydrates [94].

Quadrupole time-of-flight (QqTOF) (**Figure 10**) is one of the coupled mass spectrometers and is depicted in the most straightforward manner as a triple quadrupole with the last quadrupole area substituted by a TOF analyzer [114]. Triple quadrupole instruments have capabilities to distinguish the chemical backgrounds which result in higher sensitivity and selectivity [92]. Most quadrupole analyzers function at <4000 *m/z* and scan speeds up to 1000 *m/z* per second or more [91].

The precursor ion which is the targeted analyte is selected by the first quadrupole (Q1) based on the mass. It is then permitted to collide with argon or nitrogen collision gases in a second r.f. only quadrupole collision cell (Q2) where the collision activates the precursor ion to go through fragmentation. This process is called collision-induced dissociation (CID). The TOF as a third quadrupole mass analyzer (Q3) monitors the product ions that result from CID and gives structural facts [93].

The quadrupole is capable of monitoring different specific *m/z* values by stepping the voltages. This is essential in enhancing the detection limits of precursor ion since detector time for detecting specific ions is increased rather than wasting it doing the full scan of the analyte. Stepping the voltages is much faster [91]. When Q1 is to monitor one specific m/z ratio, it filters out other molecular ions having different m/z ratios. This is a purification step inside the MS system, removing time-consuming and complex sample purification steps before the MS analysis [93].

Multiple-reaction monitoring (MRM) is a mode of data acquisition usually utilized in ESI-MS/MS quantification analysis. Both Q1 and Q3 are static for a pre-determined pair of precursor and product ions [93]. In ion trap analyzer, ions are trapped in a 3-dimensional space by three hyperbolic electrodes using radio frequency and static voltages. A mass spectrum with regards to *m/z* ratio is created after the ejection of ions from the trap. An exciting voltage can be applied instead, while other ions are ejected, to isolate a specific ion in the trap. To induce fragmentation, an inert gas can be established. In ion trap analyzer, MS^n capabilities are produced since ion trap is able to fragment and isolate ions numerous times in series prior the attainment of the final mass spectrum [91, 115, 116].

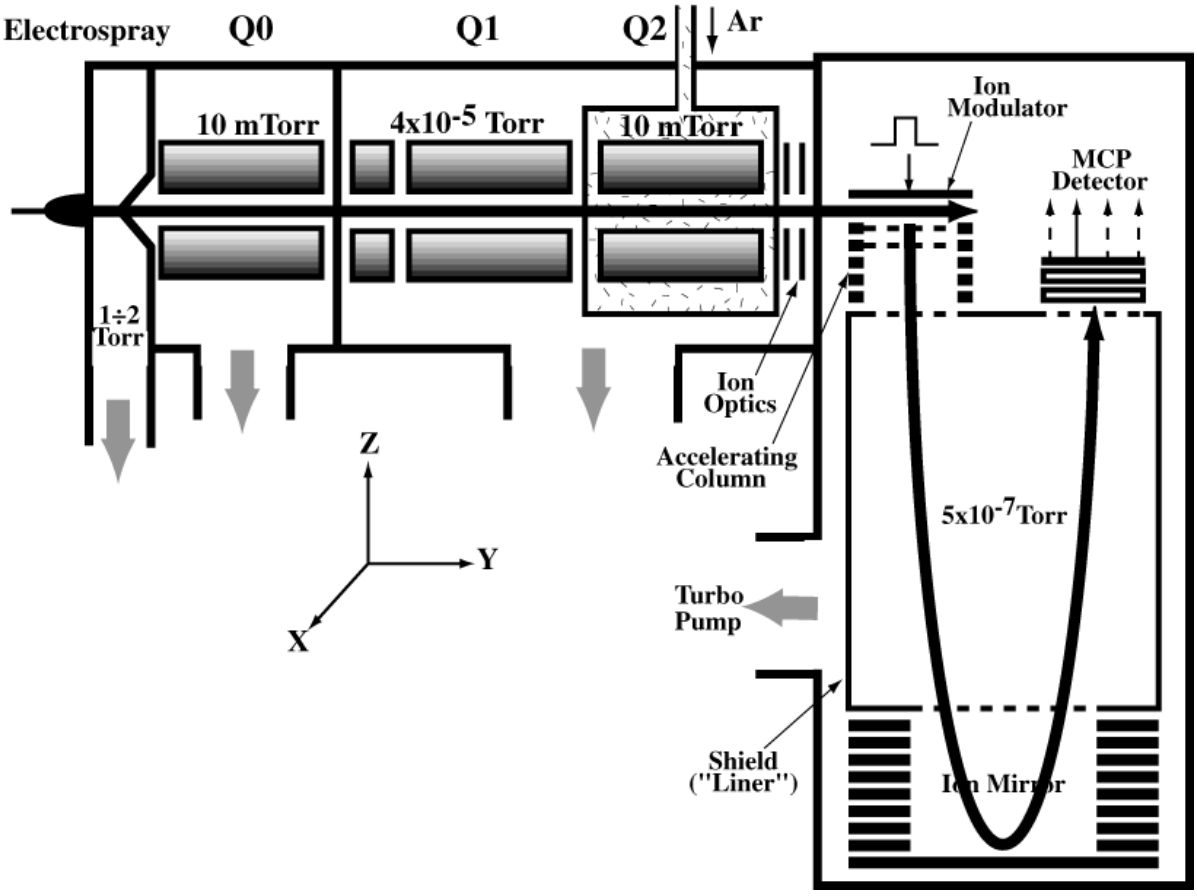

Figure 10: Schematic diagram of Quadrupole time-of-flight (QqTOF) tandem mass spectrometer (Figure is reused with permission from ref [114]). Copyright © 2001 John Wiley & Sons, Ltd

The sample is ejected through the electrospray and the targeted analyte is selected by the first quadrupole (Q1) based on the mass and then it collides with a gas (argon or nitrogen) in a second r.f. on quadrupole collision cell (Q2) where the collision activates the analyte for fragmentation. The

TOF as a third quadrupole mass analyzer monitors the product ions that result from CID and gives structural information [93].

1.7 New HPLC column matrices

The new HPLC column matrices that offer various benefits are essential in TDM studies of various diseases. Currently, available HPLC methods use old column technology that require longer runtimes and is not feasible in a point of care environment. The new HPLC column matrices have the potential of short runtimes and saving solvents. These new HPLC column matrices are commercially available from different manufacturers. These new HPLC column matrices include the pentafluorophenylpropyl (F5), octadecyl (C18), biphenyl, and reversed phase amide (RP-Amide) [117].

1.7.1 The pentafluorophenylpropyl matrix

The pentafluorophenylpropyl (F5) (**Figure 11**) bonded phase has firm dipole potential (polar interaction) from the carbon-fluorine bonds, capacity to interact through charge-exchange interactions and the pi-pi interaction potential due to the electronegativity of the fluorine atoms. It is said to give advanced shape selectivity of analytes differing in spatial size and properties due to its firmness nature [117].

The utilization of fluorinated stationary phases in liquid chromatography has turned out to be critical as of late. Fluorinated stationary phases give diverse retention systems; therefore they are time to time utilized for the separations not effortlessly acquired utilizing basic C18 phases [117].

Fluorinated phases have been appeared to show more noteworthy ion-exchange character than their alkyl associates [117]. Fluorinated phases frequently give exceptional chromatographic outcomes when analytes to be isolated vary in their ionization constants, or where some ion-exchange is vital for the retention of polar metabolites or degradation compounds [117]. Another imperative characteristic of the fluorinated phases lies in their noticeable increased shape selectivity compared to the regular stationary phase packings. Fluorinated phases, thus, are regularly better than their alkyl associates for the isolation of strongly related analytes that vary in shape and size [117].

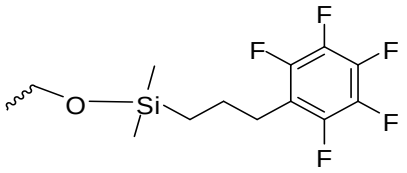

Figure 11: Chemical structure of pentafluorophenylpropyl stationary phase

1.7.2 The octadecyl matrix

The new octadecyl (C18) column has been described to function better than the normal C18 columns and it also gives amazing liquid chromatography speed [117]. When developing a new method, it is advisable to start off with this column. It has a Fuse Core molecule which gives a compact silica core encompassed by a fragile porous shell of high-purity silica [117].

Its porous shell, minute particle size, and simplistic diffusion path enable this column to display its very high column efficiency at low pressure system [117]. The new C18 column proves to be the best option within a laboratory and research environments because of its abilities of low operational pressure, increased efficiency, reduced solvent usage and runtime. The stable, reverse-phased octadecyl (**Figure 12**) Fused-Core particle platform is functional for compounds that are basic, acidic or neutral [117].

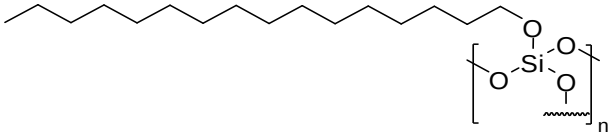

Figure 12: Chemical structure of octadecyl stationary phase

1.7.3 The biphenyl matrix

The biphenyl (**Figure 13**) phase provides alternative selectivity to drug metabolites and pharmaceutical analytes that are hard to resolve or that are not very much retained on phenyl and conventional alkyl (C18) bonded phases [117]. HPLC columns using Fused-Core particle innovation can give both the high and fast proficiencies of small particles while keeping down backpressures. The combination of low backpressure and high proficiency benefits HPLC operators [117].

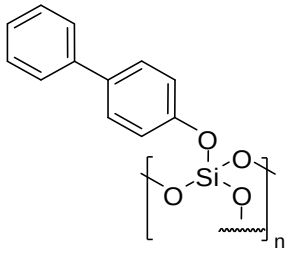

Figure 13: Chemical structure of biphenyl stationary phase

1.7.4 The reversed phase amide

The reversed phase amide (RP-Amide) (**Figure 14**) column is comprised of the combination of an embedded polar group (EPG) stationary phase with the Fused-Core particle [117]. The advantages of this column incorporate enhanced peak shape for bases, 100% aqueous compatible reversed-phase column and alternative reversed-shape selectivity to C18. The RP-Amide is a combination of modern phase technology and innovation particle technology [117]. The Fused-Core particle gives benefits regarding resolution, speed, ruggedness, and sensitivity [117].

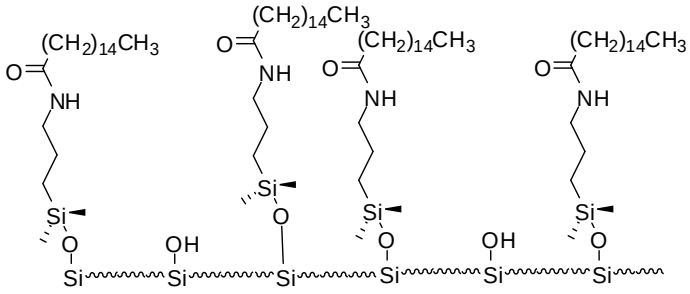

Figure 14: Chemical structure of amide stationary phase

The RP-Amide gives increased selectivity to polar analytes, particularly those that donate hydrogen bond. Amines, phenols, carboxylic acids, and to a lesser degree alcohols, indicate upgraded retention on the RP-Amide phase when contrasted with neutral, non-polar compounds [117].

The selectivity dissimilarities between the RP-Amide and the C18 can be a helpful feature in method development. Most of the times, when peaks co-elute on a C18 phase, it can be replaced with the RP-Amide. RP-Amide gives stable and reproducible analyte retention in 100% aqueous mobile phases. Numerous C18 phases are famous to experience the ill effects of phase collapse under profoundly aqueous phase conditions creating a loss of retention [117].

1.8 Bioanalytical method validation

The primary objective of bioanalytical method validation is to show the reliability of a specific method for the determination of an analyte concentration in a biological matrix, for example, urine, blood, saliva, serum or plasma. The outcomes of pharmacokinetic studies are utilized to settle on important decisions for the efficacy and safety of a therapeutic drug. It is then imperative that the bioanalytical methods utilized are validated [118, 119].

1.8.1 European Medicines Agency (EMA) guidelines

European Medicines Agency (EMA) guidelines give recommendations for the bioanalytical methods validation regarding measuring drug concentrations in biological matrices acquired in pharmacokinetic studies and clinical trials [118-120].

1.8.2 Validation parameters

1.8.2.1 Lower limit of quantification (LLOQ)

The lower limit of quantification (LLOQ) is the lowest concentration of analyte in a sample which can be reliably quantified [118-120].

1.8.2.2 Linearity

The response of the instrument regarding the concentration of analyte should be known and be assessed over a specific concentration range. There ought to be one calibration curve for each analyte examined in the method validation [118-120]. These will be discussed next.

1.8.2.3 Accuracy and precision

The accuracy of a bioanalytical method depicts the closeness of the determined value acquired by the method to the analyte's theoretical concentration. Accuracy is expressed in percentage. The precision of the bioanalytical method depicts the closeness of repeated single measures of analyte. Precision is expressed as the coefficient of variation (CV) [118-120].

1.8.2.4 Matrix effect

The interference of unintended analytes or other substances in response due to their presence in the sample should be evaluated in method validation [118-120].

1.8.2.5 Stability

Stability evaluation is completed to ensure that each step that is taken during sample preparation and sample analysis, as well as storage conditions utilized, do not affect the analyte's concentration [118-120].

1.9 Aims and Objectives

Aims:

This study aims to evaluate and compare the new column technologies (RP-Amide, F5, C18, and biphenyl) and their applicability to the simultaneous quantification of current first-line anti-HIV (efavirenz, emtricitabine and tenofovir disoproxil) and/or second-line TB (kanamycin, ofloxacin and streptomycin) treatment for the usage of TDM.

Objectives:

- To develop, optimize and validate an LC-MS/MS method for Atripla and/or second-line TB drugs by determining the lower limit of quantification, linearity, accuracy and precision in rat plasma.
- To determine resolution of the peaks in all the column technologies.
- To determine the number of theoretical plates for the four column technologies.
- To determine the peak asymmetry factor for the four column technologies.

1.10 Outline of the thesis

Chapter 1 gives background information on the topics covered in the thesis, aims and objectives.

Chapter 2 presents the submitted paper for publication "Performance comparison of fused core particle columns for the quantitation of ARVs in Atripla".

In this paper, various columns were compared to determine their efficiency based on the resolution, number of theoretical plates and asymmetry factor while developing a quantitative bioanalytical method according to EMA guidelines.

Chapter 3 presents the submitted paper for publication "LC-MS/MS method development and validation for simultaneous quantification of first-line HIV drugs and second-line TB drugs in rat plasma".

In this paper, an LC-MS-MS method for the simultaneous quantification of anti-HIV drugs (FTC, EFV, and TFV) and second-line anti-TB drugs (STR, KAN, and OFL) according to EMA guidelines was developed, optimized and validated. In the study rat plasma was used as the biological matrix. The method developed will be useful for TDM studied.

Chapter 4 gives concluding remarks and recommendations. It also gives thesis appendix.

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CHAPTER 2

Manuscript 1

Performance comparison of fused core particle columns for the quantitation of ARVs in Atripla

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2.1 Abstract

Human immunodeficiency virus (HIV), remains a global concern despite the advent of anti-retroviral (ARV) therapy. Many developing countries have now adopted the legislature that states that all HIV patients should receive ARV treatment, regardless of $CD4^+$ count. This places significant pressure on ARV stores with many developing countries running out of drugs, in addition to these patients may encounter adverse treatment outcomes. A possible solution to assist with problem is therapeutic drug monitoring (TDM) which will allow for personalized medicine and optimum dosing for each patient, thereby reducing the pressure on ARV stores and reducing potential side effects. TDM makes use of an analytical technique (commonly LC-MS) to measure drug plasma concentrations, however current methods are lengthy and cannot be applied in a point-of-care setting. Recent technological advances have led to the development of new HPLC columns with the potential to decrease run times and offer enhanced selectivity. Therefore, the aim of this study was to evaluate new chromatographic column technologies and their applicability to the simultaneous quantification of the current first-line ARV treatment. We evaluated the C18, biphenyl, pentaflourophenyl (F5) and the amide fused core columns; based on resolution, peak asymmetry factor, theoretical plates, limit of detection, linearity, accuracy and precision of the analytical method. Our results showed that each column offered its own advantages and disadvantages for the separation of the current ARV treatment, with the biphenyl column performing most consistently.

Keywords: Atripla; drug quantitation; pentaflourophenyl; amide; biphenyl; LC-MS

2.2 Introduction

Current first line combination antiretroviral therapy (ART) comprises of one non-nucleoside reverse transcriptase inhibitor (NNRTI) and two nucleoside reverse transcriptase inhibitors (NTRI's) [1]. The available treatment is a one a day pill known as Atripla, approved by the Food and Drug Administration (FDA) in July 2006 [2, 3]. This drug combination comprises of emtricitabine (FTC), tenofovir disoproxil (TFV-DP), which belong to NTRI's, and efavirenz (EFV) an NNRTI's [4]. It is considered to be the most affordable, yet effective regimen and is also available in generic forms [1]. In 2012, the World Health Organization (WHO) recommended a law compelling all HIV infected individuals to be administered ARV's as soon as they become aware of being HIV positive, regardless of their $CD4^+$ count [5]. ARV therapy can be very expensive for developing countries, which have the highest prevalence of the disease with their stores under constant pressure due to the large patient demand [6, 7]. Therefore, therapeutic drug monitoring (TDM) should be recommended for optimum dosing of infected individuals, thereby reducing pressure on ARV stores and reducing the occurrence of adverse drug effects [8].

TDM refers to the constant measurement of drug levels in blood and is used to determine the most optimal drug dosage required, with minimal toxicity [8], while also being a useful tool to monitor patient adherence [9, 10]. TDM takes into consideration factors which can also influence the drug therapy regimen, such as methods used in the laboratory for drug testing, weight and age of an individual, if individuals suffer from other co-morbidities and drug dosage [9]. It also considers drug delivery, absorption, metabolism and elimination rates, and if an infected individual is receiving any other form of medication [9]. Analysis of pharmaceuticals and other related compounds have now become more efficient due to the developments in high performance liquid chromatography (HPLC) and mass spectrometry (MS) analysis of biological samples [11]. Liquid chromatography tandem mass spectrometry (LC-MS/MS) quantification has become the gold standard tool for assessing the concentration profiles of a wide range of therapeutics in various biological matrices [12-21].

LC-MS/MS has universal applicability making it the forerunning technique in analyzing chemical and biological mixtures with great precision [11]. There are a number of established and newer column technologies available which offer greater selectivity thereby increasing the range of compounds that can be quantified [22]. In analytical chemistry, column efficiency can be evaluated by parameters such as selectivity, resolution, peak asymmetry and theoretical plates [11]. Efficiency refers to the mechanical power of separation of a column and is determined by the particle size and

length of a column [23-25]. Selectivity can be defined as the degree to which chemical dissociation occurs to determine the analytes present in a mixture [23]. Therefore, in comparing column technologies, the length of a column as well as the particle size should be kept as nominal as possible in order to decrease run times [23]. The internal diameter, volume, and column bed length are also determinants of the quantity of packing material that is contained within the column which also affects the flow rate as well as the time taken to complete the analyses [23].

Currently, the methods available for the quantification of ARV's in plasma and brain are relatively lengthy [26, 27] for routine point of care analysis. There are a number of new HPLC column technologies which offer advanced packing materials, each of them providing remarkable performance, efficiency and shortened run times [22].

Therefore, the aim of this study was to evaluate these new column technologies and their applicability to the simultaneous quantification of current first-line anti-HIV treatment. This was be done by comparing various determinants of column efficiency while developing a quantitative bioanalytical method according to European Medicines Agency (EMA) Guidelines [28].

2.3 Materials and methods

2.3.1 Reagents and standards

EFV was purchased from Adcock Ingram (South Africa), TFV-DP was purchased from Cipla Life Sciences (South Africa) and FTC was purchased from Mylan (South Africa). Internal standards, 4-(4-carboxybenzyl)-2H-1, 4-benzoxazin-3(4H)-one (CBB), adefovir dipivoxil (ADV-DP) and zidovudine (ZDV) were purchased from Sigma Aldrich, DLD Scientific (South Africa) and Aspen Pharmacare (South Africa), respectively. LC-MS grade solvents; acetonitrile (ACN) and methanol (MeOH) were purchased from Sigma Aldrich. Analytical grade formic acid (FA) was purchased from Merck Millipore (Merck, South Africa). Water was purified with a Milli-Q purification system from Millipore Corporation (Bedford, MA, USA). HLB-SPE cartridges (30 mg, 1.0 mL) were purchased from Supelco-Sigma (St. Louis, MO, USA).

2.3.2 Instrumentation

The liquid chromatography tandem mass spectrometry (LC-MS/MS) system consisted of an Agilent Technologies 1100 (Agilent, Germany) coupled to a MicroTOF-Q II electrospray ionization (ESI) time-of-flight-mass spectrometry (TOF-MS) instrument (Bruker Daltonics, Bremen, Germany). The raw data were analysed with Data Analysis 4.0 SP 5 (Bruker Daltonics).

2.3.3 Mass spectrometric analysis

The MS conditions were optimized for the detection of EFV, FTC, TFV-DP and their internal standards CBB, ZDV and ADV-DP, respectively. Acquisition parameters were: source type, ESI; ion polarity, positive; end plate source, -500 V; nebulizer, 1.8 bar; dry gas, 8.0 L/min; capillary, -5500V; dry temperature, 200 ◦C; mass range, *m/z* 100 $\rightarrow$ 600; collision cell radiofrequency, 200 Vpp; collision energy, 14 eV.

2.3.4 Preparation of calibration curve and quality control standards

Separate stock solutions of target analytes and internal standards were prepared by dissolving 1 mg of each drug in 1 mL of MeOH then stored at -20 ◦C. Appropriate serial dilutions of the stock solutions were formulated according to the working standard solutions. Calibration standards were concocted by appropriate dilutions to give final concentrations of 10, 20, 60, 100, 250, 500, 1000, 1500, and 2000 ng/mL for FTC and EFV; and 2, 10, 20, 60, 100, 250, 500, 750 and 1000 ng/mL for TFV-DP. The quality control (QC) standards at lower limit of quantification (LLOQ), low-quality control (LQC), middle-quality control (MQC) and high-quality control (HQC) were prepared by appropriate dilutions to give final concentrations of 10, 60, 250 and 750 ng/mL for TFV-DP and 20, 100, 500 and 1500 ng/mL for EFV and FTC. Levels of internal standards were 100 ng/mL for ADV-DP; and 250 ng/mL for both CBB and ZDV.

2.3.5 Chromatographic Conditions

The effectiveness of the four different reversed phase (RP) Ascentis Express columns (Supelco, Sigma Aldrich, Germany); C18, biphenyl, pentafluorophenylpropyl (F5) and amide fused core were investigated based on their resolution, number of theoretical plates and asymmetry factor. Mobile phase A was ultra-pure water (0.1% v/v FA) for both methods and mobile phase B was either MeOH (0.1% v/v FA) or ACN (0.1% v/v FA). Separation was possible within 14 min using both gradient methods at a flow rate of 0.35 ml/min and a column equilibration time of 4 min. The MeOH gradient

was started at 40% mobile phase B and increased to 99% over 12 min, held for 1 min before being returned to starting conditions in 1 min. The most optimum ACN gradient started at 10% mobile phase B, increased to 99 % over 12 min, held for 1 min and returned to 10% over 1 min.

2.3.6 Method validation

2.3.6.1 Accuracy and precision

Accuracy was determined as the ratio between the calculated concentration of the target compounds and their nominal concentrations. Intra-day and inter-day precisions were assessed by an assay of three replicates at low, medium, and high QC levels on the same day and on three consecutive days. According to the European Medicines Agency (EMA) on bioanalytical method validation, the acceptance criterion is ± 15% of theoretical concentration for both accuracy and precision (% RSD) except for the LLOQ. The QC levels were as follows; 10, 60, 250 and 750 ng/ml for TFV-DP and 20, 100, 500 and 1500 ng/ml for EFV and FTC. Internal standard concentrations were 100 ng/mL for ADV-DP and 250 ng/mL for CBB and ZDV.

2.3.6.2 Extraction recovery

Extraction recovery of the target compounds were estimated at the three QC levels (low, medium and high) in three replicates. In brief, 100 µL of rat plasma was spiked with the appropriate amount of internal standards, in order to attain the desired QC concentrations. Thereafter, the sample was made up to 1mL using LC-MS grade MeOH and vortexed for 1 minute. The mixture was then centrifuged at 15 000 rpm, 4 °C for 15 minutes. The samples were subjected to SPE using the Supel- SELECT HLB: 30mg/1 mL SPE cartridge (Supelco, Sigma Aldrich, Germany), which gave the most reproducible recoveries. The SPE cartridge was conditioned with 1 mL of MeOH. The sample was then passed (1 mL min^{-}) through the SPE cartridge and the eluent collected in an HPLC vial, prior to injection into the LC-MS system.

2.4 Results and Discussion

We evaluated four columns with different matrix active groups i.e. C18, biphenyl, F5 and amide. The F5 column technology makes use of the pentafluorophenyl functionality which utilizes fluorinated stationary phases. Early applications in the effective resolution of paclitaxel have helped such phases gain much popularity [29]. Due to the diverse retention systems of fluorinated stationary phases, it is frequently utilized for separations that are unachievable on the traditional C18 phase [22]. The amide

column stationary phase consists of an embedded polar group (EPG) and has also gained popularity as an alternative to the conventional C8 and C18 columns [22]. The advantages of this column include enhanced peak shapes for bases and 100 % aqueous compatible. The biphenyl columns are normally used when conventional phenyl and alkyl bonded phases are unsuccessful in retaining drug metabolites and other pharmaceutical agents [22]. In order to effectively evaluate these new column technologies, they were compared to the C18 equivalent which is the preferred choice of column when developing a new method [11]. All columns were fused-core particle technology, which provides a compact silica core surrounded by a delicate permeable shell of high-purity silica [22].

The gradient compositions were evaluated for optimum chromatographic retention of the analytes. Gradients of MeOH and ACN with water were evaluated for separation efficiencies on each column (**Figures 1-4**). The mass spectrometric conditions were optimized such that the maximum stable response was achieved from the precursor ion and the major product ions of each analyte. The resultant mass transitions of the analytes were optimised for the protonated molecule and obtained *via* positive electrospray ionization scanning at *m/z* 248.04 to 130.04, *m/z* 268.04 to 127.04, *m/z* 520 to 288.08, *m/z* 502 to 256.05, *m/z* 284.1 to 135.04 and *m/z* 316.3 to 168.08 for FTC, ZDV (IS), TFV-DP, ADV-DP (IS), CBB (IS) and EFV, respectively.

The chromatogram of analytes and internal standards in **Figure 1** were obtained using the C18 column. It was found that the MeOH gradient method resulted in good peak shapes even though ADV-DP and CBB couldn't be separated. The extracted ion chromatogram (EIC) was advantageous in differentiating between unresolved peaks on the total ion chromatogram (TIC).

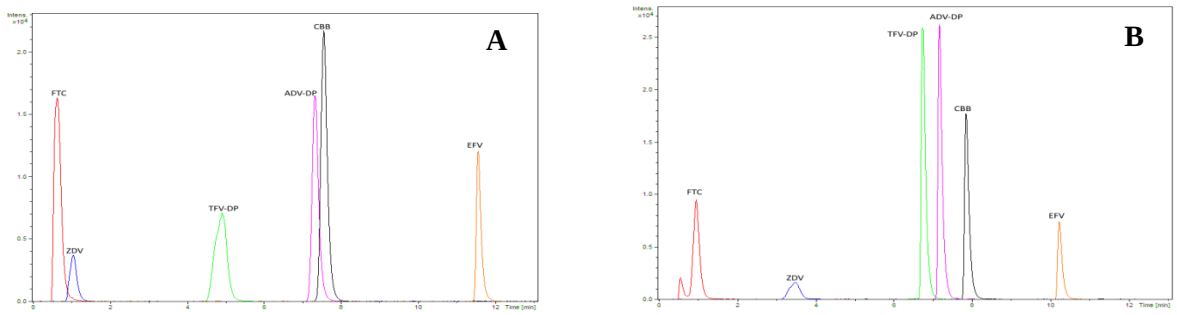

Figure 1: A MeOH gradient and **B** ACN gradient chromatogram showing FTC, TFV-DP, EFV and their respective internal standards using the C18 column (n = 6). The respective LOD's for FTC, EFV and TFV-DP was 5 ng/ml, 5 ng/ml and 1 ng/ml with R^2 values > than 0.99.

The chromatogram of analytes and internal standards in the ACN gradient method resulted in separation of FTC from ZDV but the former analyte gave peak splitting while the latter resulted in peak broadening and lower intensities.

The chromatogram of analytes and internal standards in **Figure 2** were obtained using the biphenyl column, which resulted in excellent separation of FTC, ZDV, TFV-DP, ADV-DP, CBB and EFV under both gradient systems. The ACN as the organic mobile phase however gave peak splitting for the FTC.

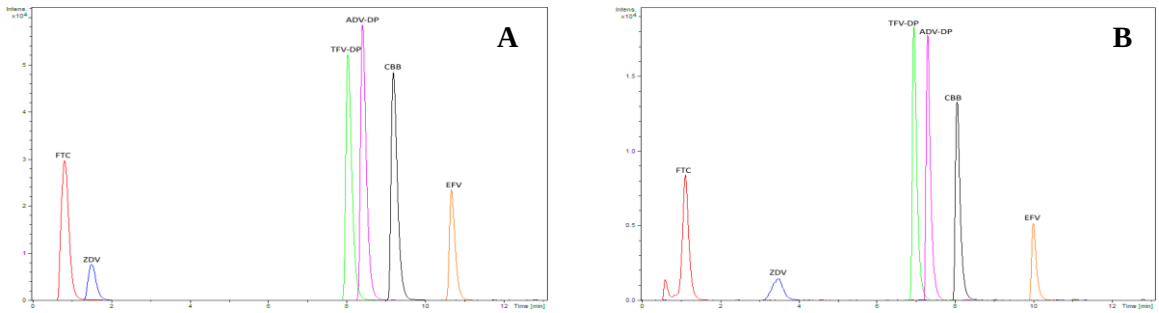

Figure 2: **A** MeOH gradient and **B** ACN gradient chromatogram of FTC, TFV-DP, EFV and their internal standards using a biphenyl stationary phase (n = 6). The respective LOD's for FTC, EFV and TFV-DP was 10 ng/ml, 15 ng/ml and 2 ng/ml with R^2 values > than 0.99.

The chromatogram of analytes and internal standards in **Figure 3** were obtained using the F5 column. The methanol gradient resulted in separation of FTC and ZDV but showed poor resolution for TFV-DP, EFV and ADV-DP. EIC analysis was performed, allowing for these analytes to be resolved.

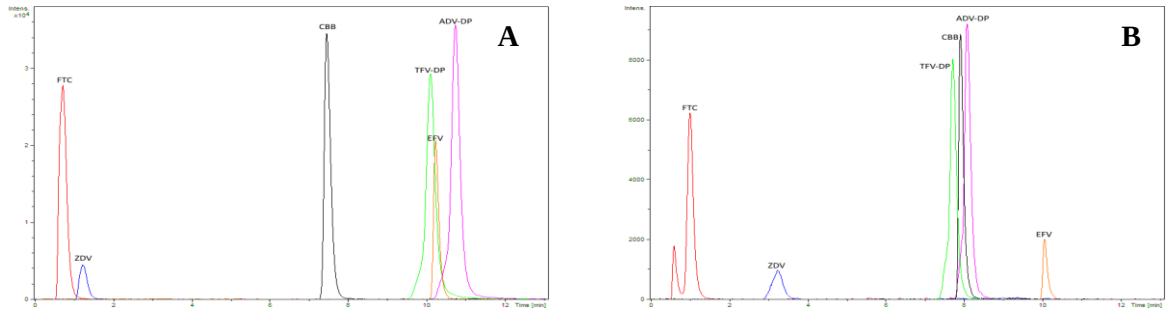

Figure 3: **A** MeOH gradient and **B** ACN gradient chromatogram of FTC, TFV-DP, EFV and their internal standards using a F5 column (n = 6). The respective LOD's for FTC, EFV and TFV-DP was 5 ng/ml, 5 ng/ml and 2 ng/ml with R^2 values > than 0.99.

The chromatogram of analytes and internal standards with the ACN gradient (**Figure 3B**) resulted in peak splitting for FTC and broadening of the ZDV peak as well as unresolved peaks for TFV-DP, CBB and ADV-DP.

Figure 4 shows the separation of atripla and its internal standards using the amide column. Separation was obtained in FTC, ZDV, TFV-DP, ADV-DP, CBB and EFV however peak broadening was observed with respect to TFV-DP under MeOH gradient conditions (**Figure 4A**).

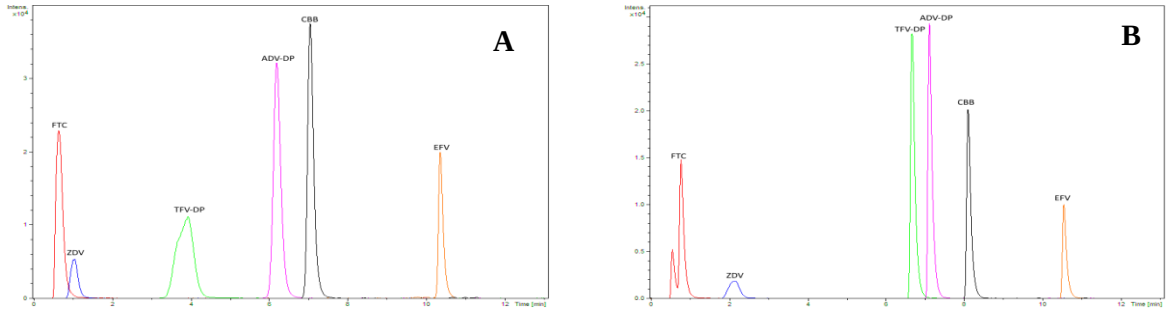

Figure 4: **A** MeOH gradient and **B** ACN gradient chromatogram of FTC, TFV-DP, EFV and their internal standards using amide column (n = 6). The respective LOD's for FTC, EFV and TFV-DP was 5 ng/ml, 5 ng/ml and 3 ng/ml with R^2 values > than 0.99.

The chromatogram of analytes and their internal standards are shown in **Figure 4B** were separated on an amide column and ACN as the mobile phase. Good separation was achieved for FTC, ZDV, TFV-DP, ADV-DP, CBB and EFV. However, peak splitting was observed for FTC together with broadening of ZDV and lower intensity.

2.4.1 Accuracy and precision

The intra- and inter-day precision and accuracy in plasma are presented in **Tables S1** to **S3** (**Supplementary information**). FTC showed intra- and inter-day precisions with an RSD percentile ranging from 0.25 to 5.22 %, and the EFV analyte in plasma ranging from 0.31 to 6.73 %. TFV-DP showed intra- and inter-day precisions of 0.26 to 8.59 % RSD. The accuracy and precision for the method was well within acceptable limits according to EMA guidelines which were within 15% for the LQC, MQC and HQC and within 20 % for the the LLOQ.

2.4.2 Extraction recovery

The plasma recovery of FTC, EFV and TFV-DP in the C18, biphenyl, F5 and amide columns are presented in **Table 1**. The % RSD on the C18 column ranged from 2.22 to 13.99 %, with the F_5

recoveries ranging from 4.46 to 9.59 % RSD. The biphenyl recoveries ranged from 0.12 to 11.45 % RSD and the amide column showed RSD percentages ranging from 0.99 to 11.92%. The accuracies and precisions determined were within acceptable limits according to EMA guidelines and reproducible in all triplicates tested. The actual recoveries ranged from 43.61 to 62.62 % for FTC, from 47.54 to 93.92 % for EFV and 52.10 to 58.19 for TFV-DF which is typical for the recovery of HIV drugs from plasma.

Table 1: Extraction recovery of FTC, EFV and TFV-DP in plasma across the different columns used in this study (data is presented as a mean ± RSD, n=3)

Column matrix	QC Level	FTC % recovery (% RSD)	EFV % recovery (% RSD)	TFV-DP % recovery (% RSD)
C18	**LQC**	47.74 (10.21)	52.05 (8.72)	58.19 (4.06)
	MQC	47.24 (2.96)	49.10 (4.32)	57.52 (2.22)
	HQC	41.36 (13.99)	51.46 (2.97)	56.47 (2.76)
F5	**LQC**	62.62 (8.43)	93.92 (11.25)	58.00 (3.83)
	MQC	69.61 (4.46)	97.94 (4.69)	57.06 (9.37)
	HQC	62.82 (4.92)	91.40 (9.59)	59.43 (6.90)
Biphenyl	**LQC**	62.16 (7.79)	47.54 (5.77)	58.35 (3.03)
	MQC	86.43 (3.64)	61.62 (5.15)	69.68 (0.55)
	HQC	40.37 (11.45)	54.34 (1.81)	69.47 (0.12)
Amide	**LQC**	43.61 (10.47)	72.65 (11.92)	52.10 (1.00)
	MQC	45.95 (2.21)	70.94 (0.99)	64.13 (7.06)
	HQC	51.18 (1.23)	86.74 (3.20)	56.73 (5.01)

2.4.3 Resolution

A resolution of 1.5 or greater represents efficient separation of two peaks [28, 30, 31]. The resolution on the C18 column was 1.28 and 0.50 for FTC & ZDV and ADV-DP & CBB, respectively. The

resolutions on the biphenyl column were calculated for FTC & ZDV, TFV & ADV and ADV-DP & CBB and were found to be 1.55, 0.94 and 1.83 respectively. The resolution on the F5 column ranged between 1.14 and 3.08. The resolutions of FTC & ZDV and ADV-DP & CBB were calculated for the amide column and was found to be 1.00 and 1.60, respectively, with the other peaks not being resolved. Overall the most acceptable resolution was observed on the F5 and biphenyl columns, using MeOH as the mobile phase.

2.4.4 Asymmetry factor and theoretical plates

The asymmetry factor (As) was one of the tools that were applied to determine the efficiencies of the column gradient methods. A value of 1 indicates symmetric peaks, while a As < 1 indicates peak fronting and As > 1 representing peak tailing. The results showed that the biphenyl column (**Table 2**) performed most consistently with only a slight peak tailing, while the other columns exhibited both tailing and fronting for different analytes.

The number of theoretical plates (N) was one of the tools used to measure column efficiencies; it shows the peak dispersion on the column which is a reflection of performance [25, 30, 31]. **Table 2** summarizes the N determined for each column. The N for each compound varied from column to column, with the amide performing best for FTC and the biphenyl and C18 for TFV-DP and EFV, respectively.

Table 2: Mean asymmetric factors and theoretical plates per metre for FTC, EFV and TFV-DP in all evaluated columns

Asymmetric factor						
	FTC	**ZDV**	**TFV-DP**	**ADV-DP**	**CBB**	**EFV**
C18	0.99	1.17	0.78	1.16	1.07	1.03
Biphenyl	1.18	1.24	1.2	1.19	1.11	1.16
F5	1.21	1.15	0.84	0.87	1.12	1.16

Amide	1.28	1.09	1.05	1.14	1.11	0.61
Number of theoretical plates						
C18	36.00	95.60	909.3	5329	5625	23511
Biphenyl	40.96	225.0	5057	6250	6688	6241
F5	31.36	144.0	3863	3738	45511	10404
Amide	49.00	121.0	676.0	2460	3136	18860

2.5. Conclusion

In this study, the efficiencies of new fused-core particle columns with the different active matrix groups which were C18, biphenyl, F5, and amide were compared based on their ability to separate the current HIV ARV one-a-day pill (Atripla) with its respective internal standards. The data obtained from this study clearly indicates that the biphenyl column is most suitable for TDM of Atripla in plasma, when using a methanol gradient method. The biphenyl phase also showed to have the most acceptable resolution and asymmetric factors when compared to the C18, F5 and, amide. However, this does not rule out the use of other columns for different applications than those intended in this study, which was the separation of the current first-line HIV therapy. Ultimately the choice of column is determined by the analyte one wishes to investigate as well as its properties, which each column having its own advantages as discussed.

2.6 Conflicts of interest

There are no conflicts to declare.

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2.8 Supplementary information

2.8.1 Methods

Formulae used in calculations of resolution, asymmetry factor and theoretical plates

The resolution was calculated based on **Eq. 1** shown below:

$$R = \frac{2\,(\,t_{R2} - t_{R1}\,)}{w_{b1} + w_{b2}} \qquad (1)$$

Where Rs is the resolutions between two peaks, t_{R1} and t_{R2} is the retention time of the first and second peak respectively, w_{b1} and w_{b2} is the width at the base of the first and second peak, respectively. The resolution was calculated in all four columns.

Eq. 2 below was used to calculate the number of theoretical plates for each stationary phase:

$$N = 16\,(\frac{t_R}{w_b})^2 \qquad (2)$$

Where N is the number of theoretical plates, t_R is the retention time, and w_b is the width at the base of the peak.

The asymmetry factor (As) is one of the tools that are applied to indicate the efficacy of the column gradient methods, where **a**, is the width of the front half of the peak, and **b**, is the width of the back half of the peak. A value of 1 indicates symmetric peaks, while a As < 1 indicates peak fronting and As > 1 representing peak tailing.

$$As = \frac{b}{a} \qquad (3)$$

2.8.2 Results

2.8.2.1 Accuracy and precision

Table S1: Intra and inter-day precision and accuracy for the analysis of FTC in plasma (n = 6).

QC Levels	Conc (ng/mL)		F5	C18	Biphenyl	Amide
LLOQ	20	Mean (%)	20.27	22.07	18.03	20.24
		Accuracy (%)	101.33	110.38	90.93	101.18
		Intra-day(% RSD)	1.43	0.35	0.77	4.53
		Accuracy (%)	103.77	111.44	104.98	101.50
		Inter-day (% RSD)	3.06	0.25	0.99	4.22
LQC	100	Mean (%)	94.27	110.29	98.36	96.09
		Accuracy (%)	94.27	110.29	97.73	96.09
		Intra-day (% RSD)	1.21	0.84	2.13	3.72
		Accuracy (%)	104.65	112.14	92.21	93.40
		Inter-day (% RSD)	1.73	0.86	1.52	5.22
MQC	500	Mean (%)	500.69	475.1	528.05	513.78
		Accuracy (%)	100.14	95.02	105.13	102.76
		Intra-day (% RSD)	3.08	1.00	3.50	4.60
		Accuracy (%)	99.81249	107.22	97.40	96.80
		Inter-day (% RSD)	2.984731	0.52	2.15	5.231245
HQC	1500	Mean (%)	1388.73	1378.87	1416.15	1544.01

	Accuracy (%)	92.58	91.92	93.71	102.93
	Intra-day (% RSD)	2.66	1.43	1.21	3.91
	Accuracy (%)	96.50618	103.96	96.22	102.94
	Inter-day (% RSD)	1.43	1.39	1.90	3.24

Table S2: Intra and inter-day precision and accuracy for the analysis of EFV in plasma. (n = 3 days, three replicates per day)

QC Level	Conc (ng/mL)		F5	C18	Biphenyl	Amide
LLOQ	20	Mean (%)	18.31	22.18	18.9	17.67
		Accuracy (%)	91.82	110.89	94.51	88.33
		Intra-day (% RSD)	6.38	1.63	1.78	8.28
		Accuracy (%)	90.62	103.25	95.14	105.40
		Inter-day (% RSD)	6.13	2.13	2.12	6.73
LQC	100	Mean (%)	110.75	87.26	110.82	95.76
		Accuracy (%)	110.75	87.26	110.82	95.76
		Intra-day (% RSD)	1.63	0.85	3.13	1.89
		Accuracy (%)	105.99	98.16	103.84	103.56
		Inter-day (% RSD)	1.35	0.49	3.33	2.47
MQC	500	Mean (%)	532.59	562.07	428.41	533.78

		Accuracy (%)	106.52	112.41	85.68	106.76
		Intra-day (% RSD)	1.83	0.58	0.31	2.82
		Accuracy (%)	103.78	106.04	88.48	110.42
		Inter-day (% RSD)	2.78	0.63	1.64	1.48
	1500	Mean (%)	1655.64	1434.83	1309.1	1510.61
		Accuracy (%)	110.38	95.66	87.27	100.71
HQC		Intra-day (% RSD)	1.79	1.32	2.29	2.52
		Accuracy (%)	99.44	94.09	86.30	103.90
		Inter-day (% RSD)	2.54	1.40	1.13	2.96

Table S3: Intra and inter-day precision and accuracy for the analysis of TFV-DP in plasma. (n = 3 days, three replicates per day)

QC Level	Conc (ng/mL)		F5	C18	Biphenyl	Amide
	10	Mean (%)	9.82	10.71	10.71	10.65
		Accuracy (%)	98.18	107.13	107.08	106.50
LLOQ		Intra-day (% RSD)	3.02	1.21	3.36	8.59
		Accuracy (%)	97.35	101.09	107.08	105.99
		Inter-day (% RSD)	3.46	0.78	3.36	2.37
LQC	60	Mean (%)	58.22	53.53	59.54	53.84

		Accuracy (%)	97.03	89.22	99.24	89.73
		Intra-day (% RSD)	0.93	0.98	7.65	2.00
		Accuracy (%)	92.95	98.37	99.24	89.50
		Inter-day (% RSD)	2.43	0.66	7.65	1.32
MQC	250	Mean (%)	270.09	233.82	277.69	219.3
		Accuracy (%)	108.04	93.53	111.08	87.72
		Intra-day (% RSD)	0.95	0.30	2.89	2.48
		Accuracy (%)	96.82	95.00	111.08	93.98
		Inter-day (% RSD)	1.85	0.61	2.89	3.64
HQC	750	Mean (%)	853.11	801.96	850.65	664.57
		Accuracy (%)	113.75	106.93	113.42	88.61
		Intra-day (% RSD)	0.26	0.57	4.33	1.01
		Accuracy (%)	105.30	97.00	113.42	90.68
		Inter-day (% RSD)	1.78	0.88	4.33	2.80

CHAPTER 3

Manuscript 2

LC-MS/MS method development and validation for simultaneous quantification of first-line HIV drugs and second-line TB drugs in rat plasma

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3.1 Abstract

Tuberculosis (TB) remains the most common opportunistic disease in HIV positive patients. TB worsens the symptoms of an HIV infection in co-infected patients and vice versa. HIV-positive patients, who are co-infected with TB, firstly use antiretroviral therapy (ART) for a few months before being introduced to TB treatment. Atripla™ is the first-line combination drug for HIV treatment, which consists of efavirenz (EFV), emtricitabine (FTC) and tenofovir disoproxil fumarate (TFV-DP). The emergence of multi-drug resistant TB (MDR-TB) has presented the need for second-line TB drugs for the effective treatment of resistant bacteria. Streptomycin (STR), kanamycin (KAN) and ofloxacin (OFL) are some of the second-line drugs commonly used to treat MDR-TB, in HIV complicated patients. Studies regarding therapeutic drug monitoring (TDM) of antiretrovirals (ARV's) and TB drugs are extremely important in order to understand the effective dose and duration of treatment, in order to maximize patients' benefits while minimizing the risk of adverse drug effects. Therefore, the aim of this study is to develop, optimize and validate a liquid chromatography-tandem mass spectrometry (LC-MS/MS) method for the simultaneous quantification of first-line anti-HIV drugs (EFV, FTC and TFV) and second-line anti-TB drugs (KAN, OFL and STR) in rat plasma for the usage of TDM. Chromatographic separation of analytes was performed on a biphenyl column (5cm x 2.1 mm, 2.7 mm; pore size of 90 Å), mobile phase A was (H_2O + 0.1% FA) and mobile phase B was (MeOH + 0.1% FA). Mass spectrometric detection was performed in positive electrospray ionization mode using multiple reaction monitoring. Evaluation of assay performance included accuracy and precision, linearity, stability, matrix effect and recoveries in plasma. The LC-MS/MS method reported here meets all the European Medicine's Agency (EMA) guidelines for use in clinical trials and for the simultaneous determination of the effective plasma concentrations of anti-TB and anti-HIV drugs, in addition its short analysis times make it a strong candidate for TDM in a point of care setting.

Keywords: Atripla; streptomycin; kanamycin; ofloxacin; biphenyl; LC-MS-MS

3.2 Introduction

Human Immunodeficiency Virus (HIV) and Acquired Immunodeficiency Syndrome (AIDS) has been recognized as a global health threat for the past three decades, in the 1980's there were only a few deaths due to this disease, however this is no longer the case as mortality has rapidly increased over the past ten years [1-4]. In 2016, there were approximately 36 700 000 and 7 100 000 HIV positive patients globally [5]. Highly active antiretroviral therapy (HAART) has become an essential treatment strategy for patients suffering from HIV infection [6]. However, this treatment has been proven to come with serious adverse effects [7]. This has posed challenges with regards to the acceptable HAART plasma concentration required to avoid toxic effects while maintaining therapeutic efficacy [8].

Patients with an HIV infection are most likely to contract tuberculosis (TB), more than any other opportunistic disease [5, 9]. Simultaneous treatment of TB and HIV is required for HIV-positive TB patients in light of the fact that TB has been recognized as the major cause of death in developing countries, especially among individuals with a compromised immune system [9, 10]. In 2015, 55% of TB patients globally were tested for HIV and amongst them, 15% were HIV positive [5]. TB contributed 35% of global mortality in individuals with HIV/AIDS in 2015 [5]. In 2015, approximately 480 000 people, globally developed multidrug-resistant TB (MDR-TB) [5]. The development of multidrug-resistant (MDR) strains of *Mycobacterium tuberculosis* (*M.tb*) has been disturbing medical experts around the world [11, 12]. These statistics prove that MDR-TB continues to be one of the major problems facing health professionals [5]. A combination of second-line TB drugs, which are expensive, are required for the successful treatment of MDR-TB [9].

There are currently five classes of ARV's available namely; nucleoside and nucleotide reverse transcriptase inhibitors (NRTIs), non-nucleoside reverse transcriptase inhibitors (NNRTIs), protease inhibitors (PIs), Integrase inhibitors (InSTIs) and fusion inhibitors. Each of these classes inhibits a specific stage in the HIV replication cycle [13, 14]. The current first-line HIV treatment consists of a triple ARV combination generally comprises of two NRTIs and an NNRTI or PI in order to target various stages of the viral cycle, this combination is a one-day-pill, known as Atripla™.

Atripla™ was approved in July 2006 by the US Food and Drug Administration (FDA). This combination presents the most effective dosing strategy and the minimal number of pills for treatment of HIV [15, 16], this combination includes two NRTIs [emtricitabine (FTC) and tenofovir disoproxil

fumarate (TFV-DP)], and one NNRTI [efavirenz (EFV)] [8]. The NRTIs mimic the normal nucleotides of DNA, inhibiting transcription of viral RNA to DNA while the NNRTIs change the conformation of the catalytic site of reverse transcriptase and completely prevents its action [13]. The major disadvantage for Atripla™ is that it combines each of its individual components' adverse effects. Some common effects include abdominal pain, rash, anxiety, back pain, paresthesia, skin discoloration particularly on the soles of the feet and palms caused by FTC. EFV is known to cause headaches, insomnia, dizziness, inability to concentrate, and nightmares while TFV is known to cause vomiting, diarrhoea, renal failure, nausea, asthenia, proteinuria, and flatulence [7, 17-19].

Streptomycin (STR), kanamycin (KAN) and ofloxacin (OFL) are second-line drugs that fall within the aminoglycoside and fluoroquinolone groups, respectively, and are commonly prescribed for the treatment of MDR-TB [9, 20-22]. Second-line TB drugs are costly and have more serious side effects than first-line TB drugs [9]. Some common side effects include anorexia, nausea, diarrhoea, skin or hypersensitivity reactions, vomiting, and central nervous system events caused by OFL. STR is known to cause a rash, vertigo, deafness, headache, sterile abscesses at injection spots, and hypersensitivity while KAN causes azotaemia, stomach pains, hearing loss, ataxia, anxiety, nystagmus, serum electrolyte abnormalities, thrush, and tremulousness headache [21, 23-25].

In a recent review, Coyne et al. (2009) stated that drug interactions among aminoglycosides and NRTIs or NNRTIs are possible. However, the use of standard doses and to monitor renal function is recommended since TFV has the risk of additive renal toxicity [25]. It further stated that interactions among fluoroquinolones and NNRTIs or NRTIs are rare but also possible. However, oral absorption of fluoroquinolones is reduced by buffered drugs, therefore, they should be taken 6 hours after or 2 hours before the use of any buffered drugs and caution should be taken when these drugs are used with drugs associated with renal dysfunction, such as TFV [25].

Investigations regarding therapeutic drug monitoring (TDM) of antiretroviral (ARV) drugs are highly important in order to understand the effective dose, frequency of dosing and duration of treatment. This could reduce toxicity levels and the cost of the therapy [8, 26, 27]. Further clinical trials are required before TDM can be accepted as a standard tool in the management of HIV therapy [28].
The treatment of HIV patients co-infected with TB can be very complicated, especially due to drug-related factors such as toxicity, adherence to therapy, drug resistance, and complex drug interactions. Therefore, it is vital to study the TDM of HIV patients co-infected with TB since TDM will be helpful in determining the effective plasma concentration levels of anti-TB and anti-HIV drugs that present

minimum drug interactions and adverse effects [29, 30]. To our knowledge, no studies exist regarding the development of an LC-MS method for the simultaneous detection of HIV and TB drugs.

Therefore, the aim of this study is to develop, optimize and validate a liquid chromatography-tandem mass spectrometry (LC-MS/MS) method for the simultaneous quantification of anti-HIV drugs (FTC, EFV, and TFV) and second-line anti-TB drugs (STR, KAN, and OFL) in rat plasma for the usage of TDM.

3.3 Materials and methods

3.3.1 Reagents and standards

EFV was purchased from Adcock Ingram (South Africa). TFV, KAN, STR and OFL were purchased from Sigma Aldrich (South Africa). FTC was purchased from Mylan (South Africa). Internal Standards, 4-(4-Carboxybenzyl)-2H-1, 4-benzoxazin-3(4H)-one (CBB), adefovir dipivoxil (ADV-DP), amikacin (AKN), dihydrostreptomycin (DHSTR) and ciprofloxacin (CFL) were all purchased from Sigma Aldrich (South Africa) except for lamivudine (3TC) which was purchased from Aspen pharmacare (South Africa). LC-MS grade solvents; acetonitrile (ACN) and methanol (MeOH) were purchased from Sigma Aldrich (South Africa). Analytical grade formic acid (FA) was purchased from Merck Millipore (Merck, South Africa). Water was purified with a Milli-Q purification system from Millipore Corporation (Bedford, MA, USA). HLB-SPE cartridges (30 mg, 1.0 mL) were purchased from Supelco-Sigma (St. Louis, MO).

3.3.2 Calibration standards and quality control samples

Separate stock solutions of target analytes and internal standards were prepared by dissolving 1 mg of each HIV drug in 1 mL of MeOH and dissolving 1 mg of each TB drug in 2 mL of MeOH and water (50:50 v/v). All prepared stock solutions were stored at –20 ◦C. The appropriate serial dilutions of the stock solutions were done to prepare the working standard solutions of target compounds and internal standards in MeOH and water (50:50 v/v). Calibration standards that were prepared ranged from 10 to 1000 ng/mL for TFV and OFL, 20 to 2000 ng/mL for EFV, FTC, STR and KAN. The quality control (QC) samples were prepared at lower limit of quantification (LLOQ), low quality control (LQC), middle quality control (MQC) and high-quality control (HQC) to give the final concentration levels of 10, 20, 250 and 750 ng/mL for TFV, and OFL, 20, 60, 500 and 1500 ng/mL

for EFV, FTC, STR and KAN. Internal standard concentrations were 250 ng/mL for ADV-DP and AKN, 500 ng/mL for CBB, 3TC, DHSTR and CFL.

3.3.3 Instrumentation and chromatographic conditions

Analysis and validation were performed using an LC-MS/MS system comprised of an Agilent 1100 series high-performance liquid chromatography (HPLC) system coupled to MicroTOF-Q II electrospray ionization (ESI) time-of-flight mass spectrometry (TOF-MS) instrument (Bruker Daltonics, Bremen, Germany). Chromatographic separation was performed using an Ascentis Express biphenyl analytical column with dimensions of 5 cm × 2.1 mm, a particle size of 2.7μm and pore size of 90 Å. The analytes and internal standards were eluted using a gradient mobile phase system comprising of mobile phase A (H_2O + 0.1% v/v FA) and mobile phase B (MeOH + 0.1% v/v FA) and the flow rate was set at 0.35 mL/min. The gradient method started from 53% mobile phase B, increased to 95% over 3 min then held for 5 min before being returned to the initial composition over 3 min. The column equilibration time was 3 min.

The MS parameters were optimized based on the mass-to-charge ratio (*m/z*) for the detection of EFV, FTC, TFV, STR, OFL and KAN including their internal standards; CBB, 3TC, ADV-DP-DP, DHSTR, CFL and AKN, respectively. Source parameters were: source type, ESI; ion polarity, positive; end plate source, -500 V; nebulizer, 1.8 bar; dry gas, 8.0 L/min; capillary, -4500V; dry temperature, 200 ◦C; mass range, *m/z* 50 $\rightarrow$ 700; collision cell radiofrequency, 200 Vpp; collision energy, 10 eV. Multiple-reaction monitoring (MRM) was performed to yield the mass transitions of analytes and internal standards shown in **Table S1**. All the data was analyzed using Data Analysis 4.0 SP 5 and Quant Analysis 2.1 (Bruker Daltonics).

3.3.4 Method validation

Method validation was conducted according to the European Medicines Agency (EMA) guidelines for bioanalytical method development [31].

3.3.4.1 Specificity and selectivity

The specificity and selectivity of the method were evaluated by analyzing six blank rat plasma samples at LLOQ level. All blank samples were analyzed in the MRM mode following the sample preparation procedure and LC-MS/MS conditions. The results were compared to chromatograms

obtained for a pure standard solution at the LLOQ level to investigate the potential interferences on the target compounds and internal standards.

3.3.4.2 Linearity and lower limit of quantification

A signal-to-noise ratio (S/N) of 3:1 was used for determining the limit of detection (LOD) and the LLOQ was determined by an S/N ratio that was 5X to that of the LOD. The calibration curves were constructed by plotting the peak area ratio of analyte to internal standard against the theoretical concentrations of the analyte. The criteria for calibration range included a correlation coefficient (R^2) ≥ 0.99 [32]. The calculated concentrations of the calibration standards should be within ±15% except for the LLOQ which should be within ±20% deviation as per EMA guidelines [31].

3.3.4.3 Accuracy and precision

Accuracy was determined as the percentage between the measured concentration of the target compounds and their nominal concentration levels. Intra-day and inter-day accuracy and precision were assessed by assay of six replicates of different QC levels (LLOQ, LQC, MQC, and HQC) the same day and on three different days. The accuracy must be within ± 15% of the nominal concentration and for precision, the percentage relative standard deviation (%RSD) must be within ±15% [31].

3.3.4.4 Matrix effect and extraction recovery

Matrix effect and extraction recovery of the analytes were estimated at the three QC levels (LQC, MQC and HQC) in six replicates. Extraction recovery in brief, 50 µL of rat plasma, 10 µL of 1.9 M ammonium acetate and 10 µL of 3 M hydrochloric acid was spiked with the appropriate amount of each analyte, in order to attain the desired QC concentrations. Thereafter, the sample was made up to 1 mL using LC-MS grade MeOH and vortexed for 1 minute. The mixture was then centrifuged at 15 000 rpm, 4 °C for 15 minutes. The supernatant was subjected to SPE using the Supel- SELECT HLB: 30mg/1 mL SPE cartridge (Supelco, Sigma Aldrich, Germany), which gave the most reproducible recoveries. The SPE cartridge was conditioned with 1 mL of MeOH. The supernatant was then passed through the SPE cartridge. The eluent was collected in an HPLC vial and vortexed, prior to injection into the LC-MS system. The matrix effect was evaluated on six lots of blank plasma from different sources, as requested by guidelines. Peak areas from blank extracts spiked with all analytes at three QC levels (post-extraction addition samples) were compared with peak areas from standard solutions

spiked with analytes at the same concentration level. The matrix effect was calculated as percentage of deviation of the peak areas obtained from the plasma extracts with the ones obtained from the standard solutions. Matrix factor should be within ± 15% [31].

3.3.4.5 Stability

Batch samples stay longer in the autosampler due to queued injections. Therefore, the autosampler stability of analytes was evaluated analyzing prepared samples stored in the auto-sampler for a period of 24 h before injection. The freeze-thaw stability was assessed after exposing QC samples to three freeze-thaw cycles. The 6-hour short term stability of the analytes on the bench top was also investigated. All stability experiments were evaluated at the four QC levels (LLOQ, LQC, MQC and HQC).

3.4 Results and Discussion

3.4.1 LC-MS/MS method optimization

The results of the mass transitions of the samples optimized and retention times obtained are shown in **Table S1**. The chromatogram is shown in **Figure 1**. Currently, there are no available studies that have developed and validated an LC-MS method for the simultaneous quantification of first-line anti-HIV drugs and second-line anti-TB drugs. To our knowledge, this current study is the first of this kind. However, numerous studies have been conducted for the LC-MS quantification of HIV and second-line TB drugs, separately. These results are similar to the ones that are already available with regard to separation. However, the total run time of this study is better compared to other similar studies.

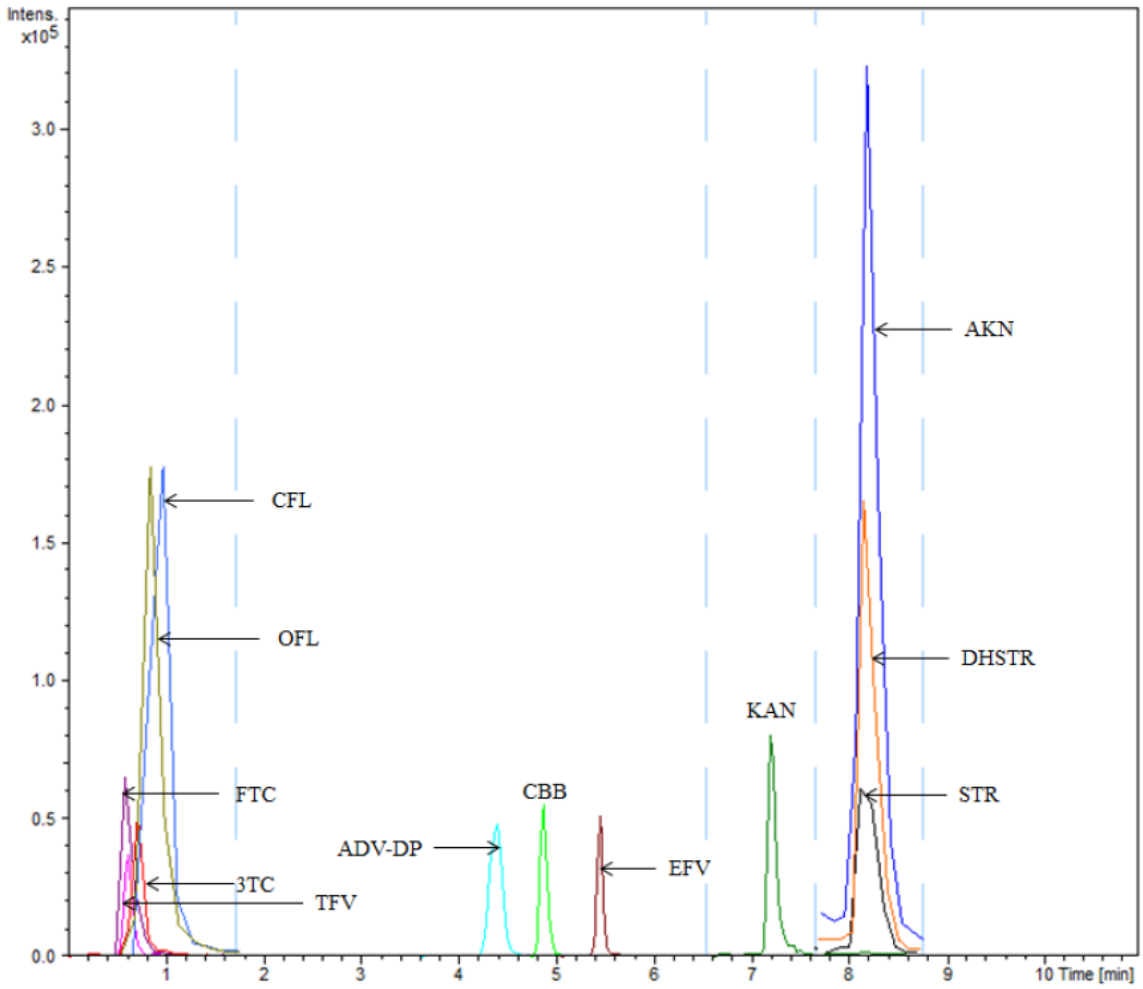

Figure 1: Chromatogram showing the simultaneous detection of three HIV and TB drugs with their internal standards

Key

FTC - Emtricitabine	3TC - Lamivudine
TFV - Tenofovir	ADV-DP - Adefovir dipivoxil
EFV - Efavirenz	CBB - 4-(4-Carboxybenzyl)-2H-1, 4-benzoxazin-3(4H)-one
STR - Streptomycin	DHSTR - Dihydrostreptomycin
OFL - Ofloxacin	CFL - Ciprofloxacin
KAN - Kanamycin	AKN - Amikacin

3.4.2 Method validation

An LC-MS/MS method for simultaneous quantification of first-line HIV drugs and second-line TB drugs in rat plasma was developed and validated with respect to specificity and selectivity, linearity and lower limit of quantification, accuracy and precision, stability, matrix effects and recovery. All criteria for bioanalytical method validation based on the EMA guidelines were satisfied [31].

3.4.2.1 Specificity and selectivity

The specificity and selectivity were evaluated by comparing the chromatograms of the drug-free rat plasma with the corresponding spiked plasma samples. No significant interfering peaks were observed due to endogenous compounds. Co-eluting compounds were differentiated from each other by the MRM's ability to deliver a unique fragment ion for each compound that is monitored and quantified (mass transition) in a matrix. A fragmented mass is also distinguished by extracted ion chromatogram (EIC). Results were within the acceptance criteria where the response was < 20% of the LLOQ for all the analytes and 5% for all the internal standards.

3.4.2.2 Linearity and lower limit of quantification

The LODs concentrations ranged from 1 to 10 ng/mL. All calibration curves had the correlation coefficients (R^2) of greater than 0.99, shown in **Table 1**. The calibration standards were also within the acceptance criteria recommended by the EMA guidelines [31]. The calibration standards ranged from 10 to 1000 ng/mL for OFL and TNV, 20 to 2000 ng/mL for EFV, FTC, KAN, and STR. The concentrations for internal standards were 250 ng/mL for ADV-DP and AKN, while it was 500 ng/mL for 3TC, CBB, CFL, and DHSTR.

Table 1: Correlation coefficient of analytes

Analyte	Correlation coefficient (R^2)
EFV	0.994720
FTC	0.997057
TFV	0.998962
KAN	0.993060
OFL	0.994408
STR	0.994246

3.4.2.3 Accuracy and precision in plasma

The results for accuracy and precision of the proposed method are shown in **Table S2**. Intra-day accuracy varied from 88.68% to 107.47% and inter-day accuracy from 90.99% to 104.99%. The intra-day precision varied from the %RSD of 0.64% to 9.71% and inter-day from 1.56% to 12.77%. These values are within the acceptance criteria of ±15% recommended by the EMA guidelines [31].

3.4.2.4 Stability

The results for the stability of the analytes are shown in **Table 2**. The stability in all evaluated conditions varied from 87.25% to 107.85% with the %RSD varying from 0.22% to 11.36%. These values are within the acceptance criteria of ±15% recommended by the EMA guidelines [31]. This confirms that all analytes were stable in all evaluated conditions.

Table 2: Stability of EFV, KAN, FTC, STR, OFL and TFV in plasma (n = 6)

			QC levels			
			LLOQ	LQC	MQC	HQC
Storage condition	Analyte	Concentration (ng/mL)	20	60	500	1500
Bench-top, 6 h, RT	EFV	Mean	18.68	58.67	529.00	1562.40
		Accuracy (%)	93.38	97.78	105.80	104.16
		(% RSD)	2.37	7.16	3.64	1.42
	FTC	Mean	20.67	57.07	507.00	1442.40
		Accuracy (%)	103.36	95.11	101.40	96.16
		(% RSD)	5.45	3.96	2.14	3.67
	KAN	Mean	20.71	60.65	497.75	1336.95
		Accuracy (%)	103.55	101.08	99.55	89.13
		(% RSD)	6.66	6.91	6.16	1.43

	STR	Mean	18.27	60.64	509.80	1611.00
		Accuracy (%)	91.34	101.06	101.96	107.40
		(% RSD)	3.25	0.22	2.52	1.00
Three freeze-thaw cycles, -80ºC						
	EFV	Mean	20.74	61.65	534.85	1406.85
		Accuracy (%)	103.71	102.75	106.97	93.79
		(% RSD)	1.18	1.05	0.88	0.91
	FTC	Mean	18.176	62.322	502.40	1555.65
		Accuracy (%)	90.88	103.87	100.48	103.71
		(% RSD)	1.17	1.10	0.90	0.75
	KAN	Mean	21.82	52.35	509.95	1355.40
		Accuracy (%)	95.07	87.25	101.99	90.36
		(% RSD)	11.36	1.61	1.55	1.02
	STR	Mean	20.706	64.932	472.20	1607.70
		Accuracy (%)	103.53	104.94	94.44	107.18
		(% RSD)	1.65	1.34	3.93	0.83
Autosampler, 24 h, RT	EFV	Mean	18.48	57.16	484.35	1562.85
		Accuracy (%)	92.38	95.26	96.87	104.19
		(% RSD)	1.80	1.47	5.69	1.30
	FTC	Mean	21.18	56.03	511.50	1463.25
		Accuracy (%)	105.89	93.38	102.30	97.55
		(% RSD)	1.78	0.59	0.93	0.65
	KAN	Mean	20.34	58.48	517.75	1367.10
		Accuracy (%)	101.72	97.47	103.55	91.14
		(% RSD)	9.29	2.03	6.66	0.88

	STR	Mean	18.198	59.50	503.90	1614.45
		Accuracy (%)	90.99	99.16	100.78	107.63
		(% RSD)	3.08	0.73	2.59	1.08
Stability	Analyte	Concentration (ng/mL)	10	20	250	750
Bench-top, 6 h, RT	TFV	Mean	9.72	19.66	261.05	729.90
		Accuracy (%)	97.21	98.28	104.42	97.32
		(% RSD)	1.24	4.26	5.16	6.28
	OFL	Mean	9.22	19.45	239.4	744.98
		Accuracy (%)	92.17	97.25	95.76	99.33
		(% RSD)	3.35	2.20	2.34	3.57
Three freeze-thaw cycles, -80ºC	TFV	Mean	9.66	21.57	241.63	762.68
		Accuracy (%)	96.63	107.85	96.65	101.69
		(% RSD)	5.23	1.13	1.38	1.25
	OFL	Mean	10.62	18.51	259.98	780.08
		Accuracy (%)	106.23	92.56	103.99	104.01
		(% RSD)	1.30	1.37	1.21	3.33
Autosampler, 24 h, RT	TFV	Mean	9.72	19.36	266.4	709.95
		Accuracy (%)	97.16	96.82	106.56	94.66
		(% RSD)	1.22	1.33	0.89	1.32
	OFL	Mean	9.15	19.61	236.98	736.28
		Accuracy (%)	91.45	98.04	94.79	98.17
		(% RSD)	1.66	1.06	1.68	1.17

RT: Room temperature

3.4.2.5 Matrix effect and extraction recovery

The results for matrix effect and extraction recovery are shown in **Table 3**. The recovery of all six analytes ranged from 85.3% to 107.4% with the %RSD ranging from 0.21% to 7.66%. These recoveries indicate that the method was reproducible for each compound. The matrix factor of all six analytes ranged from -12.98% to 5.87% with the %RSD ranging from 0.28% to 12.62%, which was within the acceptable range of ±15% determined by the EMA guidelines [31]

Table 3: Matrix effect and extraction recovery of EFV, KAN, FTC, STR, OFL and TFV in plasma (n=6)

Analyte	QC Level	Concentration (ng/mL)	Mean recovery (%)	%RSD	Matrix effect (%)	%RSD
EFV	Low	60	96.9	2.75	-6.71	3.65
	Middle	500	100.9	0.89	0.12	0.28
	High	1500	100.3	0.07	1.07	0.77
FTC	Low	60	95.7	2.16	-6.83	2.52
	Middle	500	90.1	7.46	-10.46	7.50
	High	1500	95.3	1.00	-3.36	4.41
TFV	Low	20	86.3	0.21	-9.15	4.20
	Middle	250	104.2	2.22	0.65	3.06
	High	750	85.3	2.46	-12.98	4.03
KAN	Low	60	98.9	1.59	-1.92	2.38
	Middle	500	97.6	0.81	-2.49	0.33
	High	1500	106.2	2.02	5.87	11.71
STR	Low	60	89.8	3.32	-7.21	4.32
	Middle	500	105.7	5.51	1.60	12.62
	High	1500	99.1	2.66	-8.25	0.77
OFL	Low	20	91.3	4.85	-11.35	0.40
	Middle	250	95.2	7.66	-7.76	10.70
	High	750	107.4	2.12	5.85	3.86

TDM has been utilized in the management of treatment of different health background conditions to guarantee ideal therapeutic advantages while limiting adverse effects [33, 34]. For a very long time, TDM for anti-HIV drugs and anti-TB drugs has been identified as a critical tool required for the optimization of HIV and TB treatment programs [33, 34]. A validated LC-MS/MS method is the primary requirement for successfully determining the plasma drug concentrations and pharmacokinetic parameters in TDM [33-35]. Validated LC-MS/MS methods for quantification of anti-HIV and anti-TB drugs, individually, are available [8, 33, 36-39].

The linearity, accuracy and precision of assay must be determined, and evaluated to verify that the analytical range of the method is adequate to cover the accepted plasma drug concentrations. The R^2, for all analytes was greater than 0.99 with the accuracy and precision ranging from 88.68% to 107.47%, all of which were well within the specifications of the EMA. Specificity and sensitivity studies was performed to determine the extent of interference from the drug combination and drug metabolites, this analytical method showed no significant interference with respect to the separation of the analytes. Optimization of sample preparation was done to limit matrix effects in the assay using solid phase extraction [34]. Matrix effect occurs when components in the sample matrix co-elute with the analytes of interest, which changes the signal, leading to low detection of the analytes, quantitation inaccuracy and low method robustness. The plasma recovery ranged from 85.3% to 107.4% which is within the ±15% recommended by EMA guidelines, thus suitable for TDM studies with the matrix effects ranging from -12.98% to 5.87%. All the parameters monitored in this study were well within the acceptable range as recommended by the EMA.

All these parameters are essential in method validation were performed to validate the optimized method presented in this study. In this study, we present the LC-MS/MS method with the total run time short to 11 minutes which is convenient for TDM of a large number of HIV positive TB patients. In addition to TDM, this method has applications for clinical trials and pharmacokinetic studies of HIV positive and MDR-TB patients.

3.5 Conclusion

The LC-MS/MS method developed, optimized and validated in this study allows for the precise, rapid and reproducible simultaneous quantification of first-line antiretroviral drugs (EFV, FTC, and TFV) and second-line TB drugs (KAN, STR and OFL) in plasma. This is the first such LC-MS/MS method to be developed and validated for simultaneous quantification of first-line HIV drugs and second-line TB drugs aimed at TDM. TDM is useful in improving the combined efficacy of TB and HIV treatment. This ensures that each patient is given suitable drug doses in

order to maintain plasma drug concentration within the therapeutic window, minimizing the incidence of drug-drug interactions and adverse effects.

3.6 Conflicts of interest

There are no conflicts to declare.

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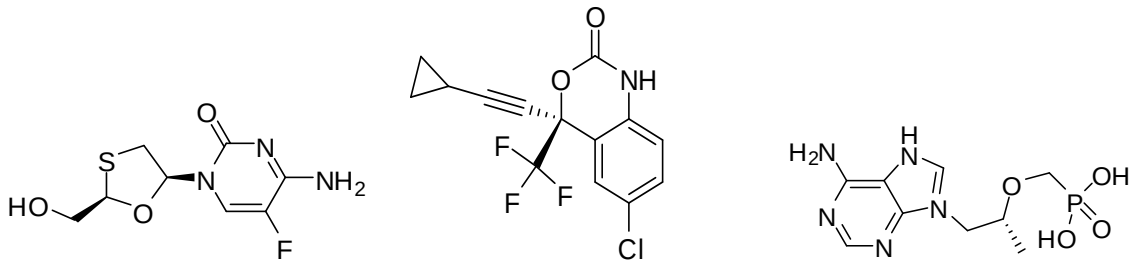
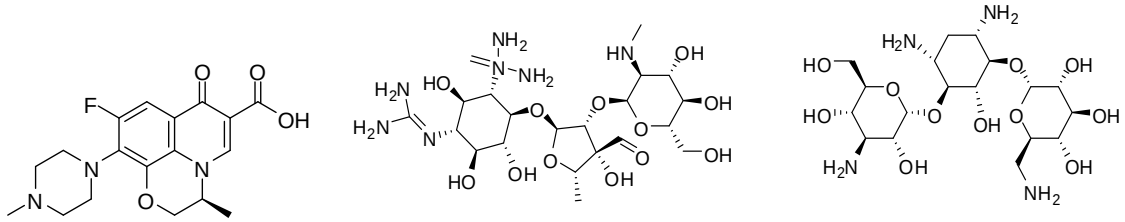
3.8 Supplementary information

3.8.1 Introduction



EMT EFV TFV

Figure S1: Anti-HIV drug structures constituting Atripla™



OFL STR KAN

Figure S2: Chemical structures of second-line anti-tuberculosis drugs

3.8.2 Methods

The matrix effect was calculated based on equation shown below:

$$ME\ (\%) = \left(\frac{Area\ of\ the\ post\ extracted}{Area\ of\ the\ solution} - 1\right) \times 100$$

Where ME is the matrix effect.

3.8.3 Results

Table S1: MRM mass transitions and retention times of analytes with their internal standards

Analyte	Precursor ion $[M^+H]^+$	Product ion $[M^+H]^+$	Retention time (min)
EFV	316	168	6.1
FTC	248	130	0.8
TFV	288	176	0.9
KAN	485	133	7.2
OFL	362	261	0.7
STR	582	311	8.0
CBB (IS)	284	135	5.6
3TC (IS)	230	112	0.8
ADV-DP (IS)	502	256	5.0
AKN (IS)	586	582	8.0
CFL (IS)	332	231	0.8
DHSTR (IS)	584	298	8.0

IS: Internal standard

Table S2: Accuracy and Precision of EFV, KAN, FTC, STR, OFL and TFV in plasma (n = 6)

		QC level			
		LLOQ	LQC	MQC	HQC
Analyte	Concentration (ng/mL)	20	60	500	1500
EFV	Mean	18.58	58.72	526.05	1515.90
	Accuracy (%)	92.88	97.87	105.21	101.06
	Intra-day (% RSD)	1.07	1.71	1.00	0.89
	Mean	20.83	62.99	509.85	1572.30
	Accuracy (%)	104.13	104.99	101.97	104.82
	Inter-day (% RSD)	9.59	5.92	7.07	7.50
KAN	Mean	17.90	62.36	490.35	1462.80
	Accuracy (%)	89.50	103.94	98.07	97.52
	Intra-day (% RSD)	8.08	2.57	1.35	1.25

	Mean	19.29	57.10	503.85	1523.85
	Accuracy (%)	96.47	95.16	100.77	101.59
	Inter-day (% RSD)	1.56	10.51	7.69	5.19
FTC	Mean	21.49	59.27	464.05	1330.20
	Accuracy (%)	107.47	98.78	92.81	88.68
	Intra-day (% RSD)	0.96	0.88	0.91	0.86
	Mean	20.00	60.69	472.30	1414.65
	Accuracy (%)	99.99	101.15	94.46	94.31
	Inter-day (% RSD)	7.81	6.20	4.64	6.41
STR	Mean	21.39	58.66	524.95	1596.9
	Accuracy (%)	106.96	97.77	104.99	106.46
	Intra-day (% RSD)	3.12	2.61	0.64	1.65
	Mean	20.63	62.35	504.20	1591.95
	Accuracy (%)	103.16	103.91	100.84	106.13
	Inter-day (% RSD)	3.36	5.31	9.17	4.33
Analyte	Concentration (ng/mL)	10	20	250	750
OFL	Mean	9.47	18.15	229.83	765.23
	Accuracy (%)	94.74	90.77	91.93	102.03
	Intra-day (% RSD)	9.71	0.90	0.99	1.39
	Mean	9.90	19.27	242.73	713.63
	Accuracy (%)	98.96	96.36	97.09	95.15
	Inter-day (% RSD)	3.98	12.64	12.77	7.03
TFV	Mean	10.08	18.2	238.23	688.35
	Accuracy (%)	100.83	90.99	95.29	91.78
	Intra-day (% RSD)	1.42	1.01	1.00	0.97
	Mean	10.27	19.34	237.48	727.58
	Accuracy (%)	102.73	96.71	94.99	97.01
	Inter-day (% RSD)	4.50	7.43	5.97	4.95

CHAPTER 4

4. Conclusion

4.1 Summary of the study

In chapter 1, it was demonstrated that HIV and TB continue to be major health threats in the world, especially as a co-infection. In 2016, WHO estimated the number of HIV positive individuals to be approximately 36 700 000, globally. WHO also estimated the global prevalence of multi-drug resistant tuberculosis (MDR-TB) to be 490 000 in 2016. In 2016, WHO approximated that HIV positive individuals have a 26-fold-higher chance of TB disease progression from latent TB infection (LTBI) to active TB when compared with HIV negative individuals. TB accounted for 22% of worldwide mortality in people with HIV/AIDS in 2016. HIV positive TB patients receive both HIV and TB treatments. Drug-drug interactions are likely to occur for the patients receiving at least three drugs for combined treatment. TDM is the ideal tool to quantify these interactions and to determine the correct dosage that a specific patient requires. TDM is the measurement of specific drug concentrations at time intervals in the patients' plasma. The need to develop an LC-MS/MS method for the simultaneous quantification of anti-HIV and anti-TB drugs using the best performing HPLC column was identified. This method will potentially assist HIV positive TB patients from suffering the serious adverse effects and reduce the time spent by these patients in hospitals.

The main aim of this study was to evaluate the performance of the new column technologies and use the one with optimum efficiency to develop and validate an LC-MS/MS method for the simultaneous quantification of anti-HIV drugs [emtricitabine (FTC), efavirenz (EFV), and tenofovir (TFV)] and second-line anti-TB drugs [streptomycin (STR), kanamycin (KAN), and ofloxacin (OFL) in rat plasma for the usage of therapeutic drug monitoring (TDM). FTC, EFV, and TFV are the constituents of a one-day pill, Atripla^{TM}, which presents the most effective dosing plan. STR, KAN, and OFL are used for the treatment of MDR-TB, which is defined as the resistance at least to rifampicin (RMP) and isoniazid (INH) first-line anti-TB drugs.

Numerous studies have been conducted for the LC-MS quantification of HIV and second-line TB drugs, separately. These studies are useful in the understanding of HIV and MDR-TB treatment, individually. These studies focus on a wide range of HIV and second-line drugs, they are not only focusing on one group but cover all groups that are available. However, the increasing number of HIV and TB co-infected patients makes it hard for clinicians to choose the treatment that will not cause drug interactions and side effects for these patients. Therefore, this left a room for

researchers to perform studies that combine these two major diseases in order to provide the best treatment plan for HIV and TB co-infected patients.

In chapter 2, evaluation of four new HPLC column technologies was done based on the resolution, theoretical plates, asymmetry factor, limit of detection, linearity, accuracy and precision. This was accomplished by developing and validating an LC-MS/MS method of simultaneous quantification of current first-line anti-HIV treatment (FTC, EFV and TFV) according to EMA guidelines. Mobile phases of MeOH and ACN with water were evaluated using a gradient method on all columns. The analytes' limit of detection (LOD) ranged from 1 ng/ml to 15 ng/ml. Linearity was determined by the correlation coefficient (R^2) which was greater than 0.99 for all the analytes in all the columns. FTC, EFV and TFV-DP showed intra- and inter-day precisions with an RSD percentage of 0.25 to 5.22 %, 0.31 to 6.73 % and TFV-DP 0.26 to 8.59 %, respectively. The recoveries % RSD on the C18, F5, biphenyl and amide column ranged from 2.22 to 13.99 %, 4.46 to 9.59 %, 0.12 to 11.45 % and 0.99 to 11.92%, respectively. The biphenyl column showed good resolution for FTC & ZDV and ADV-DP & CBB and was found to be 1.55 and 1.83, respectively. The biphenyl column also showed good peak asymmetry factor, which ranged from 1.11 and 1.24. Biphenyl column showed consistency again with regards to the number of theoretical plates when compared to the C18, F5 and, RP-amide. However, it was concluded that other columns (C18, F5 and, RP-amide) may still be used for applications other than those intended in this study, for example, the separation of the current drugs (FTC, EFV and TFV) used for first-line HIV therapy. The other columns (C18, F5 and RP-amide) that were used in this study have their own advantages depending on the objective intended to be accomplished. In addition, these new HPLC column matrices offer various benefits such as the potential of saving solvents and short runtimes, essential in TDM studies.

Looking at the short run time and number of compounds in this study, the separation is adequate compared to similar studies that have longer run times with only a few compounds being studied.

In chapter 3, an LC-MS/MS method for the simultaneous quantification of first-line anti-HIV drugs (EFV, FTC and TFV) and second-line anti-TB drugs (KAN, OFL and STR) was developed and validated according to EMA guidelines using a biphenyl column, which showed optimum performance and consistency in chapter 2. Currently, there are no available studies that have developed and validated an LC-MS method for the simultaneous quantification of first-line anti-HIV drugs and second-line anti-TB drugs. To our knowledge, this current study is the first of this kind. Mass spectrometric detection was performed in positive electrospray ionization mode using multiple reaction monitoring. Evaluation of assay performance included accuracy and precision,

linearity, stability, matrix effect and recoveries in plasma. Intra-day accuracy ranged from 88.68% to 107.47% with the %RSD of 0.64% to 9.71% and inter-day accuracy from 90.99% to 104.99% with the %RSD of 1.56% to 12.77% for all the analytes. Linearity was determined by the R^2 which was greater than 0.99 for all the analytes. Stability conditions that were evaluated include the 6 h bench-top, 24 h autosampler and three freeze-thaw cycles, which together ranged from 87.25% to 107.85% with the %RSD of 0.22% to 11.36%. Concluding from these results, all the analytes are stable. Matrix effect ranged from -12.98% to 5.87% with the %RSD ranging from 0.28% to 12.62%. The recoveries ranged from 85.3% to 107.4% with the %RSD of 0.21% to 7.66%. Those anti-TB drugs that were structurally similar and belonged to one group had poor separation. However, these results are similar to those of other studies that performed an LC-MS/MS method for the simultaneous quantification of second-line anti-TB drugs.

The LC-MS/MS methods reported in this thesis meets all the EMA Guidelines for use in clinical trials and for the simultaneous determination of the effective plasma concentrations of anti-TB and anti-HIV drugs, making it a strong candidate for TDM in a point of care setting. Effective TDM studies will ensure that patients spend less time in hospital thus saving money and time. This study covered nucleoside reverse transcriptase inhibitors (NRTIs) and non-nucleoside reverse transcriptase inhibitors (NNRTIs) antiretroviral therapy classes as well as fluoroquinolone and aminoglycoside TB drug groups. Therefore, future studies should cover more drugs from various antiretroviral therapy classes and more anti-TB drugs from different groups that are available. Future research should also perform pharmacokinetic studies of combined HIV and TB drugs in animal models before taking it further to clinical trials.

4.2 Recommendations

The LC-MS/MS methods developed and validated herein can readily be used for future studies intending to perform simultaneous quantification of first-line HIV and/or second-line TB drugs in rat plasma and is well suited for therapeutic drug monitoring. Thereafter, these methods can be used for clinical trials. This current study only covered sufficient for the scope of master's degree.

APPENDICES

APPENDIX 1

Conference poster

The South African Annual Pharmacology Conference (SAPHARM-2017), Faculty of Health Sciences, University of the Free State, 01st – 04th October 2017

The development of a rapid LC-MS/MS method for simultaneous quantification of first-line HIV and TB drugs

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Introduction

The Human Immunodeficiency Virus (HIV) is a rapidly proliferating infection occurring in the body, which further develops into Acquired Immune Deficiency Syndrome (AIDS) causing destruction to the immune system. To date, there is no known cure to exist for the virus; however, medication available on the market hinders the damage caused by the viral disease, allowing the immune system to improve and increase patients lifespan. The 2015 statistics on UNAIDS, reports that approximately 6 700 000 to 7 400 000 people in South Africa who are living with HIV/AIDS [1]. Due to the HI-virus supressing the immune system, co-morbidities may occur, one of the most common opportunistic infections being Tuberculosis (TB) [2]. Optimum treatment of TB with responsible patient practices can prompt the fruitful treatment and lessen mortality. However, some patients fail to adhere to treatment plans and develop drug resistance. In 2015, an estimated 480 000 individuals on the planet developed multidrug -resistant TB (MDR-TB). South Africa had 65% new cases of individuals who developed MDR-TB in 2015 [3]. Therapeutic drug monitoring (TDM) of antiretroviral and TB drugs is critical in clinical maintenance, in selecting the best dose regimen personalised to each patient to lessen the danger of virological failure from low plasma drug concentration and to restrain the toxicity associated with high plasma concentrations. Thus far, there has been no rapid method developedto quantify both HIV and TB drugs simultaneously [4].

AIM: To develop and validate a rapid and sensitive lc-ms/ms method for quantification of first-line HIV drugs (emtricitabine, efavirenz, and tenofovir disoproxil) and second-line TB drugs (streptomycin, kanamycin, and ofloxacin) in rat plasma.

Methodology

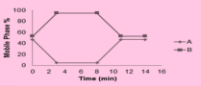
This study was conducted for the simultaneous LCMS/MS quantification of Atripla (Efavirenz, Emtricitabine, and Tenofovir Disoproxil) and second line TB drugs (Streptomycin, Kanamycin, and Ofloxacin) together with their internal Standards (4-(4-Carboxybenzyl)-2H-1,4-benzoxazin-3(4H)-one, Lamivudine, and Adefovir Dipivoxil) and (Dihydrostreptomycin, Amikacin, Ciprofloxacin) respectively.

Validation parameters evaluated were recovery in plasma, linearity, stability, accuracy and precision.
The Ascentis Express biphenyl column with the dimensions of 5cm x 2.1 mm, 2.7 mm and pore size of 90 Å was used for evaluation.

The liquid chromatography tandem mass spectrometry (LC–MS-MS) system comprised of a Agilent 1100 series HPLC system coupled to a MicroTOF-Q II electrospray ionization (ESI) time-of-flight-mass spectrometry (TOF-MS) instrument (Bruker Daltonics, Bremen, Germany). All results were analyzed with Data Analysis 4.0 SP 5 (Bruker Daltonics).

Table 1: LC method

Time(min)	A(%)	B(%)
0.00	47	53
3.00	5	95
8.00	5	95
11.00	47	53



Mobile phase A – H2O (0.1% FA)
Mobile phase B – MeOH (0.1% FA)

Results

LODs in plasma ranged from 5-15 ng/ml for the analytes, recoveries in plasma, linearity, stability, accuracy and precision, all fell within the acceptable range of EMA guidelines [5].

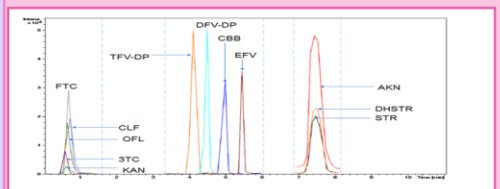


Figure 1: Representation of lc-ms/ms chromatograms for Emtricitabine, Tenofovir disoproxil, Efavirenz, Streptomycin, Kanamycin, Amikacin and their internal standards

LODs and LLOQs in solution

Analyte	LOD (ng/ml)	LLOQ (ng/ml)
EFV	10	20
FTC	10	20
TFV-DP	5	10
OFL	5	10
KAN	10	20
STR	10	20

Table 3: Recoveries in plasma

Analyte	Recovery (%)
EFV	100.3
FTC	95.3
TFV-DP	85.3
OFL	107.4
KAN	111.1
STR	99.1

Table 4: Calibration curve equations and correlation coefficient (R)

Analyte	Calibration curve equation	R
Emtricitabine	y= 4.645901 x - 0.223660	0.99
Efavirenz	y=0.221562 x + 0.190779	0.99
Tenofovir disoproxil	y=1.136485 x +0.787099	0.99
Streptomycin	y= 7.031895 x + 2.095695	0.99
Kanamycin	y= 0.355753 x + 0.081389	0.99
Ofloxacin	y= 0.706034 x + 0.052114	0.99

Discussion

Simultaneous treatment of TB and HIV is required for HIV-positive individuals with TB in light of the fact that new incidents of opportunistic infections may arise within the first few months after ART with TB being the most common in this circumstance. An integrated treatment of both HIV and TB considering the available evidence of investigations from these two diseases has appeared to be possible and proficient in managing the diseases and gives better survival in different clinical backgrounds. To screen and quantify the most commonly used antiretroviral agents and second-line TB drugs, globally , a novel analytical method with high-throughout and low cost is necessary [6]. Thus far, there has been no rapid method developed to quantify both HIV and TB drugs simultaneously.

LODs in plasma ranged from 5 to 15 ng/ml for the analytes. Recoveries in plasma ranged from 85.3% to 107.4%, linearity gave the correlation coefficient of 0.99 for all, and stability, accuracy and precision, all fell within 15% acceptable range given by EMA guidelines [5].

Conclusion and Recommendations

The LC-MS/MS method described here allows an accurate and reproducible simultaneous quantification of first-line antiretroviral agents and second-line TB drugs in plasma. The LC-MS/MS running time is short to 11 minutes. Good recovery in plasma and low limit of quantification make this a suitable method for use in clinical trials and for TDM of HIV and TB co-infected patients.

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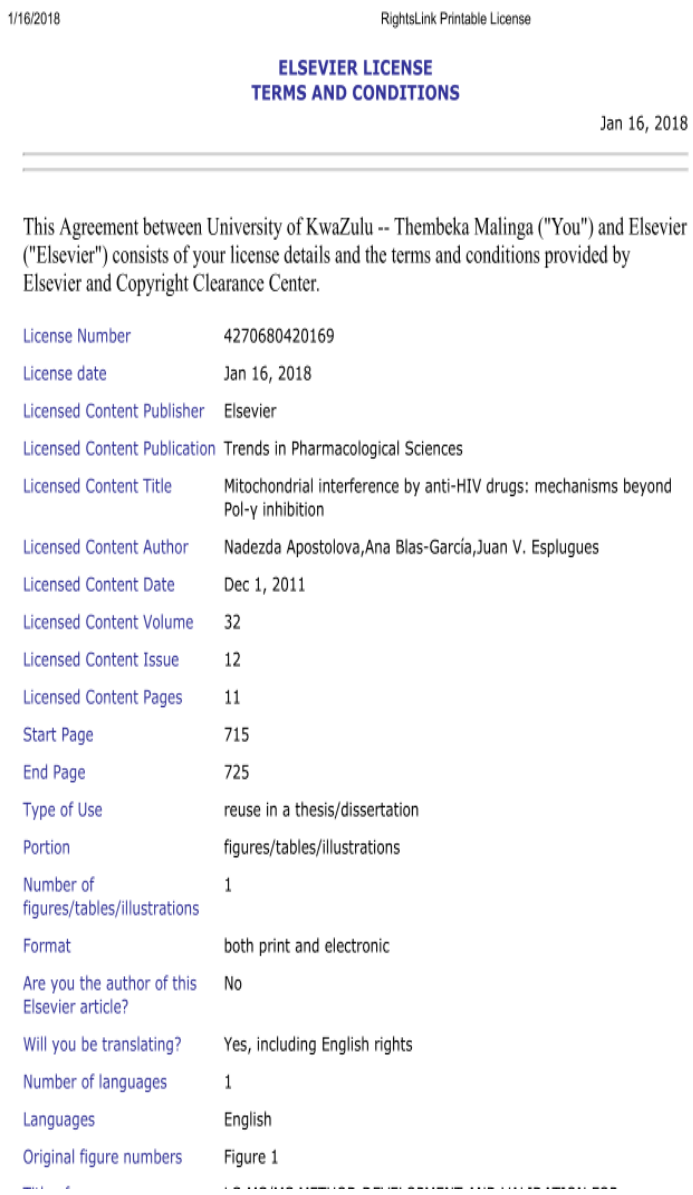

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Requestor Location	University of KwaZulu-Natal E-block, 6th floor, Room E1-06-011 Catalysis and Peptide Research Unit Discipline of Pharmaceutical Sciences Durban, KwaZulu-Natal 4000 South Africa Attn: Thembeka Malinga
Publisher Tax ID	EU826007151
Total	0.00 USD
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