GRADIENT HIGH PERFORMANCE LIQUID CHROMATOGRAPHIC METHOD FOR THE SIMULTANEOUS ANALYSIS OF EFAVIRENZ, EMTRICITABINE AND TENOFOVIR

by

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ABSTRACT

In 2014, approximately 6.8 million people in South Africa were HIV-positive, and the majority of those affected are aged 15 or older. A fixed-dose combination (FDC) antiretroviral (ARV) dosage form containing one non-nucleotide reverse transcriptase inhibitor (efavirenz) and two nucleotide reverse transcriptase inhibitors (emtricitabine and tenofovir) was licensed in South Africa in April 2013. New consolidated guidelines for HIV management and prevention of mother to child transmission (PMTCT) were published by the South African Department of Health in December 2014 and the FDC is now the recommended first-line treatment for HIV-positive patients. According to these guidelines all such people aged 15 and older, and weighing more than 40 kg, with a CD4 count of ≤ 500/ µl will be eligible for antiretroviral therapy (ART) using the FDC. In addition every pregnant and breastfeeding woman is eligible for lifelong ART regardless of CD4 count and EFV can be used as first-line treatment for pregnant women regardless of the length of gestation state of the pregnancy at that time. The use of this simplified regime is likely to promote much needed and improved adherence to therapy.

An investigation into the development of a stability-indicating reversed-phase high performance liquid chromatography (RP-HPLC) method for the simultaneous quantitation of EFV, FTC and TNF was undertaken. Isocratic HPLC analysis was found to be unsuitable due to the highly polar FTC molecule eluting in the void. Therefore a gradient HPLC method was developed and validated. The method was validated according to the International Conference on Harmonisation, now known as International Council for Harmonization (ICH). Correlation coefficients > 0.999 were obtained for each assessment of linearity and FTC, TNF and EFV are linear in the range 0.4-40 µg/ml, 0.6-60 µg/ml and 1.2-120 µg/ml. The equation of the best-fit least squares regression lines for FTC, TNF and EFV were y = 0.0191x+0.0007, y = 0.0163x+0.0116 and y = 0.01x+0.016, respectively. The method is accurate as the y-intercept was < 2% of the detector response for all ARV, and the method is precise in terms of intra- and inter-assay precision as all % RSD < 2%.

The stability-indicating nature of the method was demonstrated under acidic, alkaline and oxidative stress in addition to UV exposure and elevated temperatures, and the individual chromatograms were overlaid using Empower® 3 Software to establish whether there was interference with the peaks of interest. The forced degradation studies demonstrated the selectivity of the method for the ARV compounds.

The method was applied to assay and *in vitro* dissolution studies of commercially available tablets. The amount of each active ingredient released from Atripla® was determined and compared to the amount of each drug released from Aspen Efavirenz® and Truvada® (a combination of FTC and TNF).

The percent FTC released from Atripla® and Truvada® was similar based on the acceptance criteria for immediate-release BCS class 1 compounds. Statistical analysis was undertaken to compare the dissolution profiles of FTC, TNF and EFV. The percent of these compounds released in these studies indicate that bioequivalence testing would be required to declare these products interchangeable.

The validated RP-HPLC and *in vitro* dissolution test method are suitable for routine quality control testing of solid oral dosage forms containing EFV, FTC and TNF, and as the dissolution method can discriminate between different formulations of the same molecule, these tools can also be used for analysis during formulation development studies. The method is not suitable for the analysis of the ARV plasma due to lack of sensitivity and an inability to quantitate the compounds at the required concentration levels. The use of HPLC with mass spectroscopy for quantitation would enhance the sensitivity of the method and may eliminate the quantitation of the molecules in the presence of interference that was observed when using UV detection.

Fixed dose combination tablets are convenient for patient therapy and it is likely that in the future more molecules will be formulated into such dosage forms. However formulations such as these can pose significant difficulties when developing and using analytical methods for the quantitation of all compounds in the dosage form at the same time, in particular when the compounds have vastly different physico-chemical properties that impact the quality of a separation and therefore the analysis. Therefore when embarking on the development of FDC product cognisance of the difficulties of developing single methods for the analyses is required and approaches to overcome these difficulties should be considered.

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STUDY OBJECTIVES

EFV in a non-nucleotide reverse transcriptase inhibitor (NNRTI) and FTC and TNF are nucleotide reverse transcriptase inhibitors (NRTI). They are used in combination for highly active antiretroviral therapy (HAART) and are recommended as first-line treatment for HIV-1-positive patients in South Africa. The combination of EFV, FTC and TNF is available as a FDC tablet and the simplified dosing regimen is more convenient and promotes adherence to therapy.

The widespread use of these antiretroviral compounds in combination requires a simple RP-HPLC method for the simultaneous analysis of all compounds. The possibility of a single RP-HPLC method with multiple applications would reduce the analytical workload and has not yet been explored.

The objectives of this study were therefore to:

1. Compare isocratic and gradient RP-HPLC methods for the simultaneous quantitation of EFV, FTC and TNF.

2. Develop and validate an appropriate RP-HPLC method following the comparison of isocratic and gradient HPLC methods.

3. Apply the RP-HPLC method for quality control purposes and for the analysis of EFV, FTC and TNF in biological fluids.

CONTENTS

ABSTRACT ..... **I**

ACKNOWLEDGEMENTS ..... **III**

STUDY OBJECTIVES ..... **IV**

CONTENTS ..... **V**

LIST OF TABLES VIII
LIST OF FIGURES IX
LIST OF ABBREVIATIONS XI

CHAPTER ONE **12**

EFAVIRENZ, EMTRICITABINE AND TENOFOVIR **12**

1.1 INTRODUCTION 12
1.1.1 Overview *12*
1.2 PHYSICO-CHEMICAL PROPERTIES 16
1.2.1 Description *16*
1.2.2 Dissociation Constant (pKa) *16*
1.2.3 Partition coefficient *16*
1.2.4 Biopharmaceutical Classification System (BCS) *17*
1.2.5 Solubility *17*
1.2.6 Melting Range *17*
1.2.7 Ultraviolet absorption spectrum *17*
1.2.8 Infrared spectra *19*
1.3 STEREOCHEMISTRY AND STRUCTURE ACTIVITY RELATIONSHIP 21
1.3.1 Efavirenz *21*
1.3.2 Emtricitabine *21*
1.3.3 Tenofovir *22*
1.4 STABILITY 23
1.5 CLINICAL PHARMACOLOGY 25
1.5.1 Mechanism of action *25*
1.5.1.1 Efavirenz 26
1.5.1.2 Emtricitabine 26
1.5.1.3 Tenofovir 26
1.5.2 Therapeutic indications *26*
1.5.3 Dosage and administration *27*
1.5.3.1 Overdose 27
1.5.3.2 Missed doses 27
1.5.3.3 Discontinuation of therapy 27
1.5.3.4 Dosage adjustment 27
1.5.4 Contraindications *27*
1.5.4.1 Contraindicated medicines for concomitant use 28
1.5.5 Precautions and use in special populations *28*
1.5.5.1 Paediatric population 28
1.5.5.2 Geriatric population 28
1.5.5.3 Renal impairment 28
1.5.5.4 Hepatic impairment 29
1.5.5.5 Pregnancy and lactation 29
1.5.5.6 HIV and hepatitis B co-infection 29
1.5.6 Adverse reactions *29*
1.5.6.1 Rash 29
1.5.6.2 Lactic acidosis/severe hepatomegaly with steatosis 30
1.5.6.3 Hepatoxicity 30

1.5.6.4 Immune system reconstitution syndrome 31
1.5.6.5 Central nervous system and psychiatric symptoms 31
1.5.6.6 Onset or worsening of renal impairment 32
1.5.6.7 Bone effects 32
1.5.7 Drug interactions *33*
1.5.8 Resistance *33*
1.6 CLINICAL PHARMACOKINETICS OF EFAVIRENZ, EMTRICITABINE AND TENOFOVIR 34
1.6.1 Absorption *34*
1.6.1.1 Effect of food on oral absorption 34
1.6.2 Distribution *35*
1.6.3 Metabolism *35*
1.6.4 Elimination *35*
1.7 CONCLUSIONS 36

CHAPTER TWO 37

DEVELOPMENT OF A RP-HPLC METHOD FOR THE SIMULTANEOUS ANALYSIS OF EFAVIRENZ, EMTRICITABINE AND TENOFOVIR 37

2.1 INTRODUCTION 37
2.1.1 Overview *37*
2.1.2 Principles of HPLC *37*
2.2 PUBLISHED METHODS FOR THE SIMULTANEOUS QUANTITATION OF EFAVIRENZ, EMTRICITABINE AND TENOFOVIR 41
2.3 METHOD DEVELOPMENT 43
2.3.1 Analytical column selection *43*
2.3.2 Column temperature *44*
2.3.3 Column evaluation and system suitability *44*
2.3.3.1 Distribution constant 44
2.3.3.2 Column efficiency 44
2.3.3.3 Void volume and void time 46
2.3.3.4 Retention factor 47
2.3.3.5 Selectivity 47
2.3.3.6 Resolution factor 48
2.3.3.7 Peak symmetry factor (tailing factor) 48
2.3.4 Method of detection *48*
2.3.5 Mobile phase selection *49*
2.4 EXPERIMENTAL 50
2.4.1 Chemicals and reagents *50*
2.4.2 Preparation of stock solutions *50*
2.4.3 Preparation of mobile phase *50*
2.4.4 HPLC apparatus *51*
2.5 RESULTS 51
2.5.1 Acetonitrile content *51*
2.5.2 Buffer concentration and pH *54*
2.5.3 Effect of change of flow rate on column efficiency *57*
2.5.4 Gradient RP-HPLC method development *58*
2.5.5 Choice of internal standard *63*
2.6 CONCLUSIONS 66

CHAPTER THREE 70

GRADIENT METHOD VALIDATION AND FORCED DEGRADATION STUDIES 70

3.1 INTRODUCTION 70
3.1.1 Overview *70*
3.2 VALIDATION 70

3.2.1 Linearity and range *70*
3.2.2 Precision *73*
3.2.2.1 Intra-assay precision (repeatability) 73
3.2.2.2 Inter-day precision (intermediate precision) 74
3.2.2.3 Reproducibility 75
3.2.3 Accuracy *75*
3.2.4 Limits of quantitation (LOQ) and detection (LOD) *76*
3.2.4.1 LLOQ 77
3.2.4.2 LOD 77
3.3 FORCED DEGRADATION STUDIES 78
3.3.1 Overview *78*
3.3.2 Experimental *78*
3.3.2.1 Acid, alkali and oxidative degradation 78
3.3.2.1.1 Acid degradation 79
3.3.2.1.2 Alkali degradation 79
3.3.2.1.3 Oxidative degradation 80
3.3.2.2 Dry heat 81
3.3.2.3 Photodegradation 82
3.4 BIOANALYTICAL METHOD DEVELOPMENT FOR ANALYSIS OF EFV, FTC AND TNF IN PLASMA 83
3.4.1 Overview *83*
3.4.2 Silica–based sorbents *84*
3.4.3 Polymer –based sorbents *85*
3.4.4 Sensitivity of RP-HPLC gradient method *85*
3.4.4.1 Ghost peaks and band compression 85
3.4.4.2 Water as a source of contamination 86
3.5 CONCLUSIONS 87

CHAPTER FOUR 89

APPLICATION OF THE GRADIENT RP-HPLC METHOD OF ANALYSIS 89

4.1 INTRODUCTION 89
4.1.1 Overview *89*
4.1.2 Dissolution apparatus *91*
4.1.3 Dissolution media *92*
4.1.4 Acceptance criteria *93*
4.1.5 Dissolution profile comparisons *94*
4.2 EXPERIMENTAL 95
4.3 RESULTS 96
4.4 CONCLUSIONS 105

CHAPTER FIVE 107

CONCLUSIONS 107
REFERENCES 111

LIST OF TABLES

Table 2.1 *RP-HPLC methods for the simultaneous quantitation of EFV, FTC and TNF* *42*
Table 2.2 *Calculation of efficiency at different peak widths [197]* *45*
Table 2.3 *Summary of system suitability tests for the isocratic separation of FTC, TNF and EFV using 0.03 M pH 4 buffer and acetonitrile in different v/v ratios* *51*
Table 2.4 *Retention times of FTC, TNF and EFV at different pH and buffer concentrations* *54*
Table 2.5 *Summary of system suitability for the isocratic separation of FTC, TNF and EFV using a 70:30 v/v buffer: acetonitrile mobile phase* *57*
Table 2.6 *Gradient scouting conditions for RP-HPLC method development* *60*
Table 2.7 *Gradient conditions used to assess steepness* *60*
Table 2.8 *System suitability results from gradient steepness test* *61*
Table 2.9 *Gradient conditions to assess slope* *62*
Table 2.10 *System suitability results from gradient slope test* *62*
Table 2.11 *Summary of optimised gradient HPLC method* *63*
Table 2.12 *Final gradient conditions used for the validation of a RP-HPLC method for the analysis of FTC, TNF and EFV* *63*
Table 2.13 *IS performance under gradient conditions* *64*
Table 3.1 *Intra-assay precision for FTC, TNF and EFV* *73*
Table 3.2 *Intra-assay precision for FTC (0.4-40 µg/ml), TNF (0.6-60 µg/ml) and EFV (1.2-120 µg/ml)* *74*
Table 3.3 *Inter-day precision for FTC, TNF and EFV* *75*
Table 3.4 *Accuracy data for FTC, TNF and EFV* *76*
Table 4.1 *Summary of specifications for USP apparatus 2 [233]* *92*
Table 4.2 *Summary of pH and transit times in the gastrointestinal tract [233]* *93*
Table 4.3 *USP general test chapter dissolution <711> acceptance criteria for immediate-release products [232]* *94*
Table 4.4 *Summary of suggested dissolution methods for in-vitro dissolution testing of EFV, FTC and TNF tablets* *96*
Table 4.5 *Average cumulative release of EFV (n=12) when no SLS and 1.0 % SLS added to 0.1 M HCl dissolution media* *96*
Table 4.6 *Mean cumulative percent FTC released for test and reference products* *98*
Table 4.7 *Mean cumulative percent TNF released from test and reference products* *100*
Table 4.8 *Mean cumulative percent EFV released from test and reference products* *103*
Table 4.9 *f1 and f2 factors for dissolution profile comparison of FTC, TNF and EFV release from test and reference products* *104*

LIST OF FIGURES

Figure 1.1 *The chemical structure of efavirenz (EFV) ($C_{14}H_9ClF_3NO_2$, 315.7 g/mol)* *13*
Figure 1.2 *The chemical structure of emtricitabine (FTC) ($C_8H_{10}FN_3O_3S$, 247.2 g/mol)* *14*
Figure 1.3 A *The chemical structure of tenofovir disoproxil fumarate (TDF)($C_{19}H_{30}N_5O_{10}P$, $C_4H_4O_4$ 635.5g/mol)* *14*
Figure 1.3 B *The chemical structure of tenofovir disoproxil (TNF) ($C_{19}H_{30}N_5O_{10}P$, 519.4 g/mol)* *15*
Figure 1.3 C *The chemical structure of tenofovir monophosphate ($C_9H_{14}N_5O_4P$, 287.2 g/mol)* *15*
Figure 1.4 *The ultraviolet absorption spectrum of efavirenz (EFV)* *18*
Figure 1.5 *The ultraviolet absorption spectrum of emtricitabine (FTC)* *18*
Figure 1.6 *The ultraviolet absorption spectrum of tenofovir (TNF)* *19*
Figure 1.7 *The infrared spectrum of efavirenz (EFV)* *20*
Figure 1.8 *The infrared spectrum of emtricitabine (FTC)* *20*
Figure 1.9 *The infrared spectrum of tenofovir disoproxil fumarate (TDF)* *21*
Figure 1.10 *The structure of tenofovir disoproxil fumarate (TDF), indicating the important stereochemistry features for antiviral activity* *23*
Figure 1.11 *Proposed base hydrolysis of cyclic carbamate of EFV (adapted from [83, 86])* *24*
Figure 1.12 *Ionisation of the amine functionality in the carbamate moiety of EFV and stabilisation of negative charge through resonance (adapted from [80])* *25*
Figure 2.1 *Guassian peak illustrating different approaches to calculating efficiency [197]* *45*
Figure 2.2 A *Typical chromatogram depicting the separation of FTC, TNF and EFV using a 0.03 M buffer of pH 4 in the mobile phase 55:45 v/v buffer-acetonitrile* *53*
Figure 2.2 B *Typical chromatogram depicting the separation of FTC, TNF and EFV using a 0.03 M buffer of pH 4 in the mobile phase 65:35 v/v buffer-acetonitrile* *53*
Figure 2.2 C *Typical chromatogram depicting the separation of FTC, TNF and EFV using a 0.03 M buffer of pH 4 in the mobile phase 70:30 v/v buffer-acetonitrile* *53*
Figure 2.3 A *Typical chromatogram depicting the separation of FTC, TNF and EFV using a 0.01 M buffer of pH 4 in the mobile phase* *55*
Figure 2.3 B *Typical chromatogram depicting the separation of FTC, TNF and EFV using a 0.01 M buffer of pH 5 in the mobile phase* *55*
Figure 2.3 C *Typical chromatogram depicting the separation of FTC, TNF and EFV using a 0.01 M buffer of pH 6 in the mobile phase* *55*
Figure 2.4 A *Typical chromatogram depicting the separation of FTC, TNF and EFV using a 0.05 M buffer of pH 4 in the mobile phase* 56
Figure 2.4 B *Typical chromatogram depicting the separation of FTC, TNF and EFV using a 0.05 M buffer of pH 5 in the mobile phase* 56
Figure 2.4 C *Typical chromatogram depicting the separation of FTC, TNF and EFV using a 0.05 M buffer of pH 6 in the mobile phase* *56*
Figure 2.5 *Typical chromatogram depicting the separation of FTC, TNF and EFV using a 0.03 M buffer of pH 4 in the mobile phase 70:30 v/v buffer-acetonitrile at 0.8 ml/min* *58*
Figure 2.6 *Typical chromatogram depicting the separation of FTC, TNF and EFV for gradient steepness test* . *61*
Figure 2.7 *Typical chromatogram depicting the separation of FTC, TNF and EFV for gradient slope test* *62*
Figure 2.8 *Ultraviolet absorption spectrum of zidovudine (100 µg/ml, 60:40 v/v buffer-acetonitrile)* *64*
Figure 2.9 *Typical chromatogram showing separation and retention times of FTC (2.9 min), ZD (4.3 min), TNF (7.9 min) and EFV (9.1 min) using a gradient method* *65*
Figure 2.10 *Graphic representation of gradient slope and gradient steepness for the separation of FTC, ZD, TNF and EFV* *66*
Figure 3.1 *Typical calibration curve for FTC over the concentration range 0.4-40 µg/ml* *71*
Figure 3.2 *Typical calibration curve for TNF over the concentration range 0.6-60 µg/ml* *72*
Figure 3.3 *Typical calibration curve for EFV over the concentration range 1.2-120 µg/ml* *72*
Figure 3.4 *Chromatogram of FTC, TNF and EFV (100 µg/ml) (A) and overlaid chromatograms (B) showing FTC, TNF and EFV following exposure to 0.1 M HCl and refluxing at 70 °C for 3 hours* *79*

Figure 3.5 *Chromatogram of FTC, TNF and EFV (100 µg/ml) (A) and overlaid chromatograms (B) showing FTC, TNF and EFV following exposure to 0.1 M NaOH and refluxing at 70 °C for 3 hours* *80*
Figure 3.6 *Chromatogram of FTC, TNF and EFV (100 µg/ml) (A) and overlaid chromatograms (B) showing FTC, TNF and EFV following exposure to 3 % H_2O_2 and refluxing at 70 °C for 3 hours* *81*
Figure 3.7 *Chromatogram of FTC, TNF and EFV (100 µg/ml) (A) and overlaid chromatograms (B) showing FTC, TNF and EFV following exposure to 100 °C dry heat for 12 hours* *82*
Figure 3.8 *Chromatogram of FTC, TNF and EFV (100 µg/ml) (A) and overlaid chromatograms (B) showing degradation of FTC, TNF and EFV following exposure to UV light at 245 nm for 12 hours* *83*
Figure 3.9 *Chromatogram showing interfering peaks following injection of mobile phase* *87*
Figure 4.1 *Dissolution profile of mean (n=12) cumulative % EFV released with 1.0 % w/v and without SLS in a 0.1M HCl dissolution fluid* *97*
Figure 4.2 *Dissolution profile of mean (n=12) cumulative % FTC released from test and reference products in a 0.1 M HCl dissolution medium* *98*
Figure 4.3 *Dissolution profile of mean (n=12) cumulative % FTC released from test and reference products in a pH 4.5 dissolution medium* *99*
Figure 4.4 *Dissolution profile of mean (n=12) cumulative % FTC released from test and reference products in a pH 6.8 dissolution medium* *99*
Figure 4.5 *Dissolution profile of mean (n=12) cumulative % TNF released from test and reference products in a 0.1 M HCl dissolution medium* *101*
Figure 4.6 *Dissolution profile of mean (n=12) cumulative % TNF released from test and reference products in a pH 4.5 dissolution medium* *101*
Figure 4.7 *Dissolution profile of mean (n=12) cumulative % TNF released from test and reference products in a pH 6.8 dissolution medium* *102*
Figure 4.8 *Dissolution profile of mean (n=12) cumulative % EFV released from test and reference products in a 0.1 M HCl dissolution medium* *103*
Figure 4.9 *Dissolution profile of mean (n=12) cumulative % EFV released from test and reference products in a pH 4.5 dissolution medium* *104*
Figure 4.10 *Dissolution profile of mean (n=12) cumulative % EFV released from test and reference products in a pH 6.8 dissolution medium* *104*

LIST OF ABBREVIATIONS

ALT – *alanine aminotransferase*
API *– active pharmaceutical ingredient(s)*
ART *antiretroviral therapy*
ARV *– antiretroviral(s)*
AST *– aspartate aminotransferase*
AUC *– area under concentration curve*
BCS *– biopharmaceutics classification system*
BMD *– bone mineral density*
$\boldsymbol{Cl_{CR}}$ *– creatinine clearance*
CNS *– central nervous system*
EFV *– efavirenz*
eGFR *– estimated glomerular filtration*
FDA *– Food and Drug Administration*
FDC *– fixed dose combination*
FS *–Fanconi syndrome*
FTC *– emtricitabine*
GIT *– gastrointestinal tract*
HAART *– highly active antiretroviral therapy*
HBV – *hepatitis B*
HCV *– hepatitis C*
HIV-1 *– human immune deficiency virus type 1*
HETP *– height equivalent to theoretical plates*
HPLC *– high performance liquid chromatography*
ICH *– International conference on Harmonisation/ International Council for Harmonisation*
IEX *– ion-exchange chromatography*
IR *– infrared*
IS *– internal standard*
IVIVC *– in vitro-in vivo correlation*
LC *– liquid chromatography*
LOD *– limit of detection*
LLOQ *– lower limit of quantitation*
MCC *– Medicines Control Council*
NNRTI *– non-nucleotide reverse transcriptase inhibitor(s)*
NRTI *– nucleotide reverse transcriptase inhibitor(s)*
NNIBP *– non-nucleoside inhibitor-binding pocket*
NP *– normal phase*
NVP *– nevirapine*
PAR *– peak area ratio*
PEP *– post exposure prophylaxis*
PMTCT – *prevention of mother to child transmission*
PrEP *– pre-exposure prophylaxis*
PT *– proximal tubule*
RP *– reversed phase*
RT *– reverse transcriptase*
SEC *– size-exclusion chromatography*
SLS *– sodium lauryl sulfate*
SPE *– solid phase extraction*
TDF *– tenofovir disoproxil fumarate*
TLC *– thin layer chromatography*
TNF *– tenofovir disoproxil*
ULP *– upper limit of normal*
UPLC *– ultra-high performance liquid chromatography*
USP *– United States Pharmacopoeia*
ZD *– zidovudine*
3TC *– lamivudine*

CHAPTER ONE
EFAVIRENZ, EMTRICITABINE AND TENOFOVIR

1.1 INTRODUCTION

1.1.1 Overview

The development of highly active antiretroviral therapy (HAART) has dramatically reduced the mortality and morbidity associated with human immune deficiency virus (HIV)-infection, thereby facilitating the management of chronic infection [1–4]. Through the use of HAART, long-term suppression of human immune deficiency virus-1 (HIV-1) infection is achievable, provided patient adherence to therapy is achieved [5, 6]. Challenges affecting the maintenance of viral suppression include life-long adherence, number of tablets to be taken in a treatment regimen, complexity of dosing frequency, drug-interactions and long-term adverse effects and toxicity [3, 4].

The first single-tablet fixed-dose combination (FDC) antiretroviral (ARV) has been commercially available since 2006 and is marketed as Atripla® [7]. A generic product has been commercially available in South Africa since April 2013 [8, 9] and consists of efavirenz (EFV), emtricitabine (FTC) and tenofovir disoproxil fumarate (TDF) in a ratio of 600 mg/ 200 mg /300 mg. TDF in this quantity is equivalent to 245 mg tenofovir disoproxil (TNF) and 136 mg of tenofovir [10]. The tablet is taken once daily for the treatment of HIV-1 infection [7, 11, 12]. Once-daily FDC tablets are the simplest antiretroviral therapy available [6]. FDC ARV therapy is convenient for patients as it reduces the "pill burden" which in turn improves adherence to therapy [6, 11, 12].

FDC were initially indicated for treating HIV-positive antiretroviral naïve patients and HIV-positive pregnant women and those who are breastfeeding. It is now available to all patients on the recommendation of a physician [9]. Treatment with EFV, FTC and TNF is the preferred first-line therapy for antiretroviral naïve HIV-1-infected persons [6]. Bioequivalence between the dosage form containing a single molecule and the FDC in addition to favourable pharmacokinetics facilitates once-daily dosing of EFV, FTC and TNF [6, 7].

EFV is a non-competitive potent non-nucleoside reverse transcriptase inhibitor (NNRTI) of HIV-1 [11, 13–15]. The reverse transcriptase (RT) enzyme of the human immune deficiency virus type-2 (HIV-2) and human cellular DNA polymerases α, β, ϒ and δ are not significantly inhibited by EFV [11, 16]. A major disadvantage of EFV is the low genetic barrier to resistance. A single mutation can confer resistance to EFV and nevirapine (NVP), another NNRTI [6]. The most common side effects associated with EFV are rash and CNS disturbances. Previously it was recommended that the use of EFV in pregnancy be avoided [6, 16, 17] however, according to the latest guidelines [18], pregnant and breastfeeding women are eligible for lifelong ART regardless of CD4 count. In addition EFV is used as a first-line for treatment of pregnant women regardless of the gestation period of the

pregnancy. EFV is extensively metabolised in the liver via cytochrome P450 enzymes and thus has a high potential for interaction with other molecules also metabolised via this route [11, 19–22].

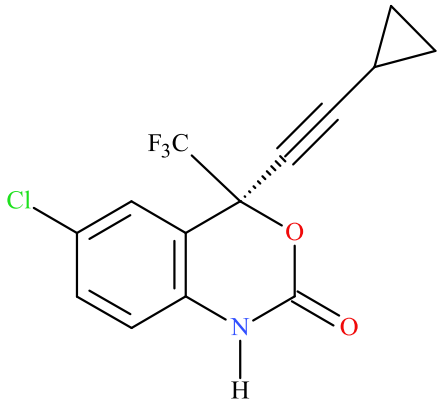


Figure 1.1 *The chemical structure of efavirenz (EFV)($C_{14}H_9ClF_3NO_2$, 315.7 g/mol)*

FTC is a synthetic nucleoside analogue of cytidine [11, 23]. TDF is a fumarate salt of the prodrug TNF [10] and TNF is absorbed and converted to tenofovir monophosphate, an acyclic nucleoside phosphonate (nucleotide) diester analog of adenosine monophosphate. FTC and TNF are nucleos(t)ide reverese transcriptase inhibitors (NRTI) [10, 11, 24, 25]. NRTI require conversion to an active metabolite for antiviral activity to be effective. The molecules mimic normal nucleos(t)ides that are incorporated into DNA at the 3' terminus. However FTC and TNF lack the 3'-OH and their incorporation at the 3' terminus of the DNA therefore terminates chain elongation by preventing incorporation of additional nucleotides [11, 23, 26]. Tenofovir diphosphate and emtricitabine 5'-triphosphate are weak inhibitors of α, β, ϒ cellular DNA polymerases and emtricitabine 5'-triphosphate weakly inhibits DNA polymerase ε [11].

The combination of TNF and FTC has been the preferred NRTI regimen since 2003 since approval by FDA [6]. A single-tablet combination has been approved by the FDA and more recently by the Medicines Control Council (MCC) of South Africa for pre-exposure prophylaxis (PrEP) although it is not widely used clinically [27, 28]. The HIV-1 reverse transcriptase (RT) mutation K65R is a common multi-drug resistance mutation that confers resistance to NRTI including TNF and FTC [11, 26, 29, 30] and this mutation may be responsible for cross-resistance between different NRTI [26, 30]. Cases of acute renal failure and Fanconi syndrome (FS) have been reported in patients treated with TNF [31–34] although clinically important renal toxicity is rare [35]. FTC and TNF undergo limited systemic metabolism [11, 36, 37].

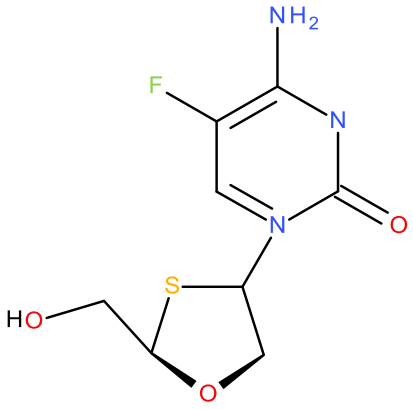


Figure 1.2 *The chemical structure of emtricitabine(FTC)($C_8H_{10}FN_3O_3S$, 247.2 g/mol)*

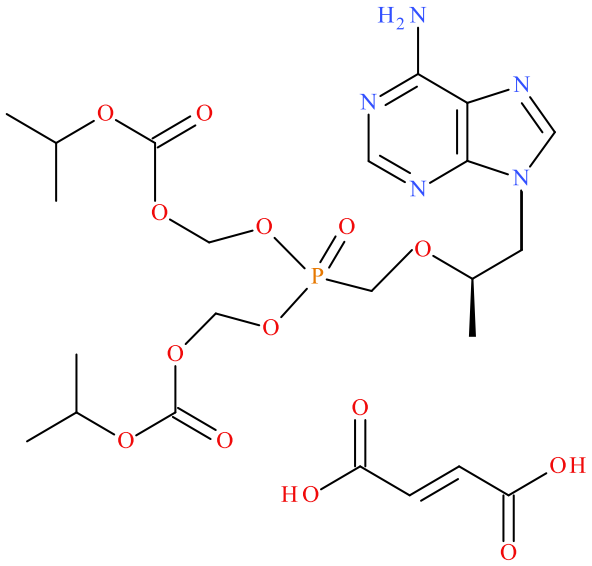


Figure 1.3 A *The chemical structure of tenofovir disoproxil fumarate (TDF)($C_{19}H_{30}N_5O_{10}P$, $C_4H_4O_4$ 635.5g/mol)*

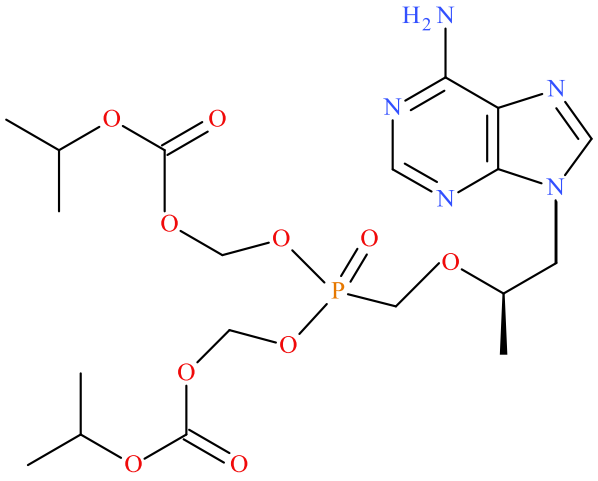


Figure 1.3 B *The chemical structure of tenofovir disoproxil (TNF)($C_{19}H_{30}N_5O_{10}P$, 519.4 g/mol)*

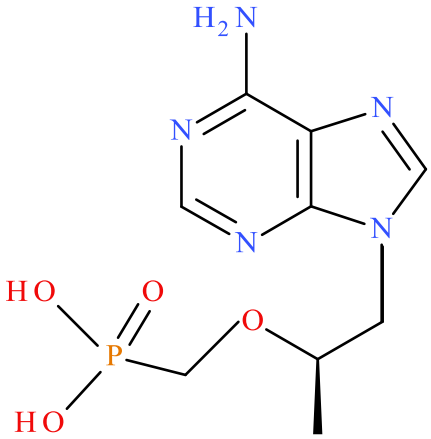


Figure 1.3 C *The chemical structure of tenofovir monophosphate ($C_9H_{14}N_5O_4P$, 287.2 g/mol)*

1.2 PHYSICO-CHEMICAL PROPERTIES

1.2.1 Description

EFV is (4*S*)-6-chloro-4-(cyclopropylethynyl)-1,4-dihydro-4-(trifluoromethyl)-2*H*-3,1-benzoxazin-2-one [11, 26, 29, 30] and occurs as a white to slightly pink crystalline powder [31, 38, 39]. The empirical formula for EFV is $C_{14}H_9ClF_3NO_2$ and the relative molar mass is 315.7g/mol [14, 31, 39]. EFV contains no less than 97.0 percent and not more than 103.0 percent of $C_{14}H_9ClF_3NO_2$ calculated with reference to the anhydrous reference material [38].

FTC is (–)-4-amino-5-fluoro-1-[(*2R, 5S*)-2-(hydroxymethyl)-1,3-oxathiolan-5-yl]pyrimidin-2(1*H*)-one; (–)-5-fluoro-1-[(2*R*,5*S*)-2-(hydroxymethyl)-1,3-oxathiolan-5- yl]cytosine [31, 40, 41] and FTC is a white to off white, crystalline powder [31, 41]. FTC has an empirical formula of $C_8H_{10}FN_3O_3S$ and a relative molecular mass of 247.2 g/mol [40, 42]. FTC contains no less than 99.0 percent and not more than 101.0 percent of emtricitabine ($C_8H_{10}FN_3O_3S$), calculated with reference to the anhydrous reference material [40].

TDF is 9-[(*R*)-2[[bis[[(isopropoxycarbonyl)oxy]- methoxy]phosphinyl]methoxy]propyl]adenine fumarate (1:1). TDF is the fumaric acid salt of the *bis*isopropoxycarbonyloxymethyl ester derivative of TNF [31, 43, 44]. TDF has an empirical formula of $C_{19}H_{30}N_5O_{10}P$, $C_4H_4O_4$ and a relative molar mass of 635.5 g/mol. It occurs as a white to almost white crystalline powder [31, 43–45]. TDF contains no less than 98.5 percent and not more than 101.0 percent of TDF ($C_{19}H_{30}N_5O_{10}P$, $C_4H_4O_4$), calculated with reference to an anhydrous reference material [31, 44]. TDF refers to the solid/raw material whereas TNF refers to TDF in solution and tenofovir peaks in chromatograms.

1.2.2 Dissociation Constant (pKa)

EFV is a weak acid with a pKa of 10.2. It is therefore ionised at high pH, at which the carbonate moiety undergoes deprotonation to form a negatively charged species [46, 47]. The trifluoromethyl and ethylene moieties are most likely responsible for the lowering of the pKa [47]. The pKa of FTC and TNF are 2.65 and 3.75 respectively [41, 42, 48]. EFV is a weak acid whereas FTC and TNF are weak bases.

1.2.3 Partition coefficient

The partition coefficient is the measure of the ability of a drug to partition into a lipophilic or hydrophilic phase prior to exerting a pharmacological effect as the drug must first cross a lipid membrane barrier and the partition to underlying aqueous environments. The logarithm of the partition coefficient (log P) is a measure of lipophilicity of a drug molecule and can be used to infer potential biological activity in addition to understanding partitioning in the context of RP-HPLC analysis.

In general,

if log P = 0, there is equal distribution of the molecule in both phases, if the
log P >0, the molecule is lipid soluble, whereas if
log P <0, the molecule is water soluble [49].

The log P of EFV is 5.4 [38] and FTC is -0.43 [42, 48]. The log P of TNF, the form of tenofovir that is absorbed, is 1.25 [48]. The hydrophilicity/hydrophobicity of molecules governs the retention characteristics in RP-HPLC where the most hydrophilic molecules elute early and hydrophobic molecules elute later [50]. Therefore the partition coefficient can be used to predict the order of elution in RP-HPLC and based on the partition coefficients of the compounds of the FDC the order of elution will be FTC, TNF and then EFV.

1.2.4 Biopharmaceutical Classification System (BCS)

The BCS provides a framework to classify molecules into categories based on their aqueous solubility and membrane permeability. Class 1 drugs have high solubility and high permeability, class 2 drugs low solubility and high permeability, class 3 drugs high solubility and low permeability and class 4 drugs low solubility and low permeability [51, 52]. EFV has low aqueous solubility and high intestinal permeability and is classified as a Class 2 molecule [53]. FTC has high aqueous solubility and high intestinal permeability and is classified as a Class 1 molecule [54]. TNF, the form of tenofovir that is aborbed, has high aqueous solubility and low intestinal permeability and is classified as a Class 3 molecule [55].

1.2.5 Solubility

EFV is practically insoluble in water (9.0 μg /ml) but is freely soluble in methanol [14, 31, 38, 39]. FTC is freely soluble in methanol and water (112 mg/ml) and is practically insoluble in dichloromethane R [31, 40–42]. TNF has a solubility of 13.4 mg/ml in distilled water at 25 °C [31, 43, 45].

1.2.6 Melting Range

EFV melts within the range of 139-141 °C [56]. FTC melts within the range of 136-140 °C [57]. TNF melts within the range of 276-280 °C [58].

1.2.7 Ultraviolet absorption spectrum

Individual stock solutions of each ARV were prepared in a water: acetonitrile mixture in a ratio of 60:40 % v/v and the UV absorption spectrum monitored between 210-400 nm. The maximum absorbance observed for EFV, FTC and TNF were 246 nm, 280 nm and respectively and the spectra are depicted in Figures 1.4, 1.5 and 1.6. Similar wavelengths of maximum absorbance have been reported [38, 59–62].

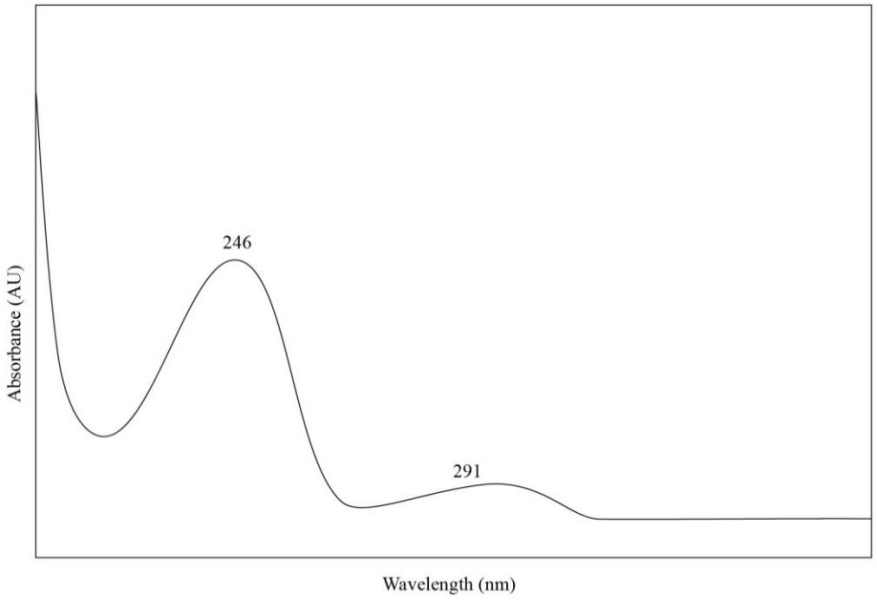


Figure 1.4 *The ultraviolet absorption spectrum of efavirenz (EFV)*

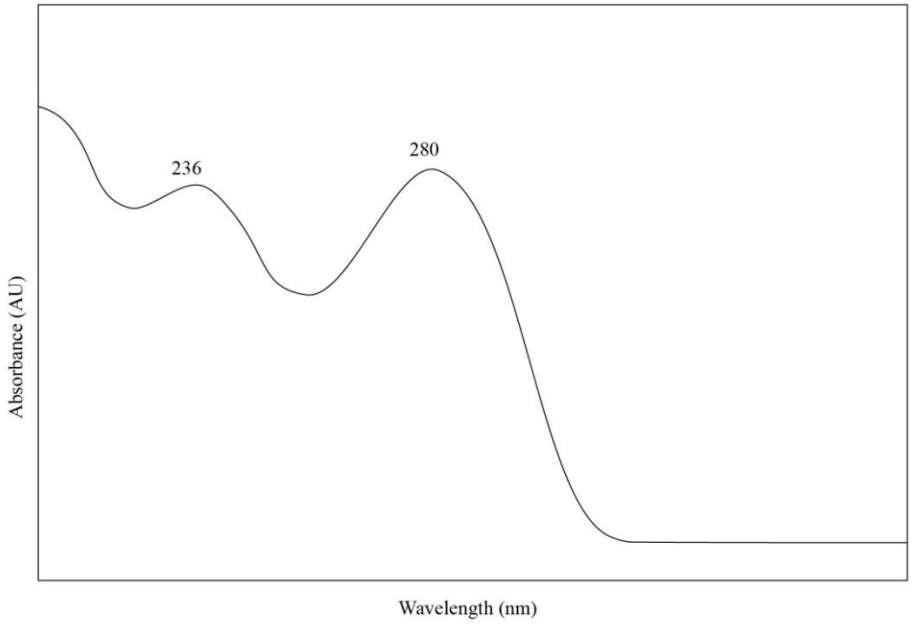


Figure 1.5 *The ultraviolet absorption spectrum of emtricitabine (FTC)*

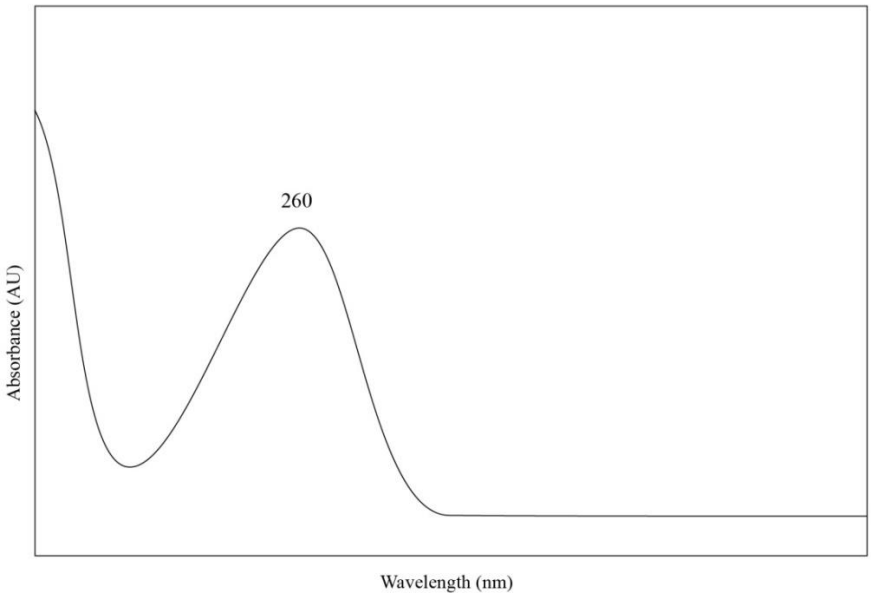


Figure 1.6 *The ultraviolet absorption spectrum of tenofovir (TNF)*

1.2.8 Infrared spectra

Infrared (IR) absorption spectroscopy is used for the identification of compounds. The spectra are compared to reference spectra of the compound and the IR spectrum can be obtained from raw materials in the solid state [63]. IR Spectra in the range 4000–650 cm^{-1} for EFV, FTC and TDF were generated using a Perkin-Elmer® FT-IR Spectrum 100 (Perkin-Elmer® Pty Ltd, Beaconsfield, England) and are depicted in Figures 1.7, 1.8 and 1.9. The The spectra generated are similar to those that have been reported [64] and the IR spectra obtained confirm the identity of EFV, FTC and TNF.

The principal bands observed in the IR spectrum of EFV (Figure 1.7) include 3312 (-NH), 2249 (C≡C), 1740 (C=O), 1604 (C=C), 1494 (C-S-C), 1350-1120 (CF_3), 1096 (C-Cl) and 900-650 cm^{-1} (aromatic ring) stretching.

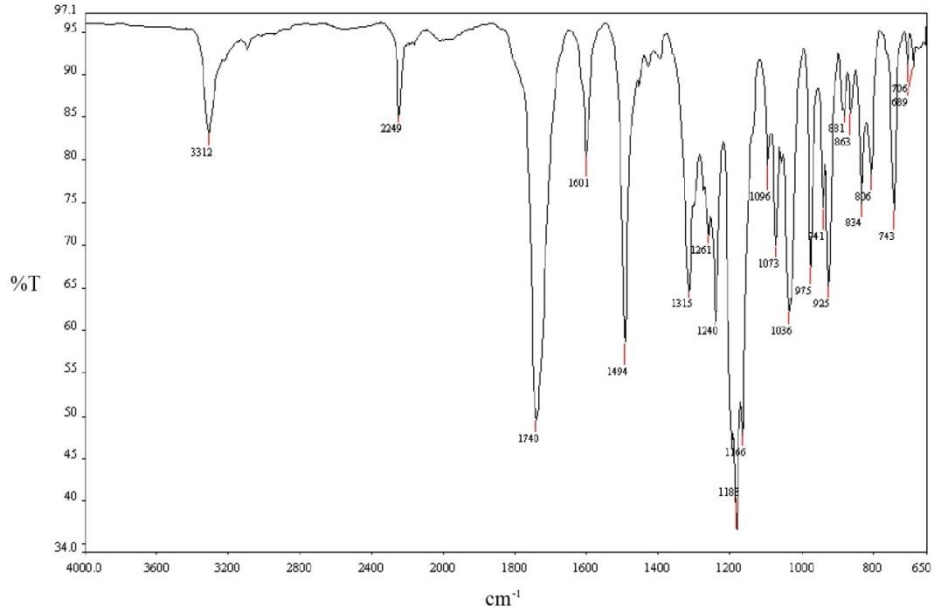


Figure 1.7 *The infrared spectrum of efavirenz (EFV)*

The principal bands observed in the IR spectrum of FTC (Figure 1.8) include 3412 (NH_2 stretch), 3072 (C=C), 1690 (C=O), 1616 (NH_2 bend), 1517 (C=C, C=N) and 774 cm^{-1} (CF) stretching.

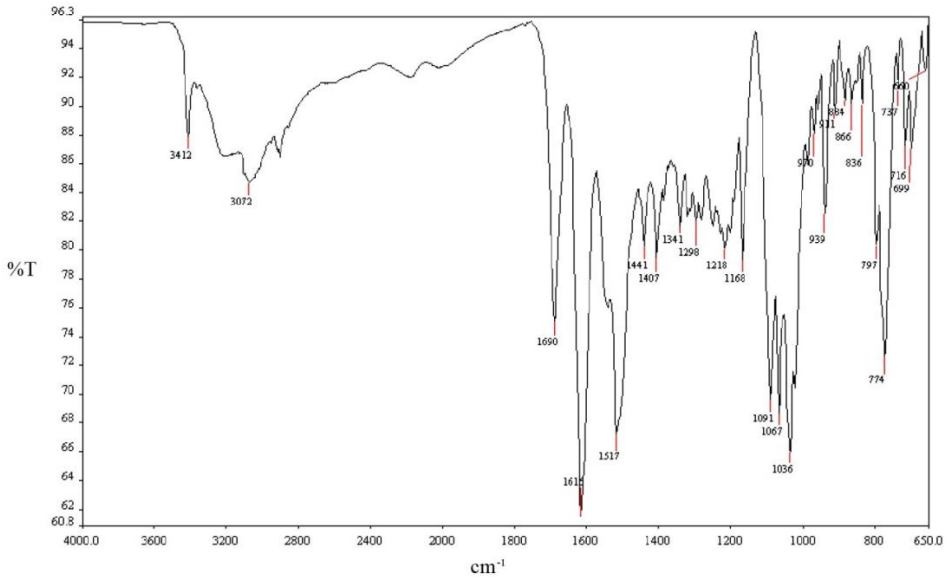


Figure 1.8 *The infrared spectrum of emtricitabine (FTC)*

The principal bands observed in the IR spectrum of TDF (Figure 1.9) include 3210 (NH), 2984 (CH), 1751 (C=O), 1673 (C=C), 1421 (C=N), 1256 (C-N), 1158 (P=O), 1050-970 cm^{-1} (P-O-P) stretching.

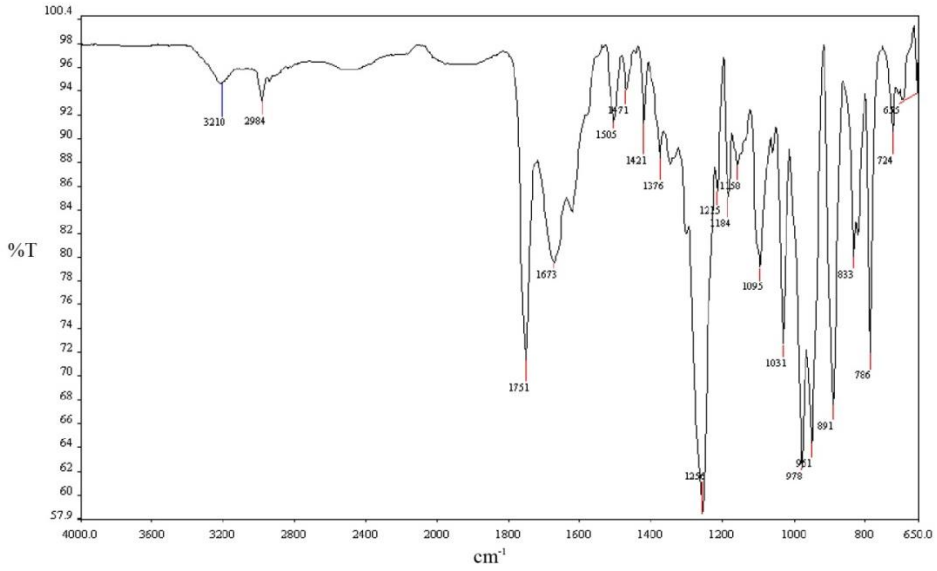


Figure 1.9 *The infrared spectrum of tenofovir disoproxil fumarate (TDF)*

1.3 STEREOCHEMISTRY AND STRUCTURE ACTIVITY RELATIONSHIP

1.3.1 Efavirenz

The NNRTI are a diverse class of molecules in terms of their chemical structure, but all interact with HIV-RT [65–67] enzyme. The non-nucleoside inhibitor-binding pocket (NNIBP) only exists in the presence of a NNRTI. The movement of the Tyr181 and Tyr188 residues from a 'down' position to an 'up' position characterises the formation of the NNIBP [67] and mutations at Tyr181 and Tyr188 residues result in loss of interaction with older generation inhibitors such as nevirapine and delavirdine. EFV has an improved resistance profile due to minimal interaction with the Tyr181 and Tyr188 residues. EFV has a small cyclo-propyl group in comparison to the bulky pyridyl ring seen with nevirapine and delavirdine [67, 68]. The small molecular size of EFV permits rearrangement within a mutated NNIBP, thereby improving its resistance profile [67]. Any substitutions with a nitrogen containing group at position C-3 (Figure 1.1) are unfavourable for antiviral activity [69]. The halophenyl and the trifluoromethyl groups are important for hydrophobic interactions within the enzyme [68], and the electronegative trifluoromethyl and acetylene groups substantially lower the pKa of EFV [47], improving the potency of the cyclic carbamate and therefore enhancing drug potency [13]. Substitution of chlorine on the benzoxazinone ring also lowers the pKa to a lesser extent [47].

1.3.2 Emtricitabine

L-deoxycytidine analogues such as FTC and lamivudine (3TC) are structurally similar molecules, only differing by a 5-fluorinated substitution on FTC. FTC and 3TC exhibit L-stereochemistry that is more effective than the D-isomer form [70–72]. Fluorine is a strong electron withdrawing group which alters π electron distribution within the cytosine moiety of FTC, affecting stacking interactions

with the primer 3'-base [70]. Fluorine substitution does not distort the geometry of hydrogens but increases biological activity [73, 74]. The sugar moiety of FTC (and 3TC) is rotated 180° with O4'-*endo* sugar puckering that is a result of the stereochemistry and Watson-Crick base-pairing [75]. The rotated sugar moiety allows for multiple triphosphate-binding conformations at the polymerase active site that can significantly decrease incorporation and therefore the efficiency in the polymerase enzyme [72]. The lack of a 3'-OH moiety results in chain termination when the moiety is incorporated into the growing DNA chain. In addition the lack of the 3'-OH moiety relieves steric hindrance that arises as a result of 180° rotation of the sugar moiety in L-nucleotides facing the primer 3'-terminus [71, 72]. Substitution at the 3'position with an electron-rich atom such as sulphur improves stacking interactions with amino acids residues within the enzyme [71, 72]. Substitution with 3'-OH results in an increase in drug resistance, while substitution with smaller atoms such as carbon or oxygen reduces drug resistance resulting from a decrease in stacking interactions between the sugar moiety and amino acid residues, in the enzyme thereby decreasing binding affinity for the enzyme [72].

1.3.3 Tenofovir

TNF is an acyclic nucleoside phosphonate in which the sugar moiety has been replaced by an acyclic moiety [76–78]. TNF differs from adefovir by a single methyl group, resulting in a lower intrinsic cytotoxicity and an increase in the therapeutic index of TNF [79]. TNF does not have sufficient oral bioavailability therefore TDF, a fumarate salt of the prodrug TNF, was developed [26, 80, 81]. TNF has a double negative charge at physiological pH and the oral bioavailability, cellular uptake and potency are increased when the negative charges are masked through the use of the disoproxil fumarate moiety [76, 77, 79, 81]. Masking of the double negative charge of TNF results in release into plasma, however a drawback is its susceptibility to concentration in the proximal tubule (PT) of the kidney through interaction with anion transporters [76, 77]. TNF is activated by phosphorylation via nucleoside kinases [26, 78], and the initial phosphorylation is a rate limiting step that is bypassed by inclusion of a phosphonate group [26, 76, 77]. The phosphonate moiety is chemically and metabolically stable, and hydrolysis back to the nucleoside is not possible for nucleoside monophosphates [79, 81]. TNF is not subject to deamination or deglycosylation [79] and the antiviral activity of dephosphorylated TNF is a consequence of higher affinity for viral DNA polymerase compared to cellular DNA polymerases [76]. The stereochemistry (*) depicted in Figure 1.10 is important for activity [76].

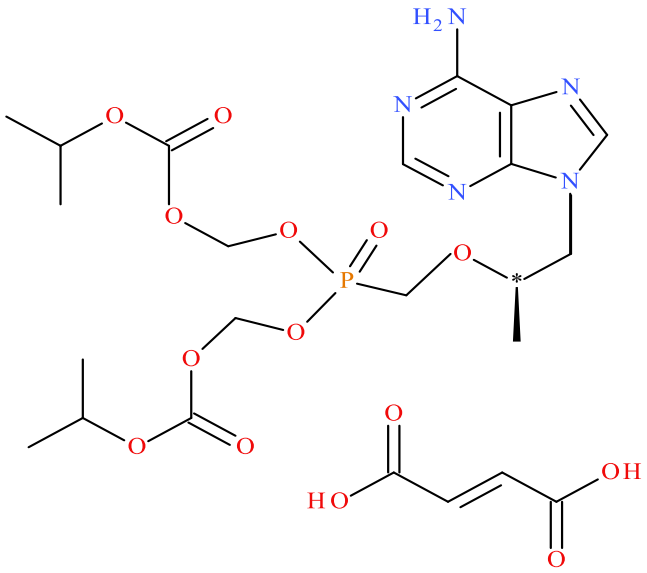


Figure 1.10 *The structure of tenofovir disoproxil fumarate (TDF), indicating the important stereochemistry features for antiviral activity*

1.4 STABILITY

Stability studies performed according to The International Conference on Harmonisation (ICH) guidelines are important measures of pharmaceutical quality control [82, 83]. Stability testing should include hydrolytic, oxidative, thermal and photolyic stability of an active pharmaceutical ingredient [83, 84].

In a study the stability of EFV, FTC and TNF individually and in combination were investigated under acidic, basic and neutral conditions [85]. All three ARV in combination were refluxed in 0.5 M HCl, 0.1 M NaOH and water for 1, 2 and 3 hours, after which the samples were neutralised. EFV, FTC and TDF degraded significantly when exposed to 0.1 M NaOH for 2 hours at an unspecified room temperature [85]. EFV has been reported to be unstable in alkaline conditions as it undergoes base hydrolysis at the carbamate amine [82, 83, 86] and degradation follows apparent first-order kinetics in the pH range 0.6-12.8 at 60 °C [5]. The proposed cyclic carbamate degradation of EFV is depicted in Figure 1.11. The pH of maximum stability for EFV is reported to be between pH 1-7 [83] and EFV was found to be stable in neutral conditions. However FTC and TNF degraded in neutral conditions [85].

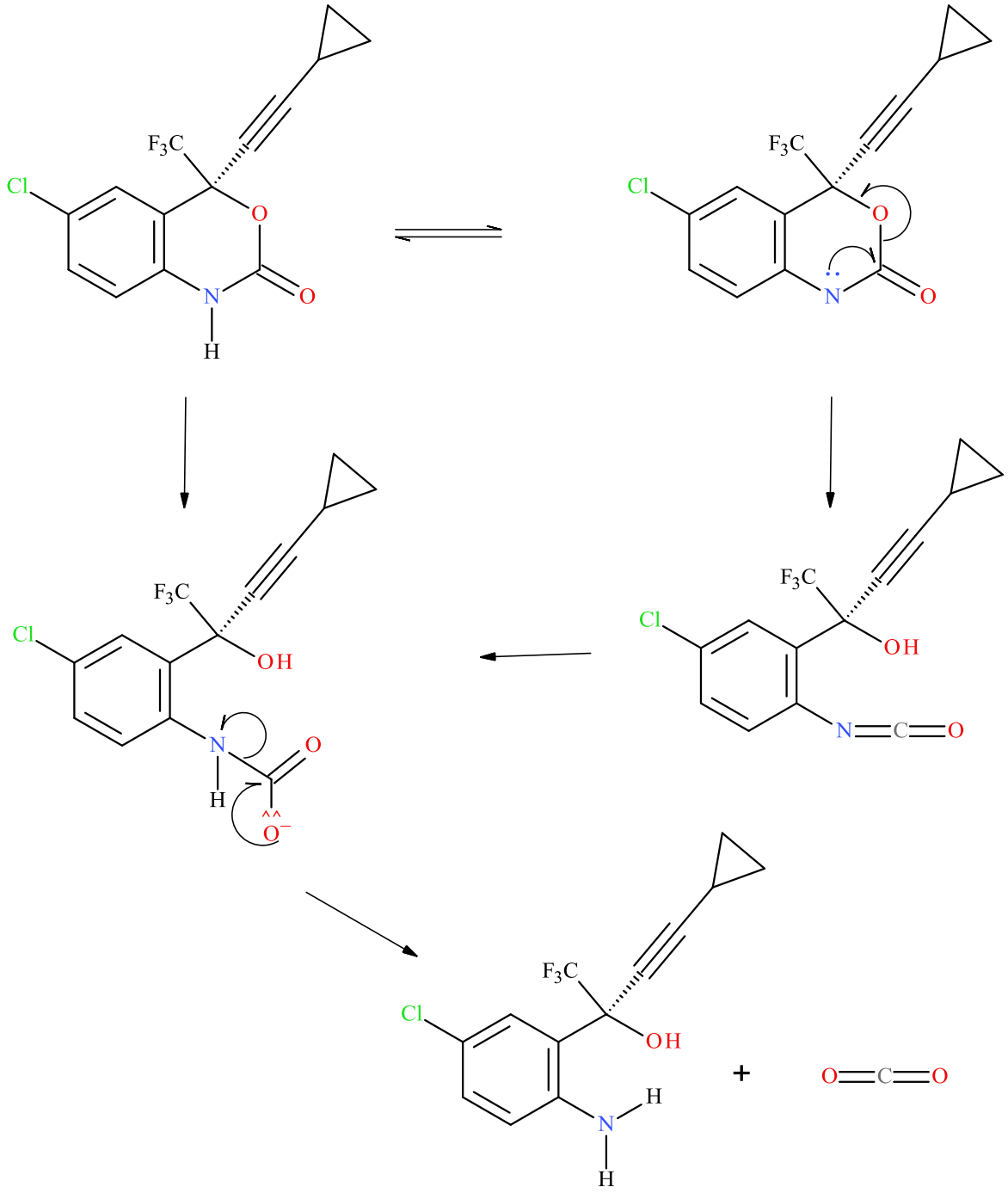


Figure 1.11 *Proposed base hydrolysis of cyclic carbamate of EFV (adapted from [83, 86])*

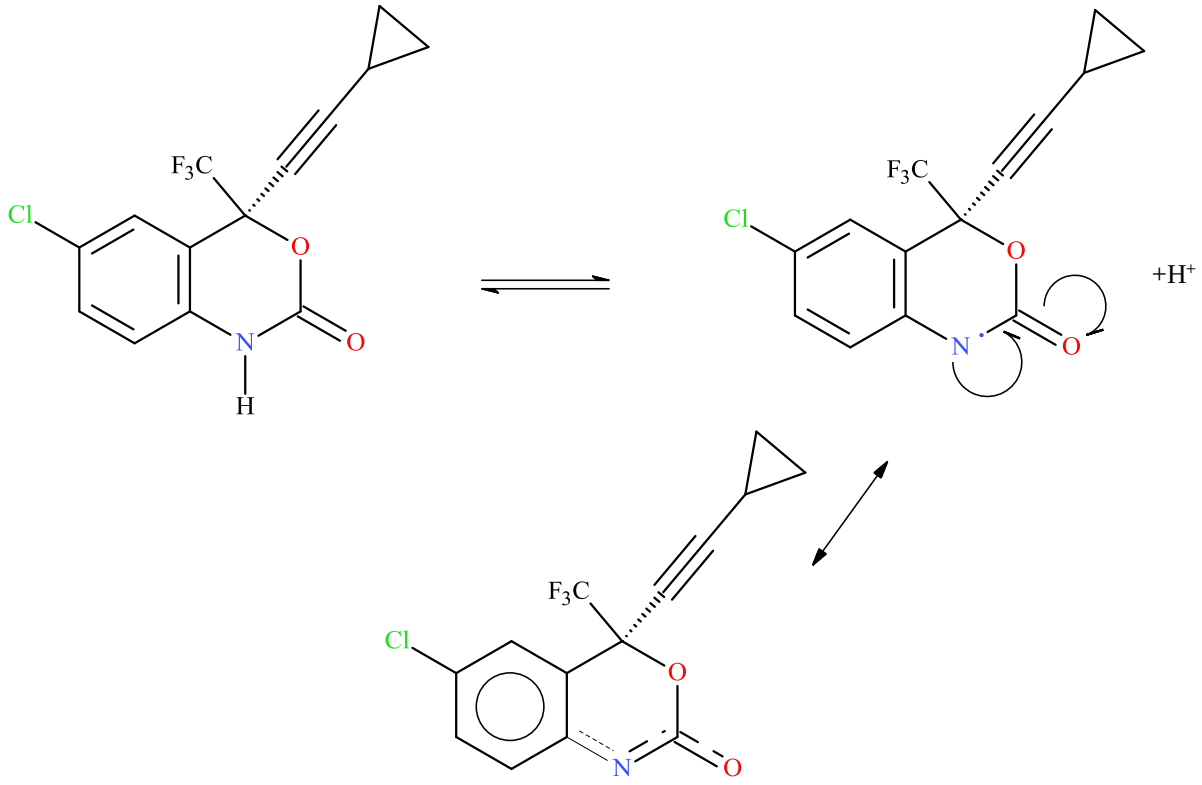


Figure 1.12 *Ionisation of the amine functionality in the carbamate moiety of EFV and stabilisation of negative charge through resonance (adapted from [80])*

A plateau in the pH profile of EFV has been reported between pH 10-11 and is a result of the ionisation of the carbamate amine which prevents base hydrolysis of EFV. The ionised carbamate is stabilised through resonance (Figure 1.12) [86].

The ARV were also subjected to oxidative, thermal and photolytic conditions. Oxidative stress in 10 % v/v H_2O_2 at an unspecified room temperature for 5 hours revealed that EFV did not oxidise under prolonged exposure to an oxidising agent. Thermal and photodegradation studies were performed on the ARV in their solid state. Thermal conditions were exposure to 60°C for 24 hours. Photodegradation conditions were exposure to 1.2 $10\times^{6}$ lux hr of fluorescent light and 200 Watt hr m^{-2} UV light. All compounds were stable under thermal- and photo- degradation conditions [85] despite photodegradation of EFV having been reported [83].

1.5 CLINICAL PHARMACOLOGY

1.5.1 Mechanism of action

Three broad classes of antiretroviral drugs are used for the treatment of HIV-1 infection. These include RT, protease, and fusion inhibitors [87]. The RT inhibitors can be further classified into NRTI and NNRTI [26]. Both NRTI and NNRTI target the HIV-1 virus RT enzyme and these classes form

the majority of approved commercially available antiretroviral drugs [88]. HIV-1 RT converts single-stranded viral RNA into double-stranded viral DNA. HIV-1 RT is a heterodimer consisting of p66 and p51 subunits [89]. The p66 subunit contains an N-terminal polymerase domain and a C-terminal RNase H domain while the p51 subunit, not directly involved in catalysis, provides structural support for subunit p66 enzymatic activities [90].

1.5.1.1 Efavirenz

EFV is a non-competitive potent non-nucleoside reverse transcriptase inhibitor of HIV-1 [11, 13–15]. The crystal structure of the p66/p51-EFV complex, EFV is bound to the p66 subunit in a small NNIBP approximately 10 Å from the polymerase active site [89]. RT changes conformation to create a NNIBP that can accommodate a NNRTI. The NNIBP does not exist unless an NNRTI is bound to RT, and binding of a NNRTI is said to block polymerisation activity of RT [13, 24, 90–92]. The RT of the HIV-2 virus and human cellular DNA polymerases α, β, ϒ and δ are not significantly inhibited by efavirenz [11, 93].

1.5.1.2 Emtricitabine

FTC, a NRTI, is a synthetic nucleoside analogue of cytidine [11, 23] and activity requires conversion of the compound to its active metabolite, emtricitabine 5'-triphosphate via phosphorylation. FTC is a competitive inhibitor of HIV-1 RT since emtricitabine 5'-triphosphate competes with deoxycytidine 5'-triphosphate, a natural substrate of RT. FTC is thus able to mimic normal nucleotides that are incorporated into DNA at the 3' terminus, however it lacks the 3'-OH and once incorporated at the 3' terminus of DNA, emtricitabine terminates chain elongation by preventing incorporation of additional nucleotides [11, 23, 26].

1.5.1.3 Tenofovir

Similar to FTC, TNF must be converted to its active metabolite for ARV activity to occur [11, 24, 25]. TNF initially undergoes diester hydrolysis and further undergoes phosphorylation by cellular enzymes to form tenofovir diphosphate that competes with deoxyadenosine 5'-triphosphate, a natural substrate of RT. Tenofovir diphosphate is incorporated into nascent viral DNA resulting in chain termination [11, 25].

Tenofovir diphosphate and emtricitabine 5'-triphosphate are weak inhibitors of cellular DNA polymerases α, β, ϒ and emtricitabine 5'-triphosphate weakly inhibits DNA polymerase ε [11].

1.5.2 Therapeutic indications

EFV used in combination with other antiretroviral agents is indicated for the treatment of HIV-1 infection in adults, adolescents and children aged 3years and older and/or those weighing 13kg or heavier [93]. EFV is an important component of HAART [1] regimens that consist of a NNRTI, such as EFV, and/or a protease inhibitor and one or two nucleoside RT inhibitors [1, 26]. EFV, TNF and FTC are preferred ARV agents for the treatment of HIV-1 infection in adults [53]. EFV, TNF and

FTC are components of a once-daily single tablet [37, 91, 94] and a FDC ARV was licenced for use in South Africa in 2013. It is first-line therapy for treating HIV-positive antiretroviral naïve patients and HIV-positive pregnant women who were breastfeeding [9, 18, 95, 96].

EFV is also indicated for use in post exposure prophylaxis (PEP) [24]. TNF and FTC are available as a once daily single-tablet regimen for the treatment of HIV-1 infection in adults [97, 98]. The single-tablet has been approved by the FDA for pre-exposure prophylaxis (PrEP), although this is not widely used clinically [27].

1.5.3 Dosage and administration

The FDC contains 600 mg/ 200 mg /300 mg of EFV, FTC and TDF, respectively (§1.1.1, Chapter one) [11, 12]. The long plasma half-life of EFV and the long intracellular half-lives of the active forms of FTC and TNF permit effective once-daily dosing [53]. The tablet is administered orally and it is recommended that the FDC be taken on an empty stomach prior to bedtime as this approach reduces the frequency of adverse effects associated with EFV and improves nervous system tolerability of EFV in patients [11, 37].

1.5.3.1 Overdose

In the event of an overdose patients should be monitored for signs of toxicity and their clinical presentation observed. Standard supportive treatment should be applied where necessary. FTC and TNF can be removed by haemodialysis but EFV is highly protein bound and therefore unlikely to be dialysed. Activated charcoal may be administered to sequester unabsorbed EFV [31].

1.5.3.2 Missed doses

If a dose is missed within 12 hours of the time it was intended to be taken then another dose should be taken as soon as the patient remembers they had missed a dose, otherwise the dose should not be taken and the patient should resume the usual dosing schedule [37].

1.5.3.3 Discontinuation of therapy

If the discontinuation of therapy with one of the components of the FDC is indicated, separate preparations of EFV, FTC and TNF are available and can be considered for use. If therapy is discontinued, the long half-life of EFV and long intracellular half-lives of FTC and TNF must be taken into consideration as this may lead to the emergence of resistance [37].

1.5.3.4 Dosage adjustment

If a FDC is used concomitantly with rifampicin (in patients weighing 50 kg or more) an additional 200 mg/day (800 mg total) of EFV may need to be administered to ensure optimal therapy [31, 37].

1.5.4 Contraindications

The use of a FDC is contraindicated in patients that exhibit significant hypersensitivity reactions such as Stevens-Johnson syndrome, toxic skin eruptions or erythema multiforme to any of the active

substances or excipients in the dosage form [31, 37]. TNF is contraindicated in patients with moderate to severe uncontrolled renal failure [99, 100] and initially evidence suggested TNF was relatively safe however there have subsequently been reports of nephrotoxicity in patients using TNF for HIV treatment [101]. EFV metabolism occurs primarily via the Cytochrome P450 enzyme system and therefore use of the FDC is contraindicated in patients with severe hepatic impairment [11].

1.5.4.1 Contraindicated medicines for concomitant use

Due to the presence of EFV in the FDC, concomitant use with agents such as terfenadine, astemizole, cisapride, midazolam, triazolam, pimozide, bepridil, or ergot alkaloids *viz.* ergotamine, dihydroergotamine, ergonovine, and methylergonovine is contraindicated [31, 37].

Concomitant use of EFV with herbal preparations containing St. John's Wort (*Hypericum perforatum*) is contraindicated due to the risk of decreased plasma concentrations of EFV which may lead to a reduction of virological suppression and may contribute to resistance to EFV or other NNRTI [31, 37].

1.5.5 Precautions and use in special populations

1.5.5.1 Paediatric population

FDC should be administered only to patients who are 15 years of age and older and who weigh ≥ 40 kg. The dosage adjustment required for younger patients who weigh < 40 kg is not possible with the FDC ARV delivery system [18, 31].

1.5.5.2 Geriatric population

Insufficient clinical data exists in patients older than 65 years of age. The FDC should be administered with caution to elderly people as this population group is more likely to exhibit a higher frequency of side effects associated with decreased hepatic, renal or cardiac function in addition to other diseases and therefore concomitant drug therapy that may exhibit drug-drug interactions [31, 37].

1.5.5.3 Renal impairment

The use of the FDC is not recommended in patients with moderate or severe renal impairment. Patients with moderate renal impairment (creatinine clearance (Cl_{CR}) of 30–49 ml/min) and severe renal impairment (Cl_{CR} of 10-29 ml/min) may exhibit an increased exposure to FTC and TNF [11, 102]. Elevated baseline serum creatinine, low body weight and advanced age can contribute to slow TNF clearance resulting in elevated TNF concentrations and an increased risk of renal toxicity [103]. The effect of renal impairment on EFV exposure is expected to be minimal but has not yet been assessed [11, 102]. Patients with moderate or severe renal impairment require dose interval adjustment for FTC and TNF, which is not possible with FDC tablets [37].

1.5.5.4 Hepatic impairment

The FDC should be used with caution in patients with liver disease, as limited clinical data is available to support this therapeutic approach, however hepatotoxicity is primarily associated with EFV [7, 31, 94]. Patients should be monitored carefully for adverse reactions with respect to EFV due to its being extensively metabolised in the liver. Patients with mild hepatic impairment may be treated with the FDC at the recommended doses and patients co-infected with HIV and hepatitis B/C or who have a history of hepatitis B/C infection should be treated with caution and monitored for signs of exacerbation of hepatitis [31, 37, 94].

1.5.5.5 Pregnancy and lactation

EFV has been associated with a potential increase in the risk of congenital defects of the central nervous system (CNS). It was recommended that EFV should not be used in pregnancy based on neural tube defects reported in animal studies, and retrospective case reports of several human infants exposed to EFV during the first trimester who were born with myelomeningocele [17, 31]. A systematic review of available evidence revealed no increased risk of overall congenital defects among infants when exposed to EFV during the first trimester of pregnancy compared to exposure to other antiretroviral drugs [17]. However a major limitation of this retrospective study was the inadequate sample size. Due to widespread use of EFV as first-line treatment in South Africa, it was previously thought that pregnancy should be avoided in women being treated with the FDC containing EFV [17, 94]. According to recently published guidelines [18] it is recommended that EFV be used as first-line treatment for all HIV-positive women who are pregnant, breastfeeding or within 12 months post-partum. EFV should be used regardless of CD4 cell count or gestation period of the pregnancy.

1.5.5.6 HIV and hepatitis B co-infection

It is recommended that patients undergo screening for chronic hepatitis B (HBV) infection prior to initiation of ARV therapy as the FDC is not indicated for the treatment of patients with chronic HBV infection. Severe, acute exacerbations of HBV have been reported in patients who discontinue therapy with FTC and TNF. Patients co-infected with HBV and HIV must be monitored for several months and anti-HBV-therapy may be initiated, if required [31, 99, 100, 104]. Liver decompensation and liver failure associated with HBV exacerbation in patients with HBV receiving FTC therapy has also been reported [31, 94].

1.5.6 Adverse reactions

1.5.6.1 Rash

Rash, a common hypersensitivity reaction with HAART, is most often associated with NNRTI [105, 106]. The eruptions can range from mild-to-moderate or life threatening [31, 106], and usually manifest as widespread maculo-papular, erythematous and pruritic rashes [31, 105, 107–109]. Fever, myalgia and arthralgia can precede the development of a rash but could occur without any sign of a

rash [107]. Rashes associated with NNRTI use typically occur within the first few weeks of initiating therapy [31, 107, 109, 110] and can resolve spontaneously on continued therapy [31, 107]. Severe cases may present as Stevens-Johnson syndrome and toxic epidermal necrolysis [111]. Reactions may persist after the cessation of therapy, particularly with drugs that have long half-lives [107].

Therapy should be discontinued in patients who develop severe rashes accompanied by blistering, desquamation, exfoliation, mucosal involvement or fever > 39 °C [31, 107]. If a patient experiences rash on the initiation of therapy, liver enzyme levels should be checked even if the patient is asymptomatic [108]. Patients should be advised about the possibility of rash when therapy is initiated [112]. Management of the rash includes the use of antihistamines and/or corticosteroids to improve the tolerability and resolution of the rash [31, 105, 109] and in severe cases therapy must be discontinued [109].

1.5.6.2 Lactic acidosis/severe hepatomegaly with steatosis

Fatal cases of lactic acidosis and severe hepatomegaly with steatosis have been reported in patients being treated with TNF and other nucleoside analogues [31]. Lactic acidosis and hepatic steatosis are thought to be a result of mitochondrial toxicity in liver and skeletal muscle cells as NRTI compounds inhibit DNA polymerase ϒ within mitochondria [113–115], leading to depletion of mitochondrial DNA and a change in the synthesis of mitochondrial proteins. Patients with hyperlactatemia associated with NRTI therapy are asymptomatic. Those developing the most severe form of hyperlactatemia present as metabolic acidosis and experience higher rates of mortality [113]. Symptoms of lactic acidosis are generally non-specific and include nausea, vomiting, abdominal pain, dyspnoea, paraesthesia, fatigue and weight loss. Women tend to be more affected than men [31, 113, 114], and the risk factors include obesity and prolonged exposure to nucleoside analogues [31]. Caution must be used when administering TNF or other nucleoside analogue in these patients [31]. Recovery includes supportive therapy and discontinuation of NRTI therapy [114].

1.5.6.3 Hepatoxicity

The AIDS Clinical Trials Group scale of liver toxicity assists to categorise the extent of hepatotoxicity in patients. Patients with normal transaminase baseline levels of < 35 U/L for aspartate aminotransferase (AST) and < 31 U/L for alanine aminotransferase (ALT) are most likely to develop hepatotoxicity when ALT and/or AST levels are above the upper limits of the normal (ULN) [116, 117]. The scale is divided into 4 grades *viz*., grade one 1.25-2.5×ULN, grade two 2.6-5×ULN, grade three 5.1-10×ULN and grade four is >10×ULN. Severe hepatotoxicity is observed in patients with grades three or four scores during ARV therapy. The severity and outcomes can vary and patients may be asymptomatic. Hepatotoxicity may resolve spontaneously or patients can suffer from liver decompensation where outcomes may be fatal [117].

Hepatotoxicity can be a consequence of a number of different mechanisms, some of which are due to direct drug toxicity, hypersensitivity reactions to the ARV, immune reconstitution in the case of HBV or hepatitis C (HCV) co-infection and mitochondrial toxicity [117]. HIV-positive patients with chronic HBV or HCV co-infection, raised baseline ALT level or hepatic dysfunction are most likely to develop severe hepatotoxicity [7, 117]. In such patients liver enzyme monitoring is recommended prior to initiation of and during the course of therapy [31, 94]. Additional risk factors for developing ARV-associated hepatotoxicity include already-existing raised transaminase levels, age, gender (female) and lack of therapeutic response to ARV or an increase in CD4+ counts following the initiation of ART [117].

It can be difficult to attribute the cause of hepatotoxicity to a single agent in HAART and the FDC containing EFV/FTC/TNF has been reported to cause hepatotoxicity. In this combination it is unlikely that the cause of hepatotoxicity is TNF or FTC as hepatotoxicity has rarely been associated with TNF and no cases have been reported with FTC, but EFV-associated hepatotoxicity has been documented [7].

1.5.6.4 Immune system reconstitution syndrome

A characteristic of HIV infection is the continuous reduction in the number CD4 T cells. The result is gradual immunodeficiency with an increased risk of opportunistic infection [118]. Immune reconstitution can occur on initiation of HAART [117, 119] as the viral load is reduced and the immune response restored through an increase in the number of CD4 T cells [117, 118]. An inflammatory immune response to residual infections or patients co-infected with diseases such as *Mycobacterium avium*, cytomegalovirus, *Pneumocystis jiroveci* pneumonia, *Mycobacterium tuberculosis* and HBV or HCV can occur. Immune reconstitution can occur within days or months of initiation of therapy [117–120] and has occurred when using active components of the FDC tablet [31]. Management of such patients depends on whether an active opportunistic infection or immune reconstitution was elicited by persisting antigens from a previous infection. In the first instance antimicrobial therapy is warranted, but in cases of residual infection that are non-replicating it is not required [118].

1.5.6.5 Central nervous system and psychiatric symptoms

Serious psychiatric adverse reactions have been reported in patients treated with EFV, including depression, suicide ideation, aggression, paranoia, mania and anxiety [31, 105, 121, 122]. Patients with a history of psychiatric disorders or substance abuse are at a higher risk of experiencing psychiatric reactions to EFV therapy [31, 121], and all patients who experience psychiatric adverse reactions should be monitored [31].

The effects of EFV on the CNS are common and approximately 50 % of patients treated with EFV experience CNS side effects that include dizziness, insomnia, impaired concentration, somnolence,

unusual dreams and/or hallucinations [31, 121–123]. These side effects can occur within a few days of initiation of therapy and usually resolve within 2-4 weeks of continued therapy [31, 107, 122, 124]. CNS side effects may lead to discontinuation of therapy of EFV [123]. Patients should be informed of the side effects associated with the use of EFV, and that alcohol and other psychoactive drugs should be avoided as they may have additive effects. Doses should be administered at bedtime to improve the tolerability of EFV to the patients [31].

1.5.6.6 Onset or worsening of renal impairment

Cases of acute renal failure and Fanconi syndrome (FS) have been reported in patients using TNF [31–34] although clinically important renal toxicity is rare [35]. TNF toxicity is located at the PT, which can lead to FS and acute injury to the kidneys [125]. TNF has also been associated with a decrease in estimated glomerular filtration rate (eGFR) and creatinine clearance Cl_{CR}, markers used to assess glomerular functioning [33, 125]. FS is described as urine wasting of solutes such as phosphate, bicarbonate, glucose, low molecular weight proteins, and amino acids that would otherwise be reabsorbed at the level of the PT. Hypophosphatemia is the most clinically significant result as it may lead to bone pain, osteomalacia and fractures [125, 126].

Potential risk factors for developing nephrotoxicity include low body mass, age, underlying renal disease, diabetes mellitus, hypertension and the concomitant use of TNF with protease inhibitors such as lopinavir/ritonavir and didanosine [32, 34, 35, 125]. Drugs eliminated by active tubular secretion may result in an increase in TNF concentrations [32].

Some patients recover renal function on cessation of TNF therapy in a short time [34, 125], but full recovery to baseline renal function may occur in only a few patients [32, 34, 125]. It is recommended that renal function be assessed prior to initiation of therapy, and patients with a Cl_{CR} <50 ml/min require dose adjustments which is not possible with a FDC product [31, 32].

1.5.6.7 Bone effects

The mechanism by which bone mineral density (BMD) changes in HIV- infected people occurs is not well understood [127–129] but it is thought that the HIV in addition to immune reconstitution and the immune response to the virus are all contributing factors [130–133]. Other risk factors include low body weight and overexposure to TNF [134], which is considered as the ARV most likely to cause BMD loss [130]. Initiation of therapy with TNF in HIV-positive adults has been associated with a decrease in BMD and an increase in risk of fracture and markers of bone turnover [31, 127–130, 133, 134].

The initial decrease in BMD is reported to be between 2-6 % in the hip and spine but appears to stabilise after 1-2 years of therapy regardless of therapy [128, 132, 133, 135]. HIV-infected people should be monitored for loss of BMD especially in cases where there is a history of bone fracture or

risk of other bone disorders such as osteopenia or osteoporosis [31]. No recommendations exist for the management of patients with osteopenia/osteoporosis and treated with TDF [130].

1.5.7 Drug interactions

The majority of drug-drug interactions reported involve EFV and are due to EFV metabolism in the liver (§1.6.3, Chapter one). EFV can induce liver enzymes and has the potential to decrease the concentration of atazanavir [31, 136], indinavir [31, 137] lopinavir/ritonavir [31, 138], saquinavir [31, [137, 139], boceprevir [1, 8, 140], telaprevir [31, 140], maraviroc [31, 141] warfarin [31, 142, 143] bupropion [31, 144] ketoconazole/itraconazole [31, 145, 146], diltiazem [31, 147], atorvastatin/ pravastatin/simvastatin [31, 148, 149], norgestimate [31, 150, 151], clarithromycin [31, 139], methadone [31, 152, 153], rifabutin [154] and artemether/lumefantrine [31, 155]. EFV can inhibit metabolism of warfarin and therefore increase warfarin concentration [31, 142, 143]. Telaprevir may increase EFV concentration [31, 140] while phenobarbital, carbamazepine, phenytoin [156–158], rifampicin and rifampin [31, 139, 154] may decrease EFV concentration.

Some drug-drug interactions have been reported for TNF where co-administration of TNF and atazanavir results in TNF decreasing the atazanavir concentration and atazanavir increasing the TNF concentration [31, 136, 159]. TNF also increases the didanosine concentration and co-administration should be avoided [31, 160–164].

FTC and TNF are eliminated via the kidneys (§1.6.4, Chapter one) and therefore, molecules that reduce renal function may result in an increase in FTC and TNF concentrations. Examples include acyclovir, adefovir dipivoxil, cidofovir, ganciclovir, valacyclovir, valganciclovir, gentamicin and NSAIDs [31].

1.5.8 Resistance

HAART is highly effective in blocking the replication of HIV, but resistance can and does develop [1, 26, 165]. Resistance to ARV occurs through the accumulation of one or more mutations in the HIV-1 RT coding region [26]. EFV has a low genetic barrier to HIV-1 resistance and HIV-1 RT is highly prone to coding errors [166]. EFV resistant mutations occur in the amino acid residues of the NNRTI-binding pocket of HIV-1 RT [87, 167]. The K103N RT mutation is the most common single mutation conferring resistance to EFV cross-resistance to other NNRTI such as nevirapine and delavirdine [1, 165, 166]. In addition EFV, a second generation NNRTI, has a greater resilience to HIV-1 RT resistance than first generation NNRTI agents such as nevirapine and delavirdine [167, 168]. Secondary mutations may follow the K103N mutation [165] and the cessation of therapy with EFV, TNF and FTC may result in functional monotherapy with EFV as it has a longer half-life than TNF and FTC therapy leading to low-frequency if development of EFV-resistant strains [166].

The HIV-1 RT mutation K65R is a common multi-drug resistance mutation conferring resistance to NRTI including TNF and FTC [11, 26, 29, 30] and may also be responsible for cross-resistance between different NRTI [26, 30]. Other mutations such as thymidine analogue mutations (TAM, M41L, D67N, K70R, L210W and T215Y/F) can affect the response to TNF [30]. The M184I and M184V RT mutations may also confer FTC and lamivudine (3TC) resistance [11, 169, 170]. This mutation occurs rapidly and results in high-level resistance to these compounds. However the prevalence of this mutation is lower in patients receiving FTC+TNF+EFV than those receiving 3TC+AZT+ EFV. FTC is more potent than 3TC *in vitro* and has a longer plasma and intracellular half-life that may account for the lower prevalence of M184I/M in patients treated with FTC over those treated with 3TC [169, 170].

1.6 CLINICAL PHARMACOKINETICS OF EFAVIRENZ, EMTRICITABINE AND TENOFOVIR

1.6.1 Absorption

EFV is highly lipophilic and has very low aqueous solubility thus it exhibits limited oral absorption and has a low bioavailability of between 40 and 45 % [171]. The peak plasma concentration for EFV is reached 3 to 5 hours after dosing and steady state is achieved within 6 to10days [1, 19].

FTC is rapidly and extensively absorbed following oral administration and exhibits a bioavailability of > 90 % with peak plasma concentrations being reached within 1.5 hours of dosing [172, 173]. The steady-state plasma concentration of FTC was 1.77 µg/ml following administration and was observed 3 hours after dosing [173].

TNF is polar and is ionised following oral administration and exhibits poor oral bioavailability. TDF masks the ionic regions of the phosphonic acid moiety to enhance absorption from the gut [36]. Following oral administration TDF is rapidly hydrolysed to TNF via plasma esterases [36, 173, 174]. The steady-state plasma concentration of TNF was 0.279 µg/ml after administration as a single dose and was obtained 2.4 hours after dosing [173].

1.6.1.1 Effect of food on oral absorption

The mean area under the plasma concentration curve (AUC) and maximum concentration (C_{max}) increased by 28 % and 79 % respectively when EFV was administered with a high fat meal as compared to that observed when administered in the fasted state. The C_{max} was delayed by approximately 0.75 hours and increased approximately 15 % when TNF was administered with a high fat or light meal and the AUC also increased by approximately 35 % in this case. The effect of food on the absorption of FTC is not clinically significant. High levels of EFV may increase the likelihood of side effects and therefore it is recommended that the fixed dose combination be taken on an empty stomach [19, 37, 94].

1.6.2 Distribution

EFV is highly plasma protein bound to albumin (approximately 99.5-99.75 %) [19]. A cohort study involving 235 HIV-infected patients revealed that the apparent volume of distribution of EFV was 252 L, indicating that EFV is widely distributed throughout the body [171]. FTC and TNF are far less protein bound than EFV (4 % and 0.7 %, respectively) [97]. The apparent volume of distribution for FTC in a study involving 161 HIV-infected adults was estimated to be 42.3 L after a 200 mg once daily dose [175], whereas in a study involving 193 HIV-infected adults it was estimated that TNF had an apparent volume of distribution of 534 L after a single 300 mg once daily dose [176].

1.6.3 Metabolism

EFV is extensively metabolised by the liver Cytochrome P450 enzymes, CYP2B6, CYP2A6, CYP3A4/5, and UGT2B7. EFV undergoes hydroxylation and then glucuronidation to form inactive metabolites [11, 19–22]. EFV has been shown to induce CYP3A4 and CYP2B6 and therefore induces its own metabolism [19, 37, 171]. As a result, EFV influences the pharmacokinetics of many co-administered drugs, increasing the risk of toxicity or therapeutic failure [22]. *In vitro* studies have shown that EFV is an inhibitor of CYP2C9, CYP2C19, and CYP3A4 within the range of plasma concentrations observed during therapy [19].

FTC is metabolised via oxidation of the thiol moiety to a 3’- sulfoxide diastereomers and conjugation with glucuronic acid to form 2'-O-glucuronide, which accounts for ≈ 9 % and ≈ 4% of the administered dose, respectively [11, 37]. *In vitro* studies have demonstrated that TNF and TDF are not substrates for CYP450 and thus hepatic metabolism is negligible. Tenofovir appears to undergo minimal systemic metabolism [36] and neither TNF nor FTC has exhibited *in vitro* inhibition of drug metabolism involving the CYP isoforms responsible for drug biotransformation [11].

1.6.4 Elimination

Approximately 14 % to 34 % of EFV is excreted in the urine, predominately as metabolites, and a further 16 % to 61 % is excreted unchanged in the faeces [19, 177]. The terminal half-life following a single dose of EFV is 52 to 76 hours. Shorter half-lives have been reported that can probably be attributed to auto-induction of EFV that increased clearance over long treatment periods [19, 178]. A G-T polymorphism at position 516 of CYP2B6 is also associated with lower plasma clearance of EFV [166].

FTC and TNF are largely excreted unchanged in the urine (60 % to 70 % and 70 % to 80 % respectively) [11, 172, 173, 178]. FTC and TNF excretion occurs via glomerular filtration and active tubular secretion [173]. The elimination half-life of FTC is 8 to 10 hours following oral administration of a single 200 mg dose [172, 173], while the elimination of TNF was 15.3 hours following oral administration of a single 300 mg dose [173]. FTC and TNF require intracellular phosphorylation to

their active forms, and concentrations persist within the cell resulting in long intracellular half-lives of 150 hours and 36 hours for TNF and FTC respectively [6, 103, 179].

1.7 CONCLUSIONS

The favourable pharmacokinetics, primarily due to the long half-lives of EFV, FTC and TNF permits the development of therapeutic technologies that enable once-daily dosing [6, 7, 19, 178, 171]. Therefore a FDC containing EFV, FTC and TNF has been marketed as a once-daily tablet that demonstrates the most simplified form of antiretroviral therapy available to date [6]. The FDC once-daily tablet is first-line therapy for ARV naïve HIV-1-infected people and reduces the pill burden which in turn can improve adherence to therapy [6, 11, 12]. A generic product has been commercially available as Tribuss® in South Africa since April 2013 [8, 9]. The wide-spread use of this FDC ARV requires the development of an analytical method for the simultaneous quantitation of these compounds in human plasma that would be useful for the application in therapeutic drug monitoring in order to check for patient adherence. Furthermore the method would be extremely useful for quality control purposes of dosage forms and would result in rapid generation of dissolution, content uniformity and assay data. EFV is weakly acidic and hydrophobic, whereas FTC and TNF are basic and hydrophilic [39, 40, 44–46]. EFV is also practically insoluble in water, whereas FTC is freely soluble in water. TNF is more soluble in water than EFV but is less soluble than FTC [14, 31, 36, 41–43]. These characteristics pose a challenge in the development of an HPLC method for analysis of these ARV in plasma and in dosage forms. The aim of this work is to simultaneously analysis EFV, TNF and FTC using RP-HPLC. Isocratic and gradient RP-HPLC will be assessed and the most appropriate method will be validated. The validated RP-HPLC method will then be applied to quality control analysis of commercially available dosage forms and bioanalysis of the ARV in plasma.

CHAPTER TWO
DEVELOPMENT OF A RP-HPLC METHOD FOR THE SIMULTANEOUS ANALYSIS OF EFAVIRENZ, EMTRICITABINE AND TENOFOVIR

2.1 INTRODUCTION

2.1.1 Overview

The widespread use of a FDC tablet containing FTC, TNF and EFV prompted the investigation into the development of a simple RP-HPLC method for the simultaneous quantitation of EFV, FTC and TNF in pharmaceutical formulations and biological fluids. A literature review revealed that HPLC is used as the primary analytical technique for the analysis of FDC tablets and UV is the predominant mode of detection [85, 180–184], One manuscript describes the use of HPLC-MS/MS [185]. Any HPLC method that is developed and validated must be simple, rapid, sensitive, selective, accurate, precise and stability-indicating.

2.1.2 Principles of HPLC

Chromatography is the science of separation of the components of a mixture and refers to a group of separation techniques. The separation process is based on analyte partitioning between two immiscible phases of which one is a mobile and the other a stationary phase [63, 186]. In liquid chromatography (LC) the mobile phase is a liquid, and this group of techniques include thin-layer chromatography (TLC), HPLC and ultra-high performance liquid chromatography (UPLC) [63]. TLC is one of the oldest forms of chromatography and is cost-effective and simple to perform but has largely been replaced by HPLC as an analytical technique due to the poor reproducibility of TLC methods [187]. HPLC is used as an analytical tool in the drug discovery, formulation development, production and marketing phases of the lifecycle of a product [188, 189]. HPLC is also used for analysis in stability studies of drug molecules during formulation development to provide information about the presence of degradation products and product instability. HPLC is also a preferred approach used to identify and quantitate drugs and metabolites in biological fluids [60, 189, 190]. HPLC separations are rugged and rapid, and advances in technology and HPLC system automation have made it an important analytical tool in the pharmaceutical industry [187, 189].

Reversed phase–HPLC (RP-HPLC), normal-phase HPLC (NP-HPLC), ion-exchange chromatography (IEX) and size-exclusion chromatography (SEC) are all types of LC. The ultimate HPLC technique used is selected based on the characteristics of the analyte or analytes of interest. For example the separation of analytes in IEX chromatography is based on the affinities of ionic analytes for counter ions located on the stationary phase backbone [5] and SEC involves separation of analytes according to their molecular size, in which larger analyte molecules are not able to partition into the stationary phase and will therefore elute faster than smaller analyte molecules [63, 189].

In NP-HPLC the stationary phase is polar and the mobile phase nonpolar. This technique is referred to as adsorption chromatography due the adsorptive nature of the stationary phase. Silica or silica gel is the typical polar stationary phase and organic solvents such as hexane are used as the mobile phase. Polar analytes have a higher affinity for the silica than the nonpolar mobile phase and are therefore retained on the stationary phase. However with an increase in the polarity of the mobile phase the retention of polar analytes will be reduced. The adsorption mechanism is reversible and is dependent on the chemical characteristics of the analyte in addition to those of the mobile and stationary phases [63]. Of all LC techniques RP-HPLC is the most common technique, and approximately 80 % of LC methods reported and used are RP methods. The stationary phase in RP-HPLC is nonpolar and the mobile phase polar [63, 188, 190] usually consisting of water in combination with organic solvents such as methanol and/or acetonitrile that are miscible with water. Modifiers such as buffers and ion-pair reagents may be added to the mobile phase to enhance chromatographic separation [188]. An increase in the proportion of organic solvent increases the elution strength of the mobile phase that may alter the retention characteristics of some analytes. Methanol is a cheap and less toxic organic solvent compared to acetonitrile, but it produces more viscous mixtures with water, thereby increasing the back pressure on the HPLC system. Acetonitrile does not produce viscous mixtures with water and is better suited for applications that require UV detection at low wavelength [63].

Sample preparation for HPLC requires that the analytes of interest be dissolved in solvents that are miscible with the mobile phase. The mobile phase facilitates the forced transport of the analyte over and through the stationary phase [186, 189]. Analytes interact with molecules of the mobile phase, the stationary phase and the interface between the two phases. Therefore the choice of mobile and stationary phases is important to ensure that adequate separation is achieved [63]. A large surface area at the interface allows for appropriate interaction of the analyte with the two phases [189].

Analytes separate by different processes, but adsorbent polar forces are the dominant type of molecular interaction occurring in NP-HPLC [189]. The process of adsorption involves interaction of an analyte with the adsorbent stationary phase during which the analyte competes with the mobile phase for adsorbent surfaces of the stationary phase [188, 189]. The stationary phase is usually porous oxides and is covered with -OH functional moieties [189]. Siloxane (SiO_2) and aluminium oxide (Al_2O_3) and the -OH moieties result in a stationary phase of high polarity [186, 189]. Various functional groups are attached to the siloxane groups of the stationary phase [189, 190] including inorganic oxides, diol, cyano, amino and dimethylamino substituents [190]. Analytes with the ability to form strong interactions or affinity with the adsorbent surface will elute more slowly than analytes with weak affinity for the surface. Differences in the strength of polar interactions determine the retention characteristics of analytes in NP-HPLC. Organic solvents used for the mobile phase sometimes have small amounts of organic modifier such as methanol or ethanol added [189]. The retention of analytes is controlled by adjusting the mobile phase by, for example, increasing the

polarity of the mobile phase through addition of organic modifiers [186]. NP-HPLC is used to separate hydrophobic analytes or analytes of low polarity that are insoluble in polar or aqueous solvents [188, 189].

RP-HPLC mainly uses dispersive hydrophobic and van der Waals forces to effect a separation. Dispersive forces are the weakest intermolecular forces governing a separation by partition [63]. Adsorption mechanisms of separation differ from partitioning mechanisms in that analytes partition into the porous stationary phase and interaction does not occur at the interface between a stationary and mobile phase [188]. Manipulation of the mobile phase composition is a powerful way to control the retention or elution of analytes by partitioning. The ratio of water to organic modifiers in addition to the pH and the ionic strength of the mobile phase may be manipulated [188]. Water is the least polar solvent that can be used in RP-HPLC and the strength of the mobile phase may be increased by increasing the amount of organic solvent in the mobile phase [186].

The composition of polar solvents to be used in a mobile phase in RP-HPLC depends on the characteristics of the analyte. The retention characteristics of neutral analytes is determined by the amount of organic modifier in the mobile phase and is not affected by pH, whereas the retention of acids and bases, i.e. analytes with ionisable functional groups, is dependent on pH and the amount of organic modifier used. The pH of the mobile phase is controlled by the addition of a buffer [63, 188] of which phosphate buffers are preferred as they exhibit low UV absorbance. A drawback of phosphate buffers is their tendency to precipitate when high concentrations of organic modifiers are used in the mobile phase [63]. Ionised forms of acids and bases are retained to a lesser extent than their unionised forms as they interact strongly with the aqueous mobile phase [63, 188]. The pKa of an ionisable analyte is an important factor as retention times vary significantly at pH around the pKa of the analyte. Therefore by controlling the pH of the mobile phase and thus the degree of ionisation it is possible to optimise the separation of the analytes of interest [63]. It is preferred if the pH of the mobile phase is not close to the pKa unless the separation involves two closely related compounds with similar pKa values. Acidic compounds are better retained at low pH whereas basic compounds are better retained at high pH [63].

Ion-pair chromatography may be useful for the separation and retention of basic analytes when using RP-HPLC. In this instance large hydrophobic ions are added to the mobile phase to form ion-pairs with the polar ions of the buffer. The chromatographic system therefore appears neutral as the ion-pairs are hydrophobic. If the ion-pair reagent is added in excess, ion-pairing with the analytes of interest may be formed thereby affecting the separation. Common ion-pair reagents for ionised bases include sulfonic acid and perfluorocarboxylic acids. Quaternary ammonium compounds are commonly used as ion-pair reagents for ionisable acids [63]. In RP-HPLC weak bases with pKa < 8 can be successfully retained using ion-suppression with acidic buffers. If this approach is ineffective

then an ion-pair reagent may be added. For basic drugs with pKa > 8 ion-pair reagents tend to be more effective than using ion suppression [187].

The stationary phases used for RP-HPLC are modified silica phases in which hydrophobic functional groups are attached to the silica particles chemically [63, 187, 189]. Octadecylsilane (ODS) or C_{18} is the most common stationary phase used for RP-HPLC separations and similarly to NP-HPLC the functional R-groups are bonded to the siloxane groups of the stationary phase. Shorter alkyl chains such as C_8 and C_4 may also be bonded to the siloxane, resulting in the formation of a stationary phase less hydrophobic than a C_{18} phase. Nonpolar analytes will be strongly retained on RP stationary phases due to the strong interaction between the analyte and stationary phase, whereas polar analytes will be eluted with the polar aqueous mobile phase [63, 190]. Phenyl and cyano functionalities have also been bonded to the silica backbone and the cyano functionality has intermediate polarity and is used in NP- and RP-HPLC [186, 187, 189].

2.2 PUBLISHED METHODS FOR THE SIMULTANEOUS QUANTITATION OF EFAVIRENZ, EMTRICITABINE AND TENOFOVIR

A summary of published methods for the simultaneous quantitation of EFV, FTC and TNF is listed in Table 2.1.

Most of the published methods report the simultaneous estimation of EFV, FTC and TNF in bulk raw material or in tablets [180–184]. Only one method has been reported for the analysis of EFV, FTC and TNF in plasma. This method differs with respect to the mode of detection as positive ion electrospray and tandem mass spectrometry was used [185], whereas UV detection using wavelengths between 252-262 nm is the preferred approach for analysis [85, 180–184]. The majority of the columns were RP C_{18} columns 150 or 250 mm in length [85, 180–182, 185]. The most recently published methods described the use of a cyano and C_8 columns [183, 184]. RP-HPLC was the mode of chromatography reported in all cases, but isocratic elution was used in only two methods [180, 184]. Acetonitrile was the organic solvent [85, 180, 181, 184, 185] of choice and the majority of methods used a buffer in the mobile phase. Several buffers were used but all were in the acidic range of pH [85, 180–184].

Table 2.1 *RP-HPLC methods for the simultaneous quantitation of EFV, FTC and TNF*

Sample	**Column/ guard column**	**Mobile phase**	**Flow rate (ml/min)**	**RT (min)**	**λ (nm)**	**Reference**
Tablet	Hypersil® BDS $C_{18,}$ 5µm, 250×4.6mm	Acetonitrile: 0.03 M KH_2PO_4 buffer (pH 3.2 adjusted with orthophosphoric acid) 60:40 % v/v	0.8	FTC 3.105 TNF 3.860 EFV 10.549	260	[180]
Plasma	Chromolith Performance® RP-18e 100× 4.6 mm	Mobile phase A 0.1 % formic acid, mobile phase B acetonitrile	1.5	-	-	[185]
Tablet	Xterra® RP-18 150 x 4.6 mm, 5µm	Mobile phase A ammonium acetate (pH 4.6 adjusted with glacial acetic acid), mobile phase B acetonitrile	1.0	FTC 4.61 TNF 7.52 EFV 9.10	260	[181]
Tablet	Inerstil® ODS C_{18} 250 x 4.6 mm, 5µm	Mobile phase A trifluro acetic acid (TFA) buffer 0.05 %, mobile phase B methanol	2.0	FTC 2.6 TNF 5.4 EFV 7.9	262	[182]
Tablet	Inertsil® ODS 3 V C_{18} 5µm 150 mm × 4.6 mm.	Mobile phase A 0.02M KH_2PO_4 buffer (pH 3.5 adjusted with orthophosphoric acid), Mobile phase B acetonitrile	1.5	FTC 2.0 TNF 5.0 EFV 7.7	256	[85]
Tablet	Zorbax® SB CN, 250×4.6 mm, 5µm	Mobile phase A methanol, mobile phase B ammonium acetate buffer (pH 4.5 adjusted with orthophosphoric acid)	1.5	FTC 4.629 TNF 14.313 EFV 17.749	260	[183]
Tablet	Zorbax® C_8, 150 mm x 4.6 mm, 5 µm	Orthophosphoric acid buffer (pH 2.4 adjusted with orthophosphoric acid): acetonitrile 70:30 % v/v	1.0	FTC 1.81 TNF 2.80 EFV 7.30	252	[184]

2.3 METHOD DEVELOPMENT

2.3.1 Analytical column selection

The stationary phase is usually packed inside a stainless steel column housing and is the site at which the chromatographic separation occurs [189]. The column hardware is stainless steel so as to be able to withstand high column pressures [186]. Columns vary in the content of the packed stationary phase and physical dimensions such as internal diameter and length. The variety of commercially available columns is extensive and the choice of column should be based on the characteristics of the analyte(s) to be separated [189, 191]. The column dimensions have an impact on the physical separation of the analytes of interest, whereas the stationary phase is the site for the chemical separation of analytes. Shorter columns reduce analytical run times and therefore also solvent consumption [186] resulting in efficient analysis times.

In RP-HPLC a typical base material to which the stationary phase is bonded is silica. Base materials determine the mechanical and chemical stability of the stationary phase [189] and silica is widely used since it is mechanically stable and can withstand high pressures. Silica particles, whilst providing a stable support, are porous and can be used to produce bonded stationary phases. Small silica particle diameters, an even size distribution and shape contribute to high column efficiency and reduced analytical run times. Uniform particle packing and high packing density also contribute to high column efficiency and low resistance to flow [186, 189]. A drawback of silica-based stationary phases is the instability of the material in mobile phases of extreme pH [63, 189, 191]. At low pH (< 2) bonded ligands are cleaved from the silica backbone and at high pH (>7) the silica particles dissolve [189].

The degree of hydrophobicity of a stationary phase is dependent on the extent of ligands or functional groups covalently bonded to the silica backbone [192]. The least hydrophobic phase is a CN, whereas C_{18} is one of the most hydrophobic stationary phases [63]. The majority of RP-HPLC is performed using C_{18}-bonded stationary phases [63, 189] and for the separation of highly polar compounds that cannot be retained on traditional C_{18} stationary phases more polar columns with C_4 or CN based phases can be used [191]. CN stationary phase columns are not as frequently used as C_8 or C_{18} columns due to their relatively low reproducibility, instability and weakly polar mobile phases are required with CN based columns in order to achieve adequate retention that is comparable to that observed with alkyl-silica based stationary phases [193, 194]. CN phases can be used for analytes that are basic, contain nitrogen or halogens [195], and a Phenomenex® Luna 5µm CN 150×4.6 mm i.d. column was selected for the chromatographic separation of FTC, TNF and EFV due to the range of polarity of these ARV molecules. FTC is a highly polar molecule, TNF is of intermediate polarity and EFV is the least polar of these compounds.

2.3.2 Column temperature

The partitioning of analytes between phases is a function of the thermodynamics of the system and maintaining a constant column temperature will ensure consistency in terms of analyte partitioning. The retention time of a compound can change as much as 5 % with a 1 °C change in the temperature of a column [2], therefore column temperature is an important consideration and 25 °C was used as the column temperature for all analyses in these studies.

2.3.3 Column evaluation and system suitability

System suitability tests are performed prior to validation studies to test the performance of a chromatographic system. The FDA provides guidelines for the acceptance criteria for each of the system suitability tests performed [196]. Empower® 3 Software was used to capture chromatographic responses and to assess system suitability for the development and optimisation of a RP-HPLC method, and the tests for system suitability include an assessment of column efficiency, selectivity and establishing the asymmetry and retention factors.

2.3.3.1 Distribution constant

The migration of analytes through a column is random and at any point in time an analyte molecule is either located in the stationary or the mobile phase. The distribution constant (K_c) is used to describe equilibrium distribution and is a ratio of the concentration of analyte in either the stationary or the mobile phase. Analytes that are not well retained or elute rapidly will have low distribution constants. In contrast the distribution constant will be high for analytes that are strongly retained on the stationary phase [63, 186]. The distribution constant can be calculated using Equation 2.1.

$$K_c = \frac{C_s}{C_m} \qquad \textbf{\textit{Equation 2.1}}$$

Where,

Kc = distribution constant
Cs = concentration of analyte in the stationary phase
Cm = concentration of analyte in the mobile phase

2.3.3.2 Column efficiency

Column efficiency is a measure of how well a column performs and establishing the number of theoretical plates, N is the most widely used approach to test the efficiency of separation of a chromatographic system [186, 189]. The higher the number of theoretical plates the higher the efficiency of the column and the number of theoretical plates can be calculated using Equation 2.2 [189].

$$N = \alpha\left(\frac{t_R}{w}\right)^2 \qquad \textbf{\textit{Equation 2.2}}$$

Where,

N = the number of theoretical plates in a chromatographic column
t_R = retention time for an analyte
w= peak width
α = constant

When calculating the efficiency of a column it is assumed that the peak will be Gaussian in shape (Figure 2.1). Gaussian peaks are ideal as they are perfectly symmetrical (bell curves) for which the constant, *α,* is equal to 5.45 when peak width is measured at half peak height. This is the easiest peak width to measure and is most often used. However this approach of calculating column efficiency is not accurate as it does not take into account peak fronting or tailing. The most stringent approach for calculating column efficiency is by measuring the width at 4.4 % of peak height, where σ is five times the standard deviation [197]. This is the preferred method of calculating column efficiency as it gives a true indication of peak shape [186]. The methods for establishing column efficiency are summarised in Table 2.2.

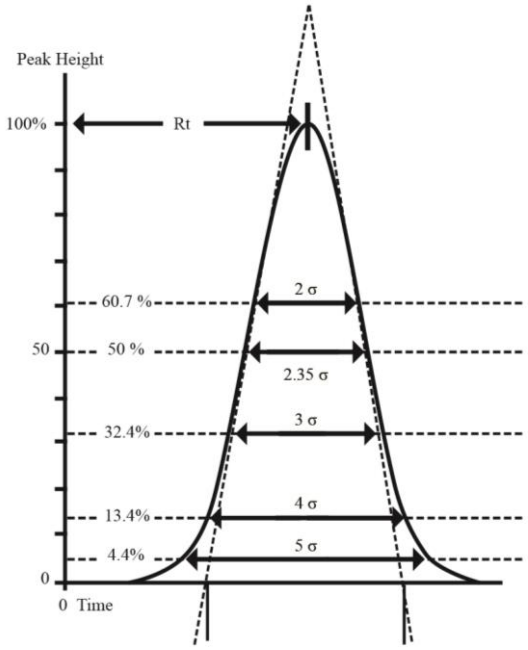


Figure 2.1 *Guassian peak illustrating different approaches to calculating efficiency [197]*

Table 2.2 *Calculation of efficiency at different peak widths [197]*

Peak width (w)	Constant (α)	Method of calculation
w_1	4	inflection
w_h	5.54	half height
w_3	9	3σ
w_4	16	4σ
w_5	25	5σ
w_{tan}	16	tangent

The efficiency of a column depends on the particle size and uniformity of packing of the stationary phase, flow rate, temperature and column length [186, 189, 190]. Smaller particle sizes and more uniform particle packing contribute to higher efficiencies and slower than optimal flow rates will result in an analyte interacting with the stationary phase for a longer period, leading to band broadening [189, 190]. Broad peaks translate to poor column efficiency. For isocratic elution, analytes with short retention times generally exhibit narrow peaks whereas analytes eluting later will be observed as broad peaks. A constant column temperature helps to maintain column efficiency [190]. The height equivalent to theoretical plate (HETP) expresses the height (H) of a single theoretical plate. It is a ratio of efficiency per unit length (L) of a given column (Equation 2.3.) [63].

$$H = \frac{L}{N} \qquad \textbf{\textit{Equation 2.3}}$$

Where,

H = height of one theoretical plate
L = length of column
N = the number of theoretical plates in a chromatographic column

The greater the efficiency of a column the smaller the value of the theoretical height will be. Measuring H is necessary when comparing the efficiency of different columns of varying length [63, 186] and an efficiency > 2000 suggests an adequate chromatographic separation efficiency [63, 196].

2.3.3.3 Void volume and void time

The void volume refers to the volume of mobile phase that travels through the stationary phase and is measured from the point of injection until detection. The void time is the rate at which an un-retained analyte will travel from the point of injection until detection [186]. The void volume (V_0) can be calculated using Equation 2.4.

$$V_0 = \Pi r^2 L p \qquad \textbf{\textit{Equation 2.4}}$$

Where,

V_0 = void volume of the mobile phase
r = internal diameter of the column
L = length of the column
p = pore size

Compounds of interest that elute within the void are considered as un-retained. The void time can be calculated by dividing the void volume by the flow rate (F) and is calculated using Equation 2.5.

$$t_0 = \frac{V_0}{F}$$ ***Equation 2.5***

Where,

t_0 = void time of the mobile phase
V_0 = void volume of the mobile phase
F = flow rate of the mobile phase

The void time is equal to the void volume when the flow rate is 1 ml/min and the void time was calculated as 1.74 min for the system used in these studies.

2.3.3.4 Retention factor

The retention factor (k') is a measure of the retention of an analyte of interest, and is calculated as a ratio of void volume to the volume of retention of the analyte of interest. Un-retained analytes will have a retention factor of 0 whereas retained analytes will have a positive retention factor [63, 189]. The retention factor is independent on flow rate and column characteristics [63] and a retention factor > 2 is recommended in order to produce peaks that are well resolved from the void [63, 191, 196]. However a retention factor of between 1 and 10 is considered acceptable for RP-HPLC [191] and the retention factor can be calculated using Equation 2.6 [63, 189].

$$k' = \frac{V_A - V_0}{V_0}$$ ***Equation 2.6***

Where,

k' = retention factor
V_A = elution volume of analyte of interest
V_0 = void volume or elution volume un-retained analyte

2.3.3.5 Selectivity

Selectivity (α) refers to the ability of a chromatographic system to distinguish between different analytes or compounds. It is calculated as a ratio of retention factors of one analyte relative to another and can be calculated using Equation 2.7 [63, 189, 198].

$$\alpha = \frac{k_2}{k_1}$$ ***Equation 2.7***

Where,

α = selectivity
k_1 = the retention factor of the first analyte
k_2 = the retention factor of the second analyte

When the selectivity is equal to one the analytes of interest are not separated and the greater the difference in retention factors of two analytes the higher the selectivity of a method. The selectivity is

independent of column length and the stationary phase. Efficiency of a separation and selectivity are complementary factors. Optimisation of selectivity or efficiency facilitates the development of otherwise difficult separations [189].

2.3.3.6 Resolution factor

The resolution factor (R_s) is a measure of the degree of separation between analytes of interest within a chromatographic run and refers to both the efficiency and selectivity of a chromatographic system, where efficiency is a measure of the mechanical separation, whereas selectivity refers to the chemical separation that is achieved. The resolution factor can be calculated using Equation 2.8 [63, 189].

$$R_s = \frac{t_{R_2} - t_{R_1}}{\frac{1}{2}(w_2 + w_1)} \qquad \textit{Equation 2.8}$$

Where,

R_s = *resolution factor*
t_{R_1} = *the retention time of the first peak*
t_{R_2} = *the retention time of the second peak*
w_1 = *the width at baseline of the first peak*
w_2 = *the width at baseline of the second peak*

One can imporve the resolution of a separation by improving either the selectivity or efficiency of a separation or both. The selection of an appropriate stationary and mobile phase is thus important to achieve adequate resolution between the analytes of interest. A resolution factor of >2 is recommened, but a resolution factor of 1.5 and 2 is considered acceptable [63, 189, 196].

2.3.3.7 Peak symmetry factor (tailing factor)

The symmetry factor (A_s) is a measure of peak shape and can be calculated using Equation 2.9.

$$A_s = \frac{w_{0.05}}{2d} \qquad \textit{Equation 2.9}$$

Where,

A_s = *peak symmetry factor*
$w_{0.05}$ = *is width at one-twentieth peak height*
d = *is the distance between the perpendicular line drawn from the peak maximum and the front of the peak corresponding to one-twentieth of the peak height*

An A_s value of 1 signifies peak symmetry, whereas when peak tailing occurs the value for A_s is > 1 and if peak fronting occurs the value for As < 1. A symmetry factor of 0.8 and 1.5 is recommended as appropriate for HPLC analyses [63].

2.3.4 Method of detection

In LC, detectors are used to provide a visual response for an analyte in solution that is under investigation. The type of detector used for an analysis is selected based on the characteristics of the

analyte(s) of interest. Several detectors are available for use, but UV detection is widely used for pharmaceutical analyses as most analytes of interest absorb light in the UV region of the electromagnetic spectrum [191, 199]. In UV spectrophotometry the functional groups of an analyte that absorb light are termed chromophores and absorption follows Beer-Lambert law if the analyte is in dilute solution [63]. Absorbance is dependent on wavelength and thus the appropriate wavelength for detection of an analyte in a sample must be identified [191]. It is important to use HPLC-grade solvents as the choice of mobile phase is important for the performance and sensitivity of the method being developed [199]. A literature review revealed that UV detection is frequently used as a method of detection for the quantitation of ARV for quality control and bioanalytical studies [200]. The wavelength of maximum absorption for each ARV was identified during method development and a wavelength of 260 nm was selected for use as all ARV absorbed UV radiation at this wavelength.

2.3.5 Mobile phase selection

The use of HPLC-grade solvents is highly recommended in order to maximise the reproducibility of a separation through the minimising of contamination. The solvents used to produce the mobile phase must be miscible to prevent high column back pressures. Acetonitrile is a commonly used organic solvent as it has a low viscosity and absorbs UV light at a wavelength of 190 nm. Methanol is also frequently used as a component of mobile phases in HPLC analyses but it is slightly more viscous than acetonitrile and absorbs UV light at 205 nm [189]. Both acetonitrile and methanol were investigated for use in the mobile phase for this separation and acetonitrile was found to produce better peak shape compared to methanol and therefore was used for all subsequent HPLC analyses. The optimum flow rate for the separation using the Phenomenex® Luna 5µ CN 150×4.6 mm i.d. stationary phase was 1 ml/min and an isocratic analysis was investigated initially as this is a simple approach compared to developing a gradient elution method.

FTC, TNF and EFV are all ionisable compounds and therefore a buffer was incorporated into the aqueous portion of the mobile phase to ensure reproducibility of the method. It is recommended that the pH of the buffer be selected based on the pKa of the analyte(s) of interest. Rapid analysis can be achieved if analytes are in their ionised form as they will predominantly interact with the mobile phase and will thus travel at a similar velocity to the mobile phase during separation. The concentration of the buffer used should fall between 0.01 M and 0.05 M [191]. Basic compounds such as FTC (pKa = 2.65) and TNF (pKa = 3.75) will be ionised at two or more pH values below their pKa. At pH 4 FTC will be largely unionised and TNF will be close to 50 % ionised. EFV (pKa 10.2) on the other hand is a weak acid and is largely unionised at two or more pH units below its pKa. At a pH 4 EFV will be largely unionised. Based on the pKa, pH of the buffer and polarity of the mobile phase the expected order of elution is FTC, TNF and finally EFV

2.4 EXPERIMENTAL

2.4.1 Chemicals and reagents

EFV, TDF and FTC and the internal standard zidovudine (ZD) were a purchased as raw material from Laurus Labs Private Ltd. (Hyderabad, India). Atripla®, Truvada® and Aspen Efavirenz® were procured from a local pharmacy. HPLC-grade acetonitrile 200 far UV Romsil-SpS® Super Purirty Solvent (Romsil® Ltd, Waterbeach, California) was used as the organic modifier. HPLC-grade water was prepared using a water purification system consisting of a Super-C carbon cartridge (activated carbon), two Ion-X® ion-exchange cartridges and an Organex-Q® cartridge and Milli-RO® 15 water purification system (Millipore®, Bedford, USA) and the reverse osmosis water was filtered through a 0.22 µm Millipak® filter (Millipore®, Bedford, USA).

2.4.2 Preparation of stock solutions

System suitability and pre-validation studies were performed using a composite standard stock solution containing all ARV at a concentration of 100 µg/ml. The standard stock solution was prepared daily by accurately weighing 10 mg of each ARV onto weighing paper using a Mettler® Model AE 163 analytical balance (Mettler® Inc., Zurich, Switzerland). The anhydrous powders were quantitatively transferred into a 100 ml A-grade volumetric flask. The powders were dissolved in a 60:40 v/v HPLC-grade water and acetonitrile (v/v %) solution. For the validation of the analytical method, a new stock solution was prepared daily by weighing 12 mg, 4 mg and 6 mg of EFV, FTC and TDF and placing directly into a 100 ml A-grade volumetric flask using a Mettler® Model AE 163 analytical balance (Mettler® Inc., Zurich, Switzerland) and were made up to volume using a solution of acetonitrile and HPLC-grade water in a ratio of 40:60 v/v. The resulting concentration of the stock solution was 120 µg/ml EFV, 40 µg/ml FTC and 60 µg/ml of TNF. The stock concentration was selected based on the quantity of each ARV in the FDC tablet and the volume of liquid likely to be used for dissolution testing. Although TDF was used to prepare stock solutions it is TNF that was monitored with HPLC.

2.4.3 Preparation of mobile phase

A fresh buffer was prepared daily as aqueous solutions are prone to bacterial growth. The buffer, a 0.03 M phosphate buffer of pH 4 (pH adjusted with NaOH pellets) was prepared as described *vide infra*. The buffer and acetonitrile were measured separately in two Schott® Duran bottles (Schott® Duran GmbH, Wertheim, Germany) and for the preparation of the buffer, 1000 ml of HPLC-grade water was measured using a 1000 ml A-grade measuring cylinder. Approximately 250 ml of water was transferred to a clean Schott® Duran bottle and 2 ml ortho-phosphoric acid (Merck® Laboratories, Wadeville, South Africa) was added to the water using a 1000 Pipetman®P (Gilson Inc., Middelton, USA) before the balance of the water was added to the bottle. The pH was measured using a Crison® Model GLP 21 pH meter (Crison® Instruments, Barcelona, Spain) and NaOH pellets were used to adjust the pH to 4 ± 0.01 units. Prior to use the buffer was filtered and degassed through a 0.45 µm

Durapore® HVLP membrane filter (Merck Millipore Ltd, Cork, Ireland). HPLC-grade acetonitrile was transferred to another Schott® Duran bottle directly from the manufacturer's bottle. As acetonitrile was used directly from the manufacturer's bottle it was not necessary to degas or filter it. Care was taken not to switch bottles, and the same bottles were used for buffer and acetonitrile. The buffer and the acetonitrile were mixed online using a Waters® Alliance® e2695 Separation module (Waters ® Corporation, Milford, USA).

2.4.4 HPLC apparatus

All HPLC analyses were undertaken using a Waters® Alliance® e2695. The separation module comprised of an Alliance® HPLC column heater, binary solvent delivery module, an auto-sampler and a Waters® 2996 PDA detector (Waters ® Corporation, Milford, USA). Empower ™ 3 software was used as the external communication interface for data acquisition (Waters® Corporation, Milford, USA). The stationary phase was a Phenomenex® Luna 5µm CN 150×4.6 mm i.d. column.

2.5 RESULTS

2.5.1 Acetonitrile content

Increasing the amount of organic modifier in a mobile phase changes the polarity and strength of the mobile phase, this in turn may alter the retention time of some analytes. The flow rate, column temperature and wavelength of detection were kept constant and a phosphate buffer (0.03 M, pH 4) was selected based on the information revealed by the literature review, and v/v ratio of acetonitrile was altered to determine the effect of a composition change on the retention times of FTC, TNF and EFV. System suitability test results are summarised in Table 2.3.

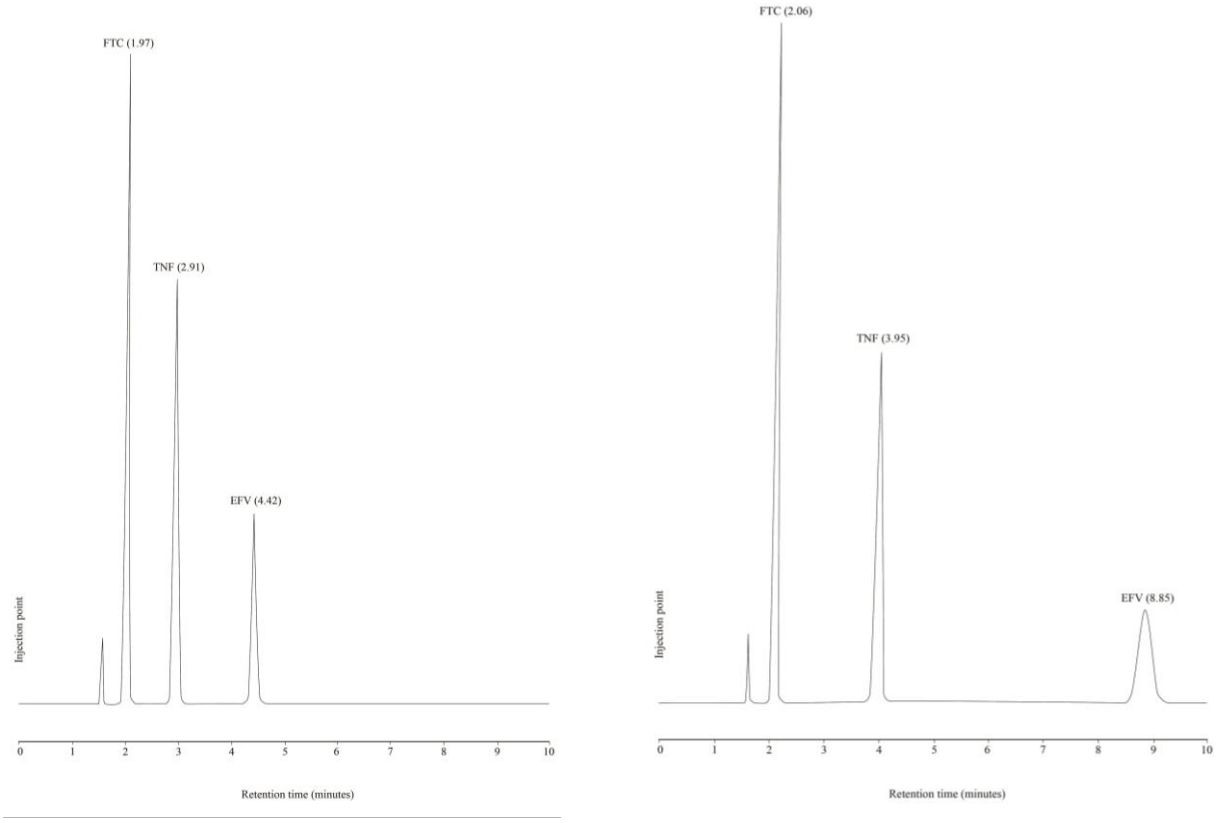
Table 2.3 *Summary of system suitability tests for the isocratic separation of FTC, TNF and EFV using 0.03 M pH 4 buffer and acetonitrile in different v/v ratios*

Buffer: ACN % v/v	ARV	Rt min	k'	Selectivity (α)	USP tailing/ symmetry factor	Plate count (5σ)
55:45	FTC	1.97	0.12736	-	1.3	7268
	TNF	2.91	0.66017	5.18344	1.1	6982
	EFV	4.42	1.52342	2.30762	1.0	8337
65:35	FTC	2.06	0.17933	2.39462	1.4	6078
	TNF	3.95	1.25955	1.49820	1.1	6687
	EFV	8.85	4.05791	3.22172	1.1	5894
70:30	FTC	2.14	0.22106	-	1.2	7690
	TNF	5.05	1.88814	8.54144	1.0	6998
	EFV	15.1	7.63843	4.04549	1.0	4458
95:5	FTC	3.00	0.71792	2.51543	1.0	2992
	TNF	-	-	-	-	-
	EFV	-	-	-	-	-

The retention times for FTC, TNF and EFV increased as the proportion of buffer in the mobile phase increased and the chromatogram depicted in Figure 2.2 A shows the retention times for a separation

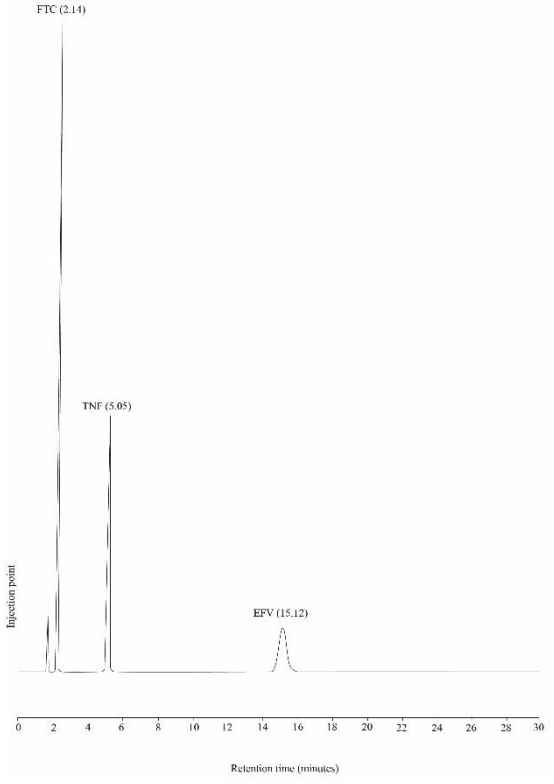
using a mobile phase with a 55:45 v/v buffer-acetonitrile composition, and Figure 2.2 B and 2.2 C shows the separation with mobile phase of 65:35 v/v and 70:30 v/v respectively. The chromatography of EFV was most affected by a change in the proportion of the aqueous component of the mobile phase and a 10 % increase in the buffer content resulted in doubling of the retention time. A further 5 % v/v increase in the buffer content resulted in the retention time increasing to almost double the previous retention time. FTC on the other hand was eluted at 3 min when the aqueous portion of the mobile phase was at 95 % v/v and both TNF and EFV did not elute after 60 min when this mobile phase composition was used.

The k' for FTC, TNF and EFV also increased as the buffer content of the mobile phase increased and the values for k' for FTC, TNF and EFV were all below the target value of 2 when a 55 % v/v buffer mobile phase was used. At a buffer concentration of 65 % v/v only EFV produced a value for k' > 2 and at 70 % v/v buffer TNF exhibited a k' of 1.9. FTC exhibited a k' of 0.7 when the buffer content was 95 % v/v, which was therefore assumed to be the maximum value for k' that could be achieved for this separation using this HPLC system. The selectivity was established for all ARV across all levels of buffer content in the mobile phase and all peaks demonstrated peak symmetry and for which the values calculated fell within the recommended ranges for symmetry. The efficiency (plate count) decreased for FTC when 95 % v/v buffer composition was used however, the plate count was still > 2000, the limit set in these studies.



A **B**

Figure 2.2A *Typical chromatogram depicting the separation of FTC, TNF and EFV using a 0.03 M buffer of pH 4 in the mobile phase 55:45 v/v buffer-acetonitrile*
Figure 2.2 B *Typical chromatogram depicting the separation of FTC, TNF and EFV using a 0.03 M buffer of pH 4 in the mobile phase 65:35 v/v buffer-acetonitrile*



C

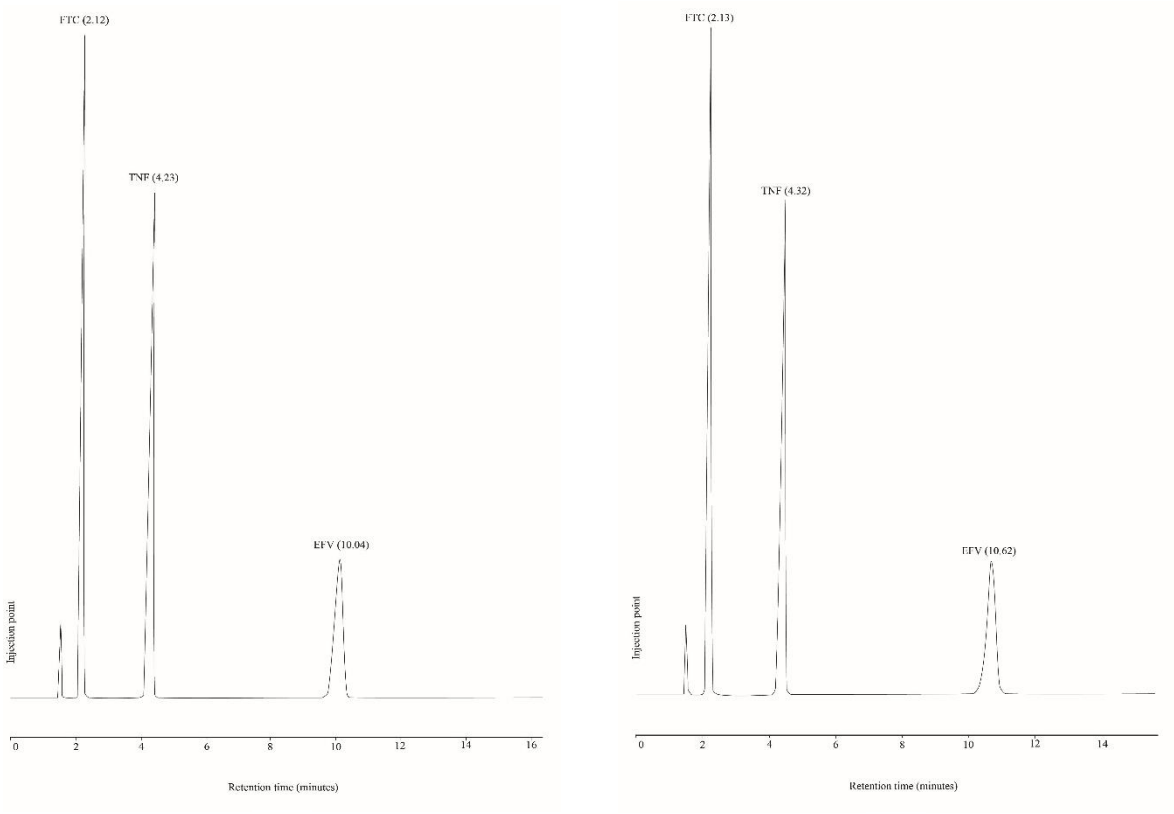
Figure 2.2 C *Typical chromatogram depicting the separation of FTC, TNF and EFV using a 0.03 M buffer of pH 4 in the mobile phase 70:30 v/v buffer-acetonitrile*

2.5.2 Buffer concentration and pH

The effect of buffer concentration on the retention times of FTC, TNF and EFV was investigated. Two buffer concentrations, representing an upper and lower concentration level, were used and the retention times of FTC, TNF and EFV evaluated at pH 4, 5 and 6 as these fall within the range that would not compromise the stability of the stationary phase. The proportion of buffer and acetonitrile (55: 45 v/v %), flow rate (1.0 ml/min), column temperature (25 °C) and wavelength of detection (260 nm) were used for all studies. The results of these studies are summarised in Table 2.4. The effect of pH on the retention characteristics of FTC, TNF and EFV is depicted in Figures 2.3 A, 2.3 B and 2.3 C and reveal chromatograms developed using a 0.01 M buffer at pH 4, 5 and 6. Figures 2.4 A, 2.4 B and 2.4 C depict chromatograms for FTC, TNF and EFV using a 0.05 M buffer at pH 4, 5 and 6 in the mobile phase. The retention time of FTC did not change with a change in buffer concentration and pH and the retention time of TNF increased slightly at pH 6 using a 0.01 M buffer as expected as TNF is mostly unionised at pH 6 and thus will partition into the stationary phase to a greater extent than at pH 4. At higher buffer concentration the retention time of TNF was not profoundly affected by a change in pH. The retention time of EFV was not expected to change significantly as the highest pH value as pH 6 is greater than two pH units below the pKa of EFV. Notably the retention time of EFV at pH 6 was longer when using a 0.01 M buffer than for that observed when using a 0.05 M buffer. However, the retention time was not significantly different at the different pH and buffer concentrations tested and since FTC, TNF and EFV differ extensively in terms of polarity to the extent where buffer concentration and pH cannot be used effectively to manipulate the retention times of all three compounds, simultaneously. This factor makes the simultaneous separation of these compounds all the more challenging.

Table 2.4 *Retention times of FTC, TNF and EFV at different pH and buffer concentrations*

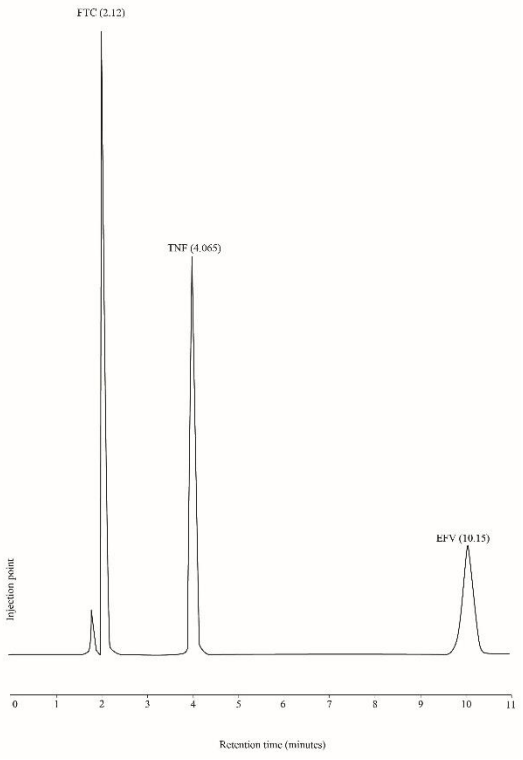
Buffer concentration (Molarity)		**Retention times (min)**		
		FTC	**TNF**	**EFV**
0.01	4	2.14	4.18	9.91
	5	2.12	4.23	10.04
	6	2.13	4.32	10.62
0.05	4	2.12	4.07	10.15
	5	2.11	4.15	9.87
	6	2.12	4.09	9.79



A **B**

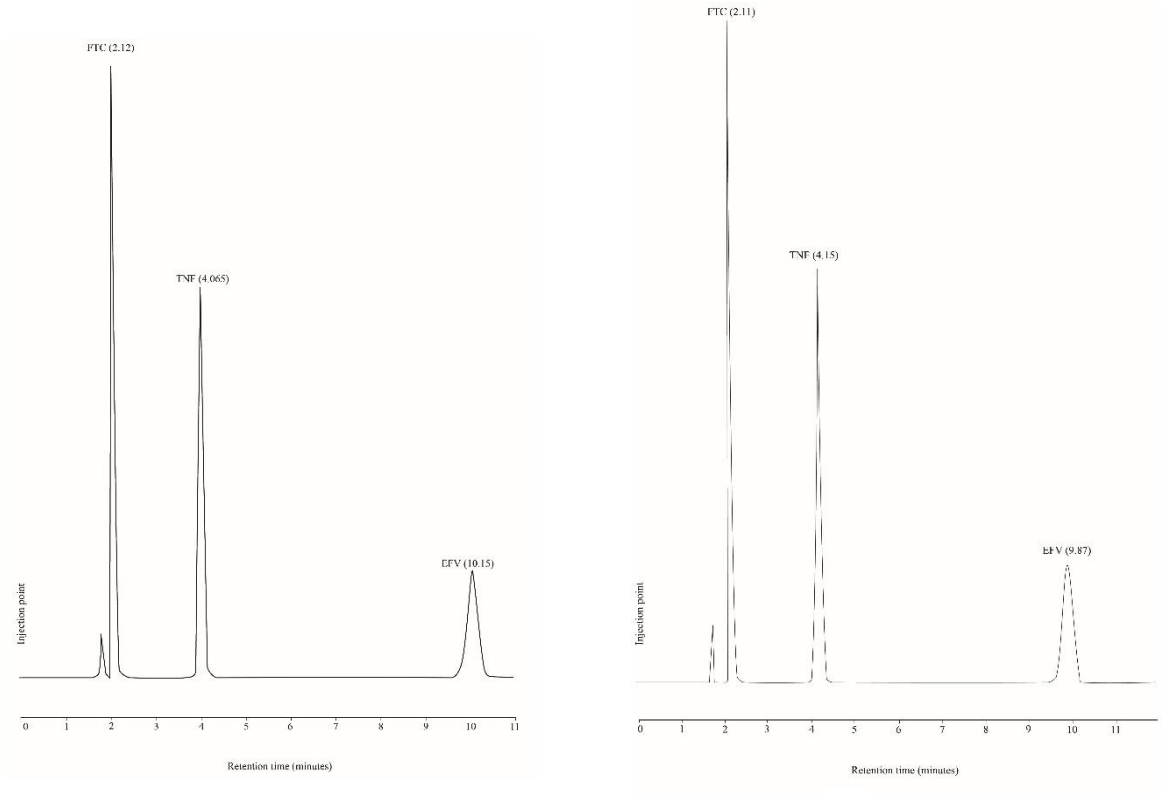
Figure 2.3 A *Typical chromatogram depicting the separation of FTC, TNF and EFV using a 0.01 M buffer of pH 4 in the mobile phase*

Figure 2.3 B *Typical chromatogram depicting the separation of FTC, TNF and EFV using a 0.01 M buffer of pH 5 in the mobile phase*



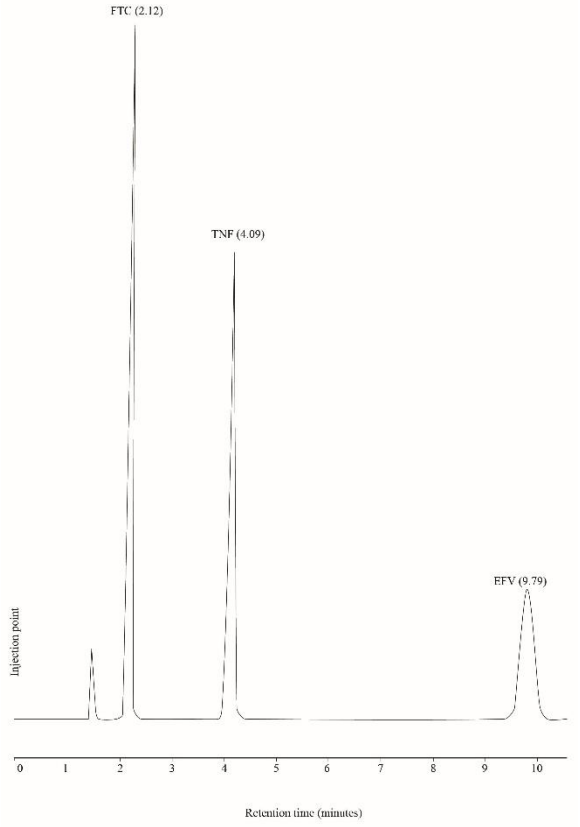
C

Figure 2.3 C *Typical chromatogram depicting the separation of FTC, TNF and EFV using a 0.01 M buffer of pH 6 in the mobile phase*



A **B**

Figure 2.4 A *Typical chromatogram depicting the separation of FTC, TNF and EFV using a 0.05 M buffer of pH 4 in the mobile phase*
Figure 2.4 B *Typical chromatogram depicting the separation of FTC, TNF and EFV using a 0.05 M buffer of pH 5 in the mobile phase*



C

Figure 2.4 C *Typical chromatogram depicting the separation of FTC, TNF and EFV using a 0.05 M buffer of pH 6 in the mobile phase*

2.5.3 Effect of change of flow rate on column efficiency

The optimum flow rate for a Phenomenex® Luna 5µ CN 150×4.6 mm i.d. is 1 ml/min, and based using the data summarised in Table 2.3, a mobile phase composition of 70:30 v/v buffer: acetonitrile was used to investigate the effect of different flow rates on column efficiency. The results of studies to determine the impact of flow rate on retention times are summarised in Table 2.5.

Table 2.5 *Summary of system suitability for the isocratic separation of FTC, TNF and EFV using a 70:30 v/v buffer: acetonitrile mobile phase*

Flow rate ml/min	ARV	Rt min	k'	Selectivity	USP tailing/ symmetry factor	Plate count (5σ)
1.0	FTC	2.137	0.221056	-	1.2	7690
	TNF	5.054	1.888136	8.541442	1.0	6998
	EFV	15.117	7.638429	4.045485	1.0	4458
0.8	FTC	2.650	0.51456	-	1.6	7620
	TNF	6.226	2.557917	1.128021	1.0	7417
	EFV	18.373	9.498762	9.498762	1.0	4862

The use of a flow rate < 1 ml/min did not result in a significantly reduced column efficiency as was expected. However the selectivity for TNF was reduced from 8.5 to 1.1 and the retention times for all ARV were longer with a slower flow rate. A change in the flow rate results in a change in the void volume and the time of a separation [186]. At a flow rate of 1 ml/min the void time is estimated to be 1.74 min based on the column specifications and when the flow rate is reduced to 0.8 ml/min the estimated void time increases to 2.2 min. The use of an even slower flow rate would result in unnecessarily long run times, reduced efficiency, and FTC would elute close to if not in the void. A chromatogram showing the separation of FTC, TNF and EFV using a flow rate of 0.8 ml/min and a mobile phase composition of 70:30 v/v buffer-acetonitrile is depicted in Figure 2.5. A comparison of the Figure 2.2 C to that of Figure 2.5 reveals the impact of a slower flow rate on the retention time and efficiency of separation particularly for EFV and in Figure 2.5 it is clear that EFV produces a much broader peak than in Figure 2.2 C.

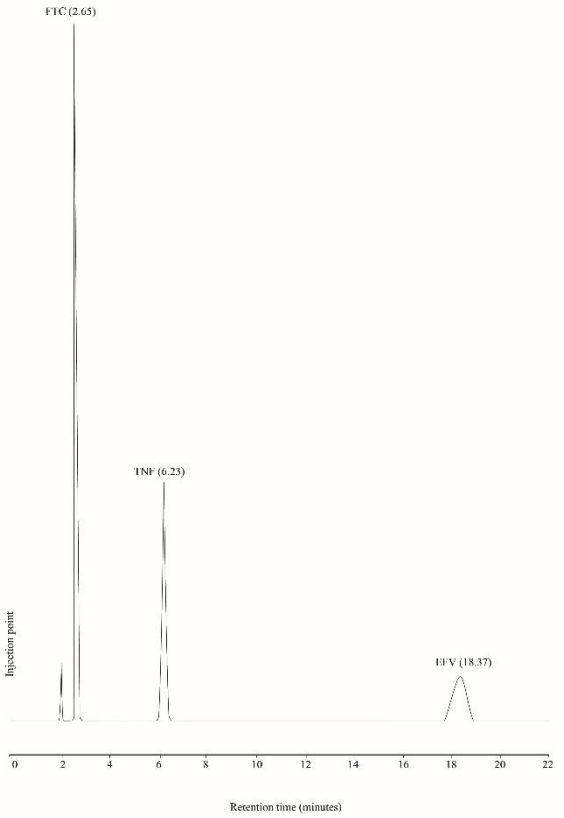


Figure 2.5 *Typical chromatogram depicting the separation of FTC, TNF and EFV using a 0.03 M buffer of pH 4 in the mobile phase 70:30 v/v buffer-acetonitrile at 0.8 ml/min*

Results from system suitability tests revealed that efficient isocratic separation of FTC, TNF and EFV is not possible thus gradient separation of FTC, TNF and EFV was investigated.

2.5.4 Gradient RP-HPLC method development

As adequate system suitability or an efficient separation was not achieved when attempting to develop and investigate the use of an isocratic HPLC method for the simultaneous analysis of FTC, TNF and EFV the use of a gradient elution approach was therefore investigated. Historically, isocratic separation were thought to exhibit better reproducibility than gradient elution methods as the mobile phase composition is constant for the duration of an analytical run and isocratic conditions also place less stress on an analytical column and/or system [189]. However the ability to successfully resolve compounds of considerably different polarity within a reasonable analytical run time is a challenge and therefore in such cases isocratic methods are limited [63, 189]. In addition the reduced peak capacity associated with isocratic conditions means the longer an analyte interacts with a stationary phase the broader are the peaks of interest in comparison to those observed using gradient elution approach [189].

Furthermore, with the advent of modern HPLC systems and technology, gradient conditions are able to produce separations with a high degree of reproducibility [189]. The term ‘gradient elution’ refers to a change of mobile phase composition during an analytical run [63, 186]. By gradually increasing the amount of organic modifier of a mobile phase during a run the separation power of the mobile phase, and therefore the method, increases. Peaks eluting late under isocratic conditions may well

elute earlier under gradient separation conditions, thereby making difficult and challenging separations possible. Gradient elution places additional stress on a column due to the constantly changing mobile phase composition and the associated properties of that mobile phase such as viscosity [63].

For gradient elution HPLC methods the peak width is dependent on the slope of the gradient used. The slope of the gradient refers to the rate of change of mobile phase composition and the retention time of the analytes of interest are controlled by changing the slope of the gradient, since different gradients result in different selectivity for different analytes. A steep gradient results in greater efficiency of a separation with narrower peak widths particularly for analytes that would elute late under isocratic conditions. Steep gradients also result in analytes eluting faster with better separations, however steep gradients require a hold-period during which the column is allowed to re-equilibrate prior to introducing the next sample. If the overall run time is too short, particularly when using steep gradients, reproducibility of the method is difficult to achieve and early eluting compounds are most affected as the column is not able to re-equilibrate to the conditions required for the start of the gradient separation [189]. The retention time of early eluting compounds is used as a guide to evaluate the re-equilibration time between injections and establish if it is sufficiently long to ensure reproducibility [186]. The flow rate is the parameter that determines the length of the hold-period, and at slower flow rates column re-equilibration takes longer. Generally three to five column volumes are recommended for re-equilibration, and the volume of the column may be estimated using Equation 2.10 [189].

$$V = 0.7\pi R^2 L \qquad \textbf{\textit{Equation 2.10}}$$

Where,

V = approximate column volume
R^2 = internal diameter of column
L = length of column

Approximately 30 % of the column volume is occupied by the stationary phase and the volume of the column is calculated in the same manner as that for a cylinder using the column specifications as the parameters of that cylinder [189].

The initial conditions for the gradient method were set at 95 % v/v buffer in order to facilitate the elution of FTC in approximately 3 min. The gradient slope and steepness were then investigated to facilitate early elution of TNF and EFV, and at a flow rate of 1 ml/min the slope and steepness of the gradient are equally important as optimal gradient steepness will result in good resolution between peaks and high separation efficiency. Excessive steepness results in a loss of resolution and less than

optimal steepness results in poor separation efficiency. The retention factor for a gradient elution separation is calculated differently from that for an isocratic separation. The first eluting peak should have a k'≤ 1. The last peak should elute when the final composition of the mobile phase reaches the end of the column [186].

A simple scouting gradient was used to set up and test gradient steepness and the conditions are summarised in Table 2.6. An initial gradient elution of 95 % v/v buffer was selected, and this was considered the upper limit for composition as TNF and EFV are not soluble under these conditions, whereas FTC is. Initially TNF and EFV are concentrated at the head of the column and will only elute when the strength of the mobile phase is such that these compounds have a higher affinity for the mobile than stationary phase and will therefore partition into the mobile phase.

Table 2.6 *Gradient scouting conditions for RP-HPLC method development*

Time min	**% v/v A 0.03 M buffer of pH4**	**% v/v B acetonitrile**
0	95	5
1	95	5
5	60	40
10	95	5

The development of a gradient method revealed that 95 % v/v buffer for the first min resulted in FTC eluting at approximately 3 min, and the capacity factor for this peak was 0.7. It was important that 95 % v/v buffer be held for at last one min prior to increasing the acetonitrile content linearly so as to ensure that retention time of FTC remained at a minimal of 3 min. These initial conditions were maintained for all subsequent experiments, and the retention times of FTC and TNF with the above gradient were 3.0 and 8.2 min, respectively. After approximately 17 min EFV had not yet eluted as the mobile phase consisted primarily of buffer. Therefore the % v/v acetonitrile had to be maintained for a longer period and a steeper gradient was required to reduce the retention times for TNF and EFV. The gradient conditions for the assessment of the impact of steepness on the separation are summarised in Table 2.7. A higher % v/v acetonitrile was used at five min in an attempt to increase the steepness of the gradient.

Table 2.7 *Gradient conditions used to assess steepness*

Time min	**% v/v A 0.03 M buffer of pH4**	**% v/v B acetonitrile**
0	95	5
1	95	5
5	30	70
10	30	70
11	95	5

Table 2.8 *System suitability results from gradient steepness test*

ARV	Retention time min	Retention factor k'	Selectivity A	USP tailing	Efficiency σ 5
FTC	2.97	0.7	-	1.0	2894
TNF	6.52	2.7	3.9	1.5	53264
EFV	7.12	3.1	1.1	1.8	57430

The system suitability results following the assessment of gradient steepness are summarised in Table 2.8 and the corresponding chromatogram is depicted in Figure 2.6. By adjusting the steepness of the gradient it was found that at 70 % v/v acetonitrile TNF and EFV eluted in a reasonable period of time, however the retention times for TNF and EFV were only 0.6 min apart and therefore a change in gradient slope was investigated. Maintaining the acetonitrile at 70 % v/v for a further three min improved the retention times of TNF and EFV. The conditions for gradient slope assessment and system suitability results for gradient slope assessment are summarised in Tables 2.9 and 2.10, respectively.

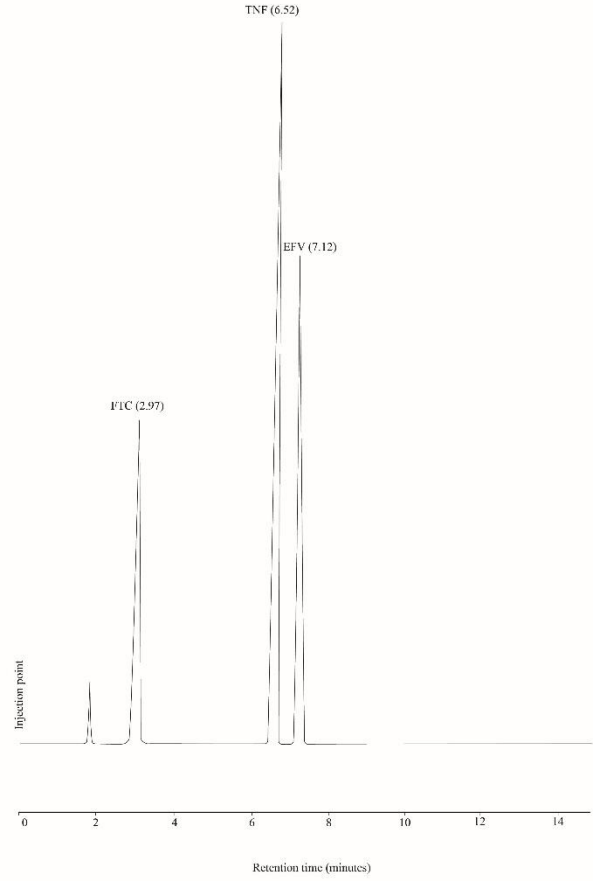


Figure 2.6 *Typical chromatogram depicting the separation of FTC, TNF and EFV for gradient steepness test*

Table 2.9 *Gradient conditions to assess slope*

Time Min	% v/v A 0.03 M buffer of pH4	% v/v B acetonitrile
0	95	5
1	95	5
8	30	70
10	30	70
11	95	5

Table 2.10 *System suitability results from gradient slope test*

ARV	Retention time Min	Retention factor k'	Selectivity A	USP tailing	Efficiency σ 5
FTC	2.9	0.7	-	1.0	2966
TNF	7.9	3.5	4.9	1.2	49925
EFV	9.1	4.2	1.2	1.5	78641

Decreasing the slope, or prolonging the rate of change of mobile phase composition resulted in longer retention times for TNF and EFV with better separation achieved between the two peaks. The last eluting compound was detected in < 10 min and the impact of gradient slope on the retention of TNF and EFV is depicted in Figure 2.7.

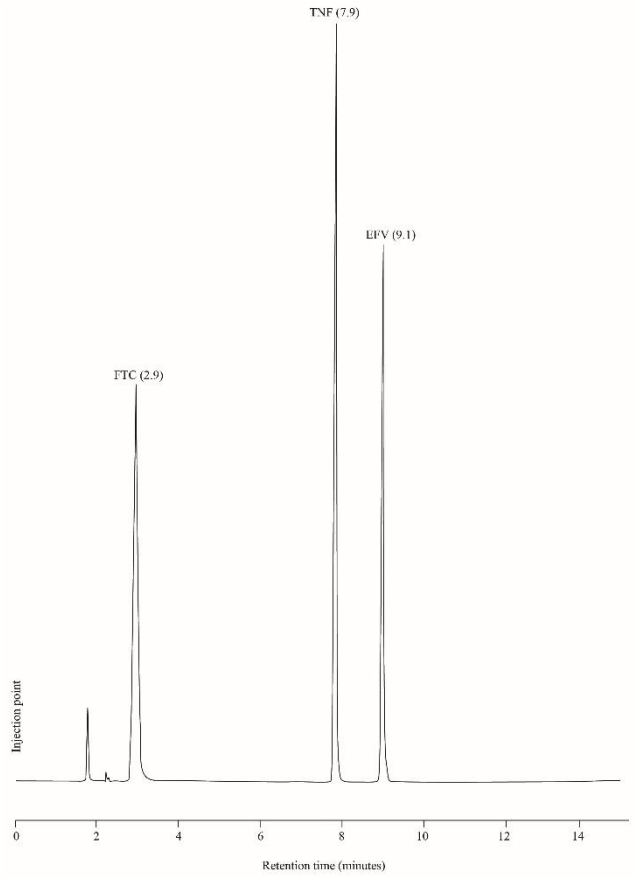


Figure 2.7 *Typical chromatogram depicting the separation of FTC, TNF and EFV for gradient slope test*

Having achieved a reasonable slope the next step requirement was to establish the re-equilibration time required between injections as unnecessarily long run times are wasteful and run times that are too short will affect the reproducibility of the retention time of fast eluting compounds such as FTC. The estimated column volume was calculate and used to approximate the equilibration time, and a run time of 15 min was selected for evaluation as it falls between the recommended three to five column volumes necessary for proper re-equilibration to be achieved between injections. The initial conditions were maintained for one min, after which the acetonitrile content of the mobile phase was increased linearly from 5 % v/v to 70 % v/v from one to eight min. These conditions were held for a further two min prior to resetting to the initial conditions, after which the column was allowed to re-equilibrate until the next injection. This approach ensured that good resolution between peaks was achieved. The optimised HPLC and gradient conditions are summarised in Tables 2.11 and 2.12, respectively.

Table 2.11 *Summary of optimised gradient HPLC method*

Column	Phenomenex® Luna 5µm CN 150×4.6 mm i.d.
Flow rate	1.0 ml/min
Run time	15 min
Injection volume	10 µl
Detection wavelength	260 nm
Temperature	25°C
Mobile phase	Mobile phase A: 0.03 M phosphate buffer at pH4 Mobile phase B: acetonitrile
Retention time min	FTC 2.9 TNF 7.9 EFV 9.1

Table 2.12 *Final gradient conditions used for the validation of a RP-HPLC method for the analysis of FTC, TNF and EFV*

Time min	**% v/v A 0.03 M buffer of pH4**	**% v/v B acetonitrile**
0	95	5
1	95	5
8	30	70
10	30	70
11	95	5

2.5.5 Choice of internal standard

An internal standard (IS) is added in a known concentration to standard working solutions containing the analytes of interest to compensate for any potential loss of analytes during sampling and/or sample manipulation. Ideally the IS should be similar in structure and chemistry to the analyte(s) of interest [63]. In other words the use of an IS can enhance the reproducibility of a method. However as the three analytes for these studies vary in their physico-chemical properties the retention times, peak symmetry, peak resolution and selectivity were used to identify an appropriate IS for HPLC analysis. 3TC, ZD and NVP were all evaluated using gradient conditions, and results are listed in Table 2.13.

Table 2.13 *IS performance under gradient conditions*

Internal standard	Retention time Min	Peak shape	Comments
Lamivudine	2.9	Peak was well resolved and symmetrical	The retention time is the same as FTC
Zidovudine	4.3	Peak was well resolved and symmetrical	
Nevirapine	6.9	Peak was well resolved but peak fronting seen	

The peaks for ZD and NVP were acceptable in terms of retention time, peak resolution and selectivity, and due to the proximity of the ZD peak to that of FTC, ZD was selected for use as an IS. The wavelength of maximum absorption of ZD was established (Figure 2.8) as 266 nm after scanning absorbance from 210n-400 nm, which is close to the wavelength of detection used for this RP-HPLC method and it would be readily detected at 260 nm.

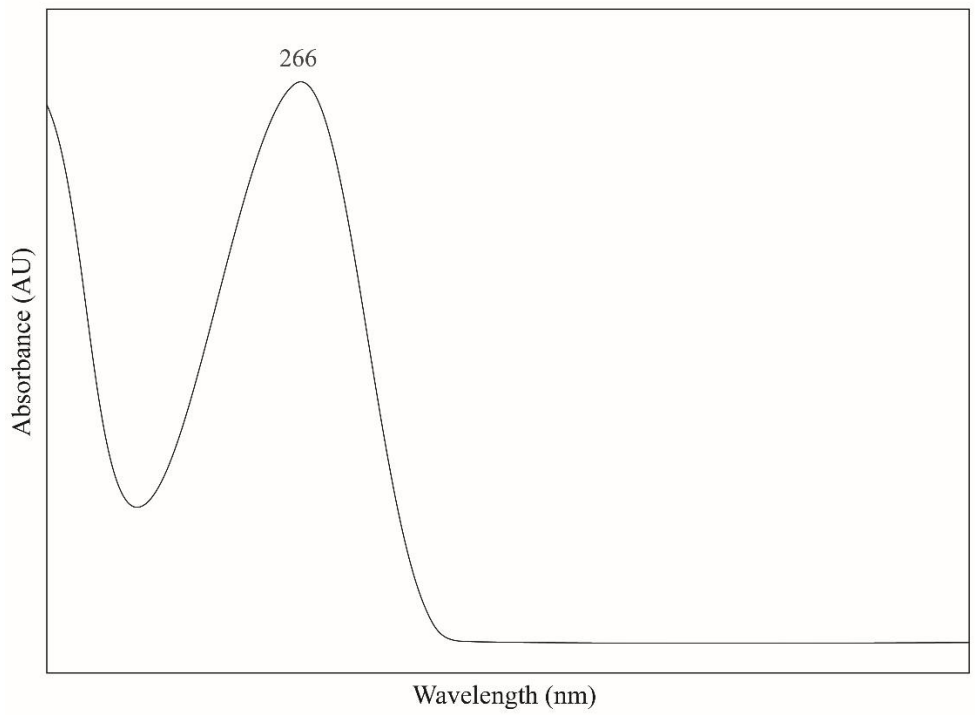


Figure 2.8 *Ultraviolet absorption spectrum of zidovudine (100 µg/ml, 60:40 v/v buffer-acetonitrile)*

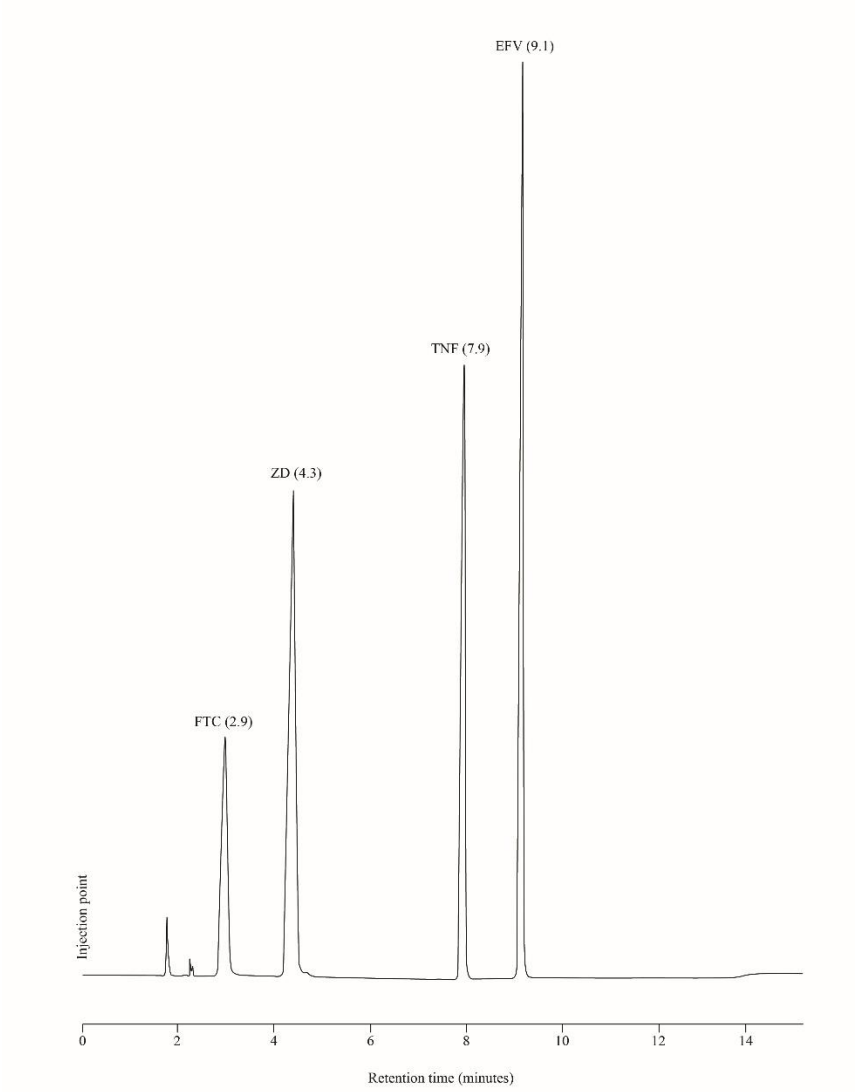


Figure 2.9 *Typical chromatogram showing separation and retention times of FTC (2.9 min), ZD (4.3 min), TNF (7.9 min) and EFV (9.1 min) using a gradient method*

A typical chromatogram of the separation of FTC, ZD, TNF and EFV is depicted in Figure 2.9, and the gradient steepness and slope are included in Figure 2.10. The red line represents the proportion of buffer in the mobile phase and the blue line the amount of acetonitrile in the mobile phase and it is clear that TNF and EFV elute when the mobile phase content has the highest proportion of acetonitrile.

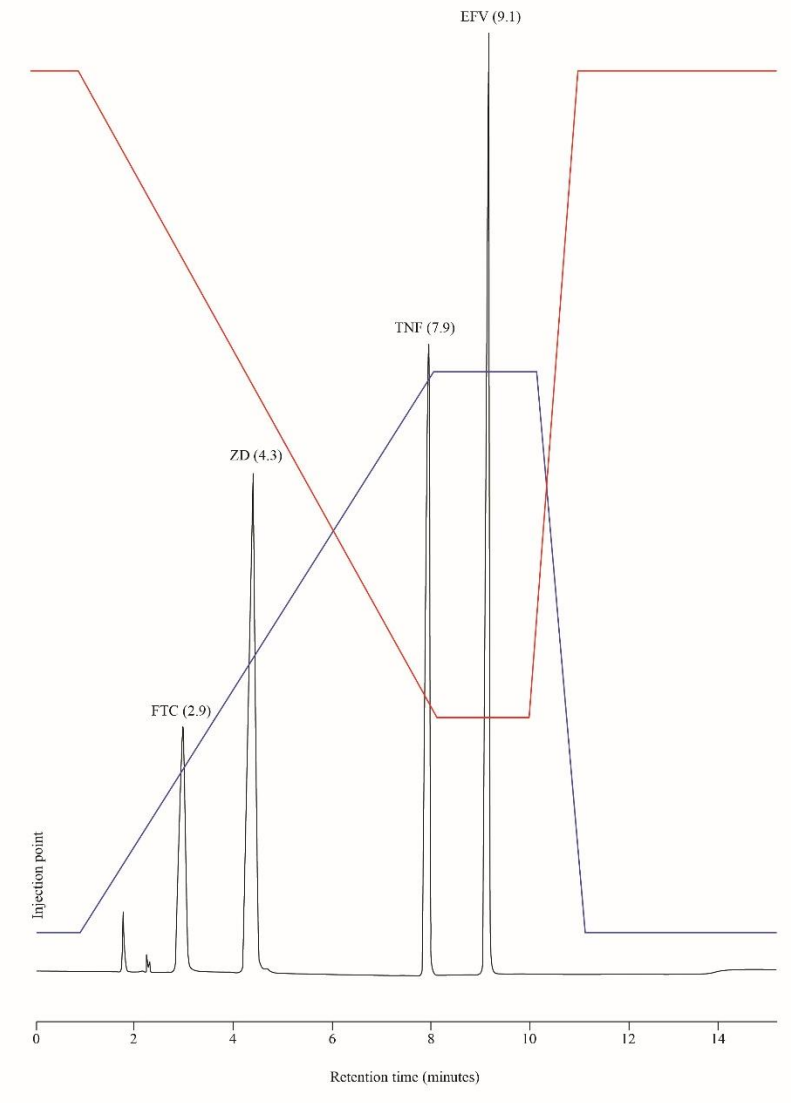


Figure 2.10 *Graphic representation of gradient slope and gradient steepness for the separation of FTC, ZD, TNF and EFV*

2.6 CONCLUSIONS

The possibility of developing an isocratic separation for the analysis of FTC, TNF and EFV was initially investigated, as isocratic separations are relatively simple to develop and operate because the mobile phase composition remains constant throughout the analysis and the separation power of the mobile phase therefore remains constant throughout the chromatographic run. As a result isocratic separations tend to be predictable and reproducible with the added advantage that limited strain is placed on the analytical column. Isocratic separations however have limited application when the analytes differ widely in physico-chemical properties, for example polarity, and these may translate into unnecessarily long run times and result in wastage of expensive solvents. In addition isocratic separation methods tend to have a low peak capacity, i.e. the longer compounds partition and remain in the stationary phase the broader and flatter will be the resultant peak, thereby contributing to a loss

of sensitivity. In contrast for peaks that predominantly partition into the mobile phase and that travel at more or less the same velocity as the mobile phase the peaks will be sharp and narrow.

The polarities of the compounds of interest vary, and FTC is extremely polar, basic and hydrophilic with pKa and log P values of 2.65 and -0.43, respectively. TNF is a basic polar hydrophilic molecule with pKa and log P values of 3.75 and 1.25, respectively. EFV is the least polar of the three and is weakly acidic and hydrophobic, with pKa and log P values of 10.2 and 5.4, respectively.

The impact of type of organic solvent, buffer characteristics, mobile phase composition and flow rate were investigated during attempts to develop an isocratic separation for FTC, EFV and TNF. The use of acetonitrile resulted in a chromatographic separation in which the analytes exhibited a good peak shape and was therefore preferred to methanol as it also produces less viscous mixtures with water than methanol does. A buffer was required as all three compounds are ionisable and the use of a buffer ensures that peak shape is maintained. For basic molecules such as FTC and TNF to be in the unionised state the pH of the buffer should be two or more pH units higher than the pKa of the molecules. Therefore FTC and TNF would be largely unionised in the pH range in which the stability of the stationary phase is maintained. However an acidic molecule such as EFV would be unionised at two or more pH units below its pKa and therefore EFV would be unionised at all pH values corresponding to that at which the stability of the stationary phase is not compromised and that would be used for an isocratic separation.

The concentration of buffer and pH used did not appear to have a significant effect on the retention times of FTC, TNF and EFV resulting in the use of an intermediate concentration of 0.03 M and a pH of 4 for the buffer to maintain conditions for the separation that would promote reproducibility of that separation. The composition of the mobile phase was then investigated and it was established that the retention time and capacity factor of FTC increased from 1.97 to 3.0 min and 0.12736 to 0.71792, respectively when the amount of buffer in the mobile phase increased. However the efficiency of the separation decreased and the plate number dropped from 7268 plates to 2992 plates, but the peak shape improved. The retention time of TNF and EFV suggest that these compounds are more likely to be affected by a change in mobile phase composition than FTC indeed the retention times of TNF and EFV increased when the amount of buffer in the mobile phase increased, as the analytes tend to partition into the stationary phase for a longer time under these conditions. Neither TNF nor EFV eluted in under an hour when the mobile phase was comprised of 95 % v/v buffer, and as these conditions produced the best retention time for FTC with an associated adequate efficiency and good peak shape the development of an isocratic separation for all three compounds would be a challenge. The effect of flow rate was also investigated, and as expected the retention time for FTC increased when the flow rate was changed from 1 ml/min to 0.8 ml/min but the void time also increased and FTC eluted close to the void. In addition column efficiency was lost, and despite remaining > 2000 it

was concluded that changing the flow rate was not efficient as a way of manipulating the retention times of these compounds.

Given that the compounds of interest are very different from a physico-chemical perspective a gradient separation was then investigated. In a gradient separation the mobile phase composition is altered during a chromatographic run by increasing the concentration of the organic solvent in the mobile phase. Gradient separation can be used to overcome some of the limitations usually observed with isocratic separations due to a significantly enhanced separation power of the mobile phase and enhanced peak capacity and resolution. In a gradient separation when the analytes in a mobile phase with a high aqueous content are injected onto the analytical column the analytes concentrate at the head of the column and will partition out of the stationary phase and into the mobile phase as the strength of the mobile phase increases. Analytes elute in the order of their polarity, whereby the most polar analytes will travel at more or less the same velocity as the mobile phase and will elute first. Consequently less polar analytes will migrate more slowly than the mobile phase and will elute later.

Initially gradient conditions were set at 95 % v/v buffer, and maintaining a 95 % v/v buffer content for one min resulted in a retention time of 2.9 min for FTC. Adequate gradient steepness was achieved when the amount of acetonitrile in the mobile phase was increased from 5 % v/v to 70 % v/v and the resolution between TNF and EFV was better when a steep gradient was applied. Adequate gradient slope was achieved by increasing the amount of acetonitrile linearly from one to eight min during the separation. The maximum acetonitrile of 70 % v/v was maintained for a further two min before decreasing the content to 5 % v/v. A 15 min run time was required for the column to re-equilibrate prior to introduction of the next sample. This approach enhanced the reproducibility of the separation and any shorter run times appeared to affect the reproducibility of retention of FTC. The retention times of FTC, TNF and EFV under these conditions were 2.9 min, 7.9 min and 9.1 min and the retention factors were 0.7, 3.5 and 4.2. A more efficient separation with a better peak shape for TNF and EFV was achieved under gradient conditions than when an isocratic method of analysis was used. ZD was selected as an internal standard due to a relatively short retention time close to that observed for FTC.

As one of the objectives was to attempt to develop a universal method of analysis for FTC, TNF and EFV there may well be problems when using this method of analysis for monitoring concentrations of FTC in biological fluids due to the presence of interfering peaks that may affect quantitation of FTC. However the use of an IS such as ZD may resolve some of the difficulties and therefore ZD was used for all future analyses.

An isocratic RP-HPLC method for which a mobile phase of 70:30 v/v buffer-acetonitrile was developed and evaluated, however the method was not suitable for the analysis of FTC, TNF and EFV simultaneously and a gradient RP-HPLC method was therefore developed and used for the analysis of

FTC, TNF and EFV as the separation was adequate and the run time reasonable. The RP-HPLC method was optimised using system suitability testing as a guide to assess the effect of manipulation of buffer concentration and pH, mobile phase composition and flow rate on the separation. The method that has been developed demonstrates suitable retention factors, selectivity, peak shape and column efficiency and is suitable for validation and application for the *in vitro* analysis of FTC, TNF and EFV in dosage forms.

CHAPTER THREE
GRADIENT METHOD VALIDATION AND FORCED DEGRADATION STUDIES

3.1 INTRODUCTION

3.1.1 Overview

Following method development and system suitability testing, analytical methods must be validated. Validation of an analytical method is undertaken to guarantee that the method is reliable and will produce accurate and precise results. Analytical method validation must also demonstrate that the method is fit and appropriate for its intended purpose [63, 186, 201]. Within the pharmaceutical industry, analytical methods are used in drug development processes, drug characterisation and manufacture and must therefore be validated to ensure that appropriate decisions are made on the basis of sound data [201, 202]. The ICH has produced guidelines pertaining to method validation and the ICH Q2 (R1) guideline document [203] is considered the primary guideline for analytical method validation [204] and is referred to by the Food and Drug Administration (FDA) and United States Pharmacopoeia (USP) [205]. The ICH Q2 (R1) guideline includes terminology and provides a framework for validation parameters and criteria for accepting validation data [189, 202]. The intended purpose of an analytical method will determine the exact validation parameters that are to be applied and the criteria for accepting those validation data [201]. The most frequently used parameters for analytical method validation include linearity, range, accuracy, precision (repeatability and intermediate precision), robustness and limits of detection and quantitation [63, 186, 189, 201].

3.2 VALIDATION

3.2.1 Linearity and range

The linearity of an analytical method is measured in a specified range and refers to the response of the detector to the concentration of the analyte in a specific sample matrix. The linearity of a method is related to the directly proportional response to analyte concentration when the analytical method is applied to samples of known dilute concentration [63, 189, 203]. The linearity of a method is particularly important when UV detection is used, as the concentration of analyte must elicit a response that obeys the Beer-Lambert law [186]. The linearity of the method was investigated over a range of analyte concentrations. The ICH guidelines recommend that a minimum of five concentrations be used [203]. The response, in this case the peak area ratio (PAR) of the relevant ARV to that of the IS, ZD, was plotted against analyte concentration. The correlation coefficient and least squares linear regression are statistical approaches used to evaluate linearity, and a correlation coefficient > 0.999 indicates a satisfactory fit of the best fit regression line to the experimental. The y-intercept should also be ≤ 2.0 % of the detector response represented as the average PAR [189, 206].

The range refers to the range between the lower and upper concentrations of analytes in the samples under investigation and within which the method should demonstrate satisfactory linearity, accuracy and precision [203].

A typical calibration curve for FTC is depicted in Figure 3.1. The equation of the best-fit least squares linear regression line for FTC over the range of 0.4–40 µg/ml was y = 0.0191x+0.0007. The correlation coefficient (R^2) was 1, indicating that the method was linear over the range investigated for FTC. The y-intercept was 0.0007 and is ≤ 2.0 % of the detector response, indicating that the method has suitable accuracy within the range investigated. Standard deviations are included but are too small to be seen as the method is precise.

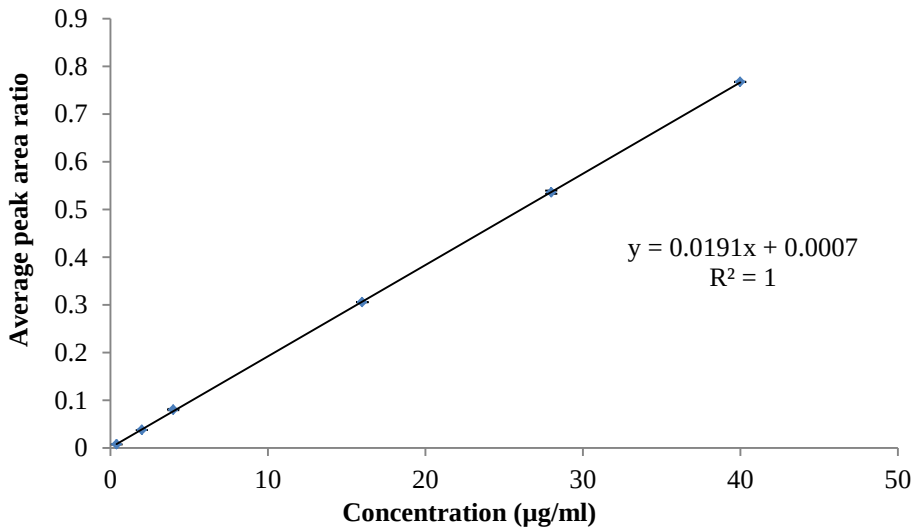


Figure 3.1 *Typical calibration curve for FTC over the concentration range 0.4-40 µg/ml*

A typical calibration curve for TNF is depicted in Figure 3.2. The equation for the best-fit least squares regression line for TNF over the range 0.6–60 µg/ml was y = 0.0163x+0.0116. The correlation coefficient (R^2) was 1, indicating that the method was linear over the concentration range of TNF investigated. The y-intercept was 0.0116, which is ≤ 2.0 % of the detector response, indicating that the method has suitable accuracy within the range of concentrations investigated. Standard deviations are included but are too small to be seen as the method is precise.

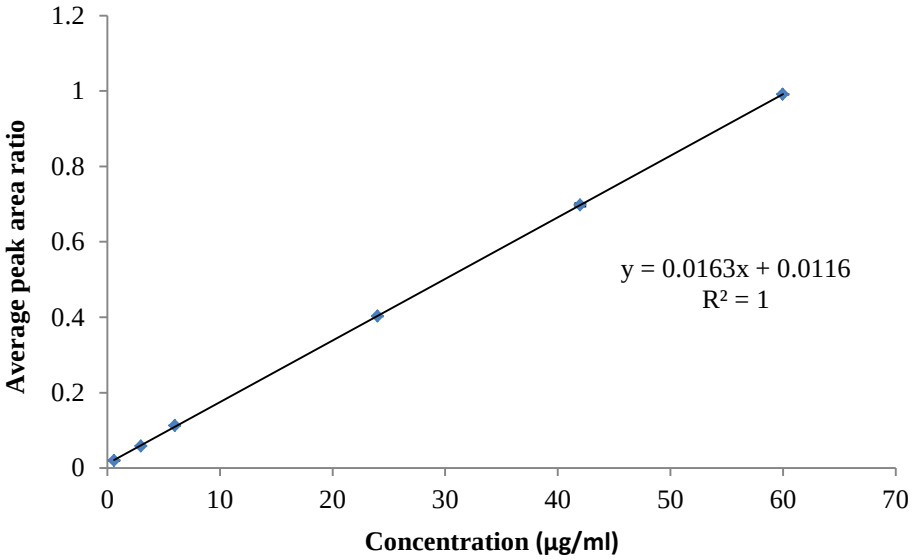


Figure 3.2 *Typical calibration curve for TNF over the concentration range 0.6-60 µg/ml*

A typical calibration curve of EFV is depicted in Figure 3.3. The equation of the best-fit least squares regression line for EFV over the range of 1.2–120 µg/ml was y = 0.01x+0.016. The correlation coefficient (R^2) of 0.9999 indicates that the method was linear over the concentration range of EFV investigated. The y-intercept was 0.016, which is ≤ 2.0 % of the detector response, indicating that the method has suitable accuracy within the range of concentrations investigated. Standard deviations are included but are too small to be seen as the method is precise.

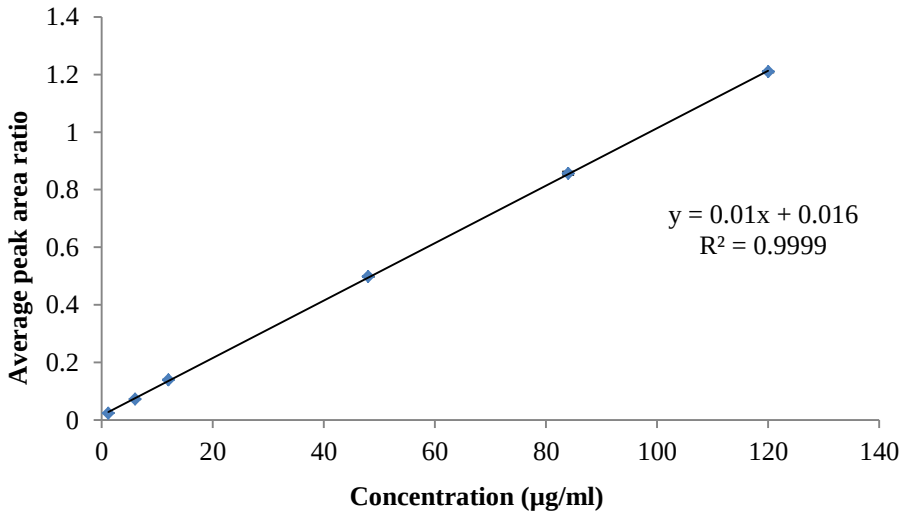


Figure 3.3 *Typical calibration curve for EFV over the concentration range 1.2-120 µg/ml*

3.2.2 Precision

The precision of an analytical method is a measure of the degree of repeatability, reliability and reproducibility of that method when applied to the same sample(s) under specified conditions [203]. The intra-assay precision (repeatability), intermediate precision (inter-day precision) and reproducibility are different levels of precision and following use of the analytical method, the extent of precision can be determined by calculating the standard deviation or the percent relative standard deviation (% RSD) of the mean of individual test results in separate samples within a series of data [63, 201]. The standard deviation is an indication of the distribution of individual results about the mean in a dataset [206]. The % RSD should be ≤ 2 % for an analytical method to be considered precise [207].

3.2.2.1 Intra-assay precision (repeatability)

Intra-assay precision is an indication of repeatability of a method and is established by replicate analysis of the same analytical sample when performed under specified conditions by the same analyst over a short period of time using the same analytical equipment [189, 203]. The ICH guidelines recommend that as a minimum requirement repeatability be assessed at three concentrations in triplicate, for a total of nine determinations [63, 203]. The three concentrations must fall within the range of the analytical method and must represent low, medium and high concentrations that may be observed during analysis of samples of unknown concentration. Intra-assay precision data for FTC, TNF and EFV are summarised in Table 3.1. Three concentrations of FTC *viz.*, 6 µg/ml, 20 µg/ml and 32 µg/ml, of TNF *viz.*, 9 µg/ml, 30 µg/ml and 48 µg/ml and of EFV *viz.*, 18 µg/ml, 60 µg/ml and 96 µg/ml were injected (n=5) using ZD as the IS. The PAR of each ARV to ZD was established for each concentration.

Table 3.1 *Intra-assay precision for FTC, TNF and EFV*

Theoretical concentration µg/ml	**Actual concentration µg/ml**	**Average PAR (FTC/ZD) *n*=5**	**Standard deviation**	**% RSD**
FTC				
6	5.90	0.113356	0.000126	0.11
20	20.1	0.384376	0.000179	0.05
32	31.5	0.601454	0.000778	0.13
TNF				
9	8.85	0.155936	0.000103	0.07
30	30.2	0.503907	0.000295	0.06
48	47.2	0.780973	0.000203	0.03
EFV				
18	17.7	0.192923	0.000217	0.11
60	60.5	0.620758	0.000525	0.09
96	94.1	0.957069	0.000693	0.07

The intra-assay precision was assessed by calculating the % RSD for the replicate analysis of each ARV at all concentrations within the respective concentration ranges. It is clear from Table 3.1 that

the % RSD for all analysis was ≤ 2 % limit set in our laboratory, and the analytical method therefore exhibits suitable repeatability for each ARV.

The results of intra-assay precision analysis of data generated for the calibration curves investigated (n=5) for FTC, TNF and EFV are summarised in Table 3.2.

Table 3.2 *Intra-assay precision for FTC (0.4-40 µg/ml), TNF (0.6-60 µg/ml) and EFV (1.2-120 µg/ml)*

Theoretical concentration µg/ml	**Average PAR (FTC/ZD) *n*=5**	**Standard deviation**	**% RSD**
FTC			
0.4	0.007569	0.000115	1.52
2	0.037758	0.000115	0.31
4	0.080548	0.001159	1.44
16	0.305893	0.000247	0.08
28	0.536042	0.003421	0.64
40	0.767287	0.000599	0.08
TNF			
1.2	0.020303	0.00025	1.23
6	0.058839	4.4E-05	0.07
12	0.113115	0.000223	0.20
48	0.402944	0.000406	0.10
84	0.697969	0.004517	0.65
120	0.991301	0.000606	0.06
EFV			
1.2	0.024489	0.000263	1.07
6	0.072726	0.000151	0.21
12	0.139795	0.000142	0.10
48	0.498216	0.000781	0.16
84	0.857161	0.005468	0.64
120	1.209793	0.002118	0.18

3.2.2.2 Inter-day precision (intermediate precision)

Inter-day precision is an indication of the extent of the precision of a method when the analytical method is used under different conditions *viz.*, different days, different analysts or different equipment yet applied within the same laboratory [203]. The assessment of inter-day precision gives an indication of the influence of different conditions and settings on the performance of an analytical method. The inter-day precision was assessed by using the analytical method over three days. Three concentrations (low, medium and high) for each ARV were analysed (n=3) on three consecutive days using the same analytical equipment. The results of inter-day precision for FTC, TNF and EFV are summarised in Table 3.3.

Table 3.3 *Inter-day precision for FTC, TNF and EFV*

	Theoretical concentration µg/ml	Actual concentration µg/ml	Standard Deviation	% RSD
FTC				
Day 1	6	5.90	0.000126	0.11
	20	20.1	0.000179	0.05
	32	31.5	0.000778	0.13
Day 2	6	5.99	0.000318	0.30
	20	20.1	0.000193	0.05
	32	32.7	0.000221	0.04
Day 3	6	6.01	0.000141	0.13
	20	19.5	0.000113	0.03
	32	32.6	0.007628	1.31
TNF				
Day 1	9	8.85	0.000103	0.07
	30	30.2	0.000295	0.06
	48	47.2	0.000203	0.03
Day 2	9	9.02	0.000332	0.24
	30	30.3	0.00025	0.05
	48	49.3	0.000181	0.02
Day 3	9	8.91	8.21E-05	0.05
	30	29.2	0.00031	0.06
	48	48.7	0.010776	1.32
EFV				
Day 1	18	17.7	0.000217	0.11
	60	60.5	0.000525	0.08
	96	94.1	0.000693	0.07
Day 2	18	18.1	0.000211	0.11
	60	60.8	0.000521	0.08
	96	98.5	0.000308	0.03
Day 3	18	18.1	0.001138	0.55
	60	58.9	0.000222	0.04
	96	97.7	0.012357	1.21

The % RSD for all ARV samples of all concentration on all days of analysis was ≤ 2 %, indicating that the analytical method demonstrates suitable intermediate precision for each ARV as defined in our laboratory.

3.2.2.3 Reproducibility

Reproducibility is the precision of an analytical method when transferred and used in another laboratory [203], and reproducibility is not required if intermediate precision has been demonstrated [207]. Furthermore, as the analytical method was to be used in only one laboratory by one analyst it was not deemed necessary to undertake reproducibility studies for this method of analysis.

3.2.3 Accuracy

Accuracy is a measure of the closeness of an unknown test concentration or amount to an accepted reference concentration or amount within a specified range. Concentrations of an analyte in solutions of unknown concentration are determined experimentally using the analytical method undergoing validation, after which the accuracy of the method is calculated and expressed as % recovery and/or %

RSD [63, 189, 203]. Assessing accuracy in the absence of precision for the method of analysis is meaningless [206]. To assess accuracy the concentration of samples should be at 80, 100 and 120 % of the API content or label claim as stated on the commercial package of a dosage form [189, 203]. Accuracy was assessed using commercially available products. Twenty Atripla® (MSD Pty Ltd, Johannesburg, South Africa) tablets containing 600 mg EFV, 300 mg TDF and 200 mg FTC were weighed and the average weight of one tablet calculated. The tablets were powdered aliquots of powder equivalent to 80, 100 and 120 % of one tablet weight were accurately weighed and dissolved in approximately 70 ml of 60:40 % v/v HPLC-grade water and acetonitrile in a 100 ml A-grade volumetric flask. Each solution was sonicated for 30 min before making up to volume with 60:40 % v/v solution of HPLC-grade water and acetonitrile. After sonication the solutions were well mixed by shaking and a small volume was removed and centrifuged (Damon IEC HN-SII Centrifuge, Needham Heights, USA) for 20 min at 1200 rpm using Kimax tubes (Kimble, Vineland, USA) to separate insoluble excipients. The supernatant was then used to make working stock solutions in 100 ml A-grade volumetric flasks. Accuracy was established by calculating the % recovery and % RSD. The results of accuracy studies for FTC, TNF and EFV are summarised in Table 3.4. The analytical method was considered to be accurate if the precision of the analysis for the samples under investigation was < 2 %.

Table 3.4 *Accuracy data for FTC, TNF and EFV*

% of label claim	Theoretical concentration µg/ml	Measured concentration µg/ml	% Recovery	Standard deviation (*n*=5)	%RSD
FTC					
80	16	13.5	84	0.000392	0.18
100	20	16.8	84	0.001294	0.50
120	24	19.95	83	0.001671	0.53
TNF					
80	24	19.07	79.5	9.62E-05	0.03
100	30	23.9	79.8	0.00183	0.48
120	36	28.44	79.0	0.001964	0.44
EFV					
80	48	42.3	88	0.000433	0.11
100	60	52.7	87.9	0.002442	0.50
120	72	62.1	86.2	0.003442	0.60

Whilst it is desirable to achieve 100 % recovery, the recovery need not be 100 % if the method is precise or the % recoveries are the same each time the assay is performed under the specified conditions. In this case the % RSD was < 2 % for each ARV at each concentration level investigated, thereby indicating that the method is suitable for use.

3.2.4 Limits of quantitation (LOQ) and detection (LOD)

The lower limit of quantitation (LLOQ) is defined as the lowest concentration of an analyte that can be accurately and precisely determined using the analytical method that has been developed [203].

The limit of detection (LOD) is defined as the lowest amount of analyte that can be detected but not necessarily quantitated [189]. The LLOQ and LOD are usually determined for the analysis of related substances, impurities and degradation products [189, 203].

Signal to noise (s/n) ratios are used to establish the LOD and LLOQ, and ratios of 3:1 or 2:1 and 10:1 are used, respectively [63, 189]. The s/n ratio is calculated by dividing the height of a signal or peak by the range of the baseline noise observed for the analytical method. The LLOQ is a compromise between the lowest concentration detected or the sensitivity and acceptable precision and accuracy for the method. Use of the s/n ratio approach is a good rule of thumb for establishing these parameters but this is not the only method recognised in the ICH guidelines for establishing this parameter [207]. The LLOQ and LOD can be established by calculating the % RSD and LLOQ should have a % RSD of ≤ 5 % and the LOD by convention 0.3 × LLOQ [208]. Alternate methods of establishing the LLOQ and LOD are described mathematically in Equations 3.1 and 3.2 [207].

$$\text{LLOQ} = \frac{10\ SD}{S} \qquad \textit{Equation 3.1}$$

$$\text{LOD} = \frac{3.3\ SD}{S} \qquad \textit{Equation 3.2}$$

Where,

SD = the standard deviation of the response
S = the slope of the calibration curve

3.2.4.1 LLOQ

The LLOQ can be determined following sequential injection of samples prepared from a standard stock solution with decreasing concentration(s) of each analyte of interest. The lowest concentration with a % RSD ≤ 5 % was used to set the LLOQ for FTC, TNF and EFV. The LLOQ for FTC, TNF and EFV were 0.4, 0.3 and 0.6 µg/ml with average PAR of 0.004, 0.008 and 0.04, respectively. The % RSD for FTC, TNF and EFV at LLOQ were 2.38, 1.53 and 0.288 %. Since the stock solution was a composite of all ARV a compromise of the lowest concentration of each ARV was used and the concentration of FTC that yielded the most suitable accuracy and precision was the limit set for this parameter as the wavelength selected for analysis was not the λ_{max} for FTC. The LLOQ of 0.4 µg/ml for FTC was therefore used, as a lower concentration of 0.32 µg/ml was not detected at all and consequently the LLOQ for TNF and EFV were 0.6 and 1.2 µg/ml, respectively.

3.2.4.2 LOD

An approximate value for the LOD for FTC, TNF and EFV was established by calculation and by convention was the product of the LLOQ and 0.3. The LOD for FTC, TNF and EFV were 0.12, 0.18 and 0.36 µg/ml, respectively.

3.3 FORCED DEGRADATION STUDIES

3.3.1 Overview

The FDA and ICH Q1A (R2) guideline provides a framework for assessing the intrinsic stability of drug substances and/or new formulations when exposed to stress conditions for specific periods of time [209, 210]. Stability studies are required as part of the registration process of new and generic drug substances and products. Long-term studies are typically performed over a 12–36 month period, whereas accelerated studies are usually performed over a 6 month period. Degradation products and degradation pathways of new compounds may be identified, more stable formulations may be developed and the shelf-life and appropriate packaging for products can be identified and tested with data generated from appropriate studies [211]. Forced degradation stress conditions are harsher than accelerated conditions and such studies are performed to establish the stability-indicating nature or specificity of a validated analytical method. In this case the purpose of these studies was not to quantitate degradation products but rather to ensure that any degradation products, if present, did not interfere with the quantitation of the analytes of interest [207, 210, 211]. Typically for forced degradation studies the compounds of interest or product are exposed hydrolytic, oxidative, thermolytic and photolytic conditions [209, 212]. Acid and alkali hydrolysis are common reactions in which ionisable functional groups are hydrolysed to form primary degradation products [213]. Hydrochloric acid and sodium hydroxide are commonly used as reagents to prepare acidic and alkaline solutions and the concentration range of these materials that are used is usually between 0.1 and 1.0 M [211]. Solutions of EFV, FTC and TNF and anhydrous EFV, FTC and TDF were exposed to different stress conditions and materials were then analysed using the RP-HPLC method undergoing validation. Chromatograms from individual experiments were overlaid to establish whether interference would occur when all compounds were tested in FDC products following dissolution or when conducting analytical testing simultaneously.

3.3.2 Experimental

3.3.2.1 Acid, alkali and oxidative degradation

Acid, alkali and oxidative degradation studies were performed using the same experimental approach. Three stock solutions (1 mg/ml) of FTC, TNF and EFV were prepared by accurately weighing 10 mg of each analyte and dissolving in 10 ml of a 60:40 % v/v solution of HPLC-grade water and acetonitrile. 0.1 M HCl, 0.1M NaOH and 3 % v/v H_2O_2 solutions were prepared and added individually in a 1:1 ratio to the three stock solutions in separate 25 ml round-bottom flasks. The solutions were refluxed at 70 °C for 3 hours. A 1 ml sample of each solution was removed from each round-bottom flask and transferred to a 10 ml A-grade volumetric flask prior to dilution with a 60:40 % v/v solution of HPLC-grade water and acetonitrile prior to analysis using HPLC (n=3).

3.3.2.1.1 Acid degradation

When the EFV solution was mixed with 0.1 M HCl at room temperature (22 °C) a precipitate formed, which subsequently disappeared after refluxing at 70 °C. A chromatogram of FTC, TNF and EFV prior to degradation is depicted in Figure 3.4 A and can be used to compare the chromatogram observed following acid degradation of FTC, TNF and EFV (Figure 3.4 B). It is clear that significant degradation of TNF occurs, as shown by the presence of two degradation products, *viz*. TA and TB. The peak areas of FTC and EFV appear to have increased but no degradation products had been formed when the data were analysed.

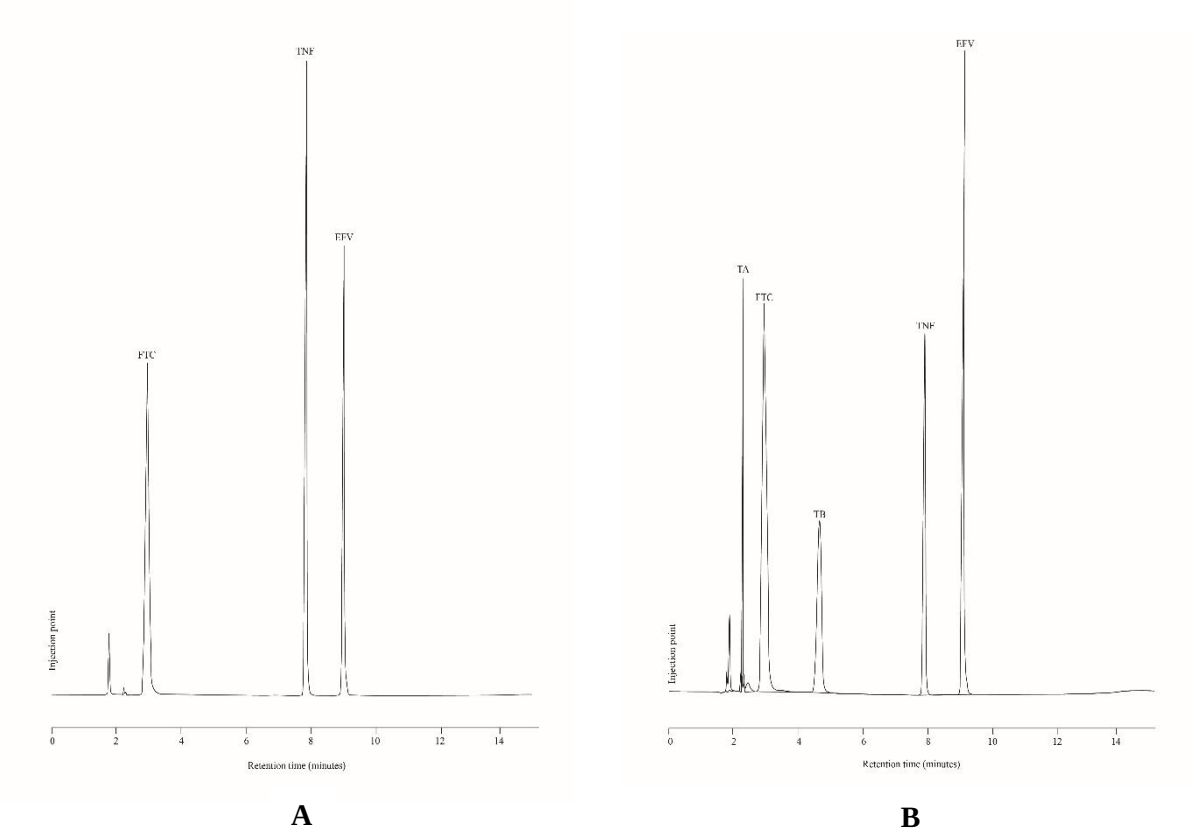


Figure 3.4 *Chromatogram of FTC, TNF and EFV (100 µg/ml) (A) and overlaid chromatograms (B) showing FTC, TNF and EFV following exposure to 0.1 M HCl and refluxing at 70 °C for 3 hours*

3.3.2.1.2 Alkali degradation

A chromatogram developed following the analysis of a solution of FTC, TNF and EFV prior to degradation is depicted in Figure 3.5 A and can be used to compare the chromatograms developed for samples analysed following exposure to alkali degradation conditions that are depicted in Figure 3.5 B. EFV precipitated from solution when it was mixed with the 0.1 M alkali solution and the precipitate disappeared on refluxing at 70 °C for 3 hours. All analytes degraded under the harsh alkali conditions and TNF completely degraded to form a new degradation product (TC). FTC and EFV also degraded to a significant extent and degradation products FA and FB were formed from FTC. EFV

produced a split peak which could indicate the presence of a degradation product however it was not adequately separated nor was it isolated.

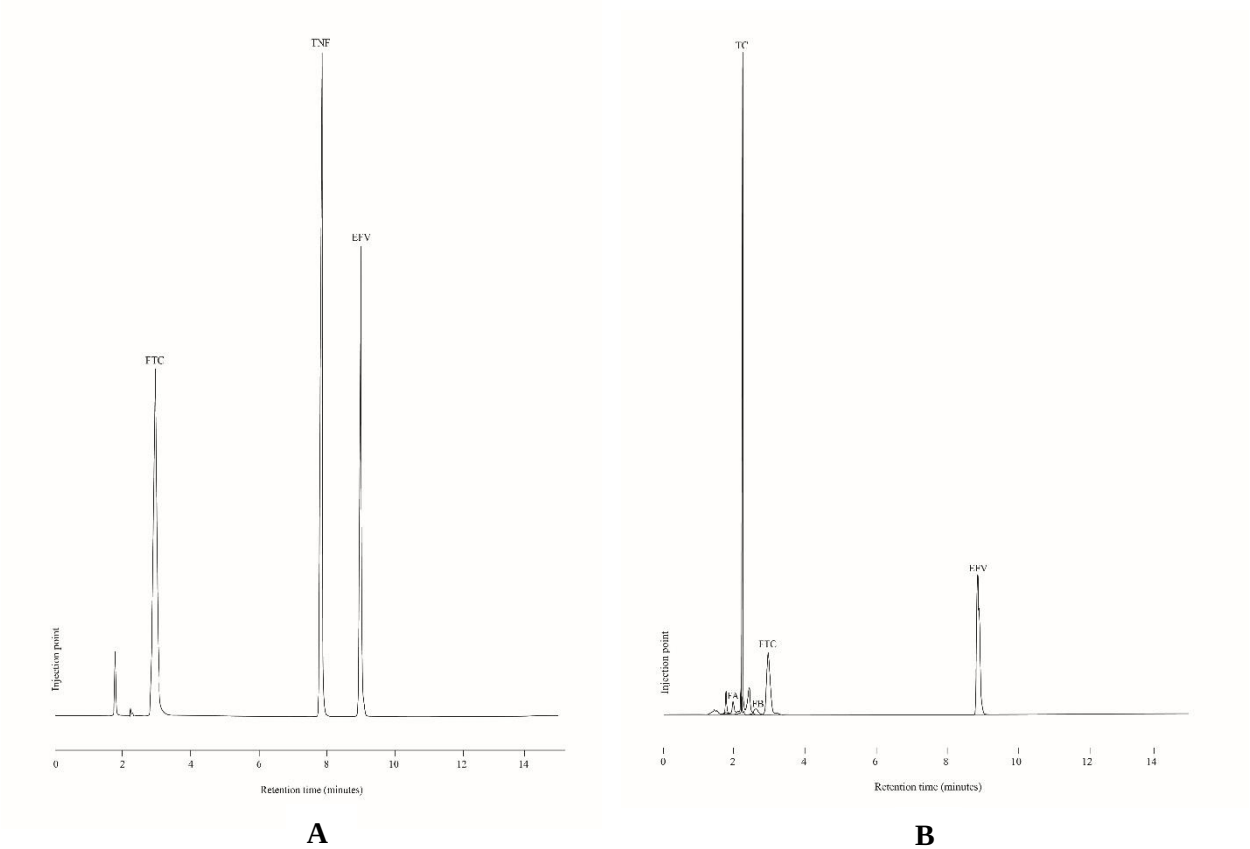


Figure 3.5 *Chromatogram of FTC, TNF and EFV (100 µg/ml) (A) and overlaid chromatograms (B) showing FTC, TNF and EFV following exposure to 0.1 M NaOH and refluxing at 70 °C for 3 hours*

3.3.2.1.3 Oxidative degradation

A chromatogram developed following simultaneous analysis of FTC, TNF and EFV is depicted in Figure 3.6 A and the chromatograms following oxidative degradation of FTC, TNF and EFV are depicted in Figure 3.6 B and can be compared to Figure 3.6 A. FTC was completely oxidised to form a degradation product (FC), whereas TNF a degraded only slightly to form products TD and TE and EFV to form product EA.

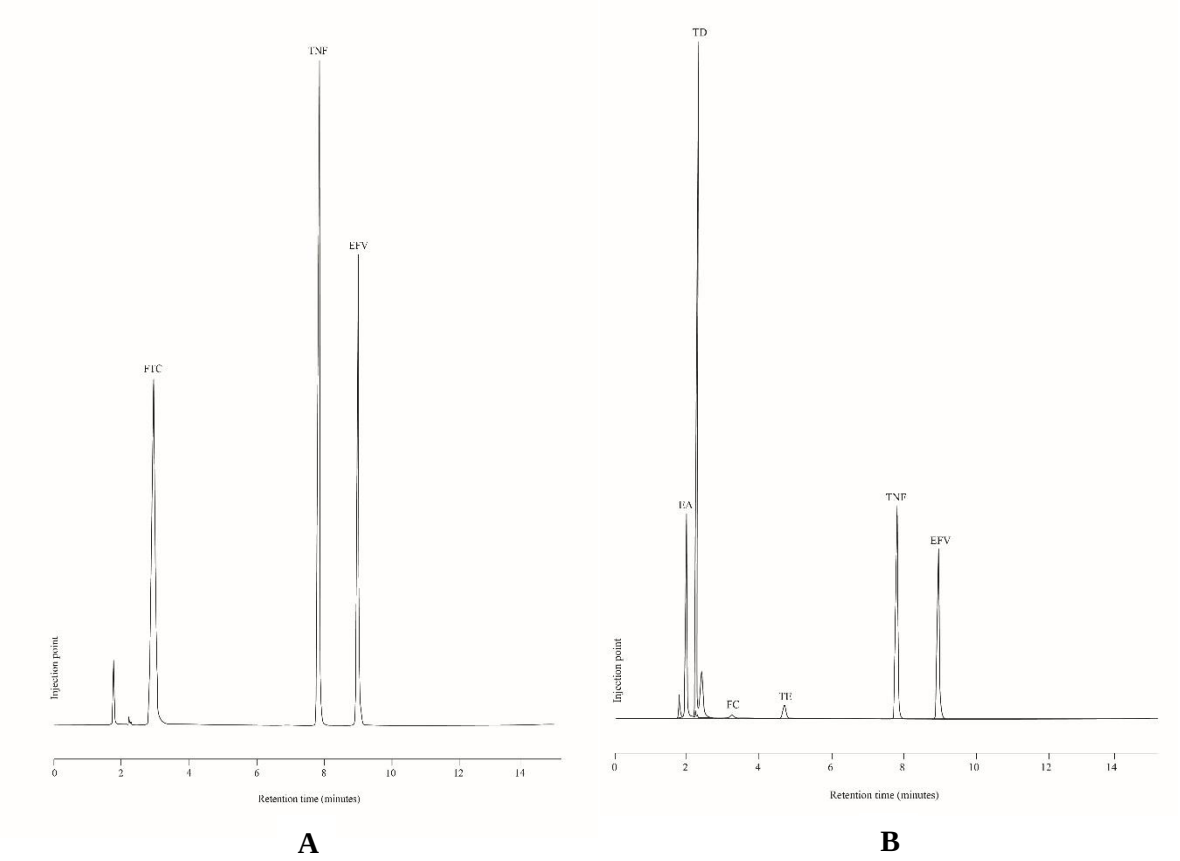


Figure 3.6 *Chromatogram of FTC, TNF and EFV (100 µg/ml) (A) and overlaid chromatograms (B) showing FTC, TNF and EFV following exposure to 3 % H_2O_2 and refluxing at 70 °C for 3 hours*

3.3.2.2 Dry heat

Dry heat studies were undertaken after accurately weighing 10 mg FTC, TDF and EFV onto three clean watch glasses and then placing the samples into an oven (TERM-O-MAT, LABOTEC, Johannesburg, South Africa) for 12 hours at 100 °C. After 12 hours each of the samples was removed and cooled to room temperature (22 °C) and then quantitatively transferred into one of three A-grade 100 ml volumetric flasks and dissolved in 60:40 % v/v HPLC-grade water and acetonitrile prior to HPLC analysis (n=3). Decolourisation of TDF was observed on visual inspection but was not observed for FTC and EFV. The chromatogram for a solution of FTC, TNF and EFV generated prior to exposure to heat is depicted in Figure 3.7 A. The resultant chromatogram following exposure of the compounds to dry heat is depicted in Figure 3.7 B in which degradation of TNF but not FTC and EFV is evident. TNF degraded significantly to produce degradation products TF, TG, TH, TI, TJ and TK. Product TK may affect the quantitation of EFV but it is unlikely that the product or API would be exposed to such harsh conditions during conventional use and handling and therefore this product is unlikely to interfere with analysis of a FDC product.

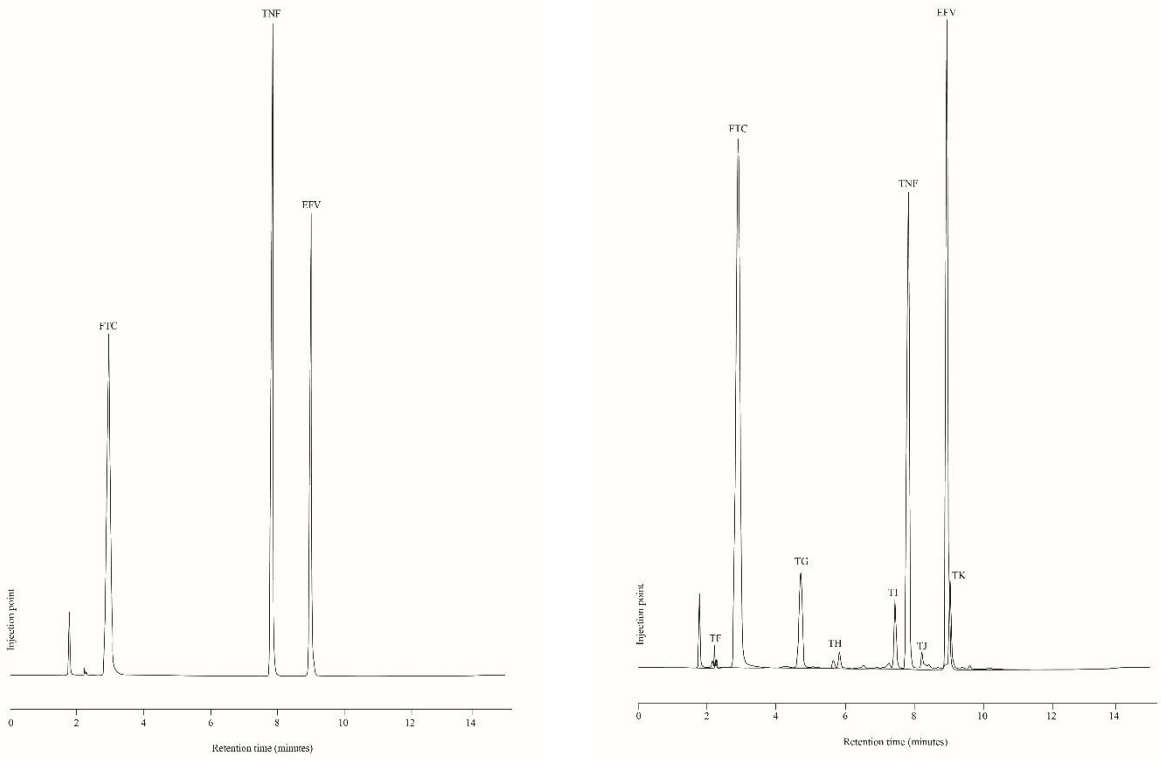


Figure 3.7 *Chromatogram of FTC, TNF and EFV (100 µg/ml) (A) and overlaid chromatograms (B) showing FTC, TNF and EFV following exposure to 100 °C dry heat for 12 hours*

3.3.2.3 Photodegradation

Photostability is evaluated by exposing API or drug product to UV or fluorescent light to establish the stability of the API or product to light. The minimum recommended exposure to UV light is 200 Wh/m^2 in a wavelength range of 300 to 800 nm to ensure photolysis, if the compound is susceptible to light [212]. Photostability studies were performed using an Atlas SUNTEST® CPS+chamber (Lisengericht, Germany) and the API were exposed to 500 Wh/m^2 using filter G and a temperature of 35 °C. After 12 hours exposure to 254 nm light the substances were removed and quantitatively transferred to each of three A-grade 100 ml volumetric flasks and dissolved in 60:40 % v/v HPLC-water and acetonitrile prior to analysis. The chromatogram for a solution of FTC, TNF and EFV generated prior to photodegradation is depicted in Figure 3.8 A. The resultant chromatogram following exposure of the compounds to UV light at 254 nm for 12 hours is depicted in Figure 3.8 B in which no degradation of TNF was seen but peak area for FTC and EFV decreased suggesting that FTC and EFV degraded.

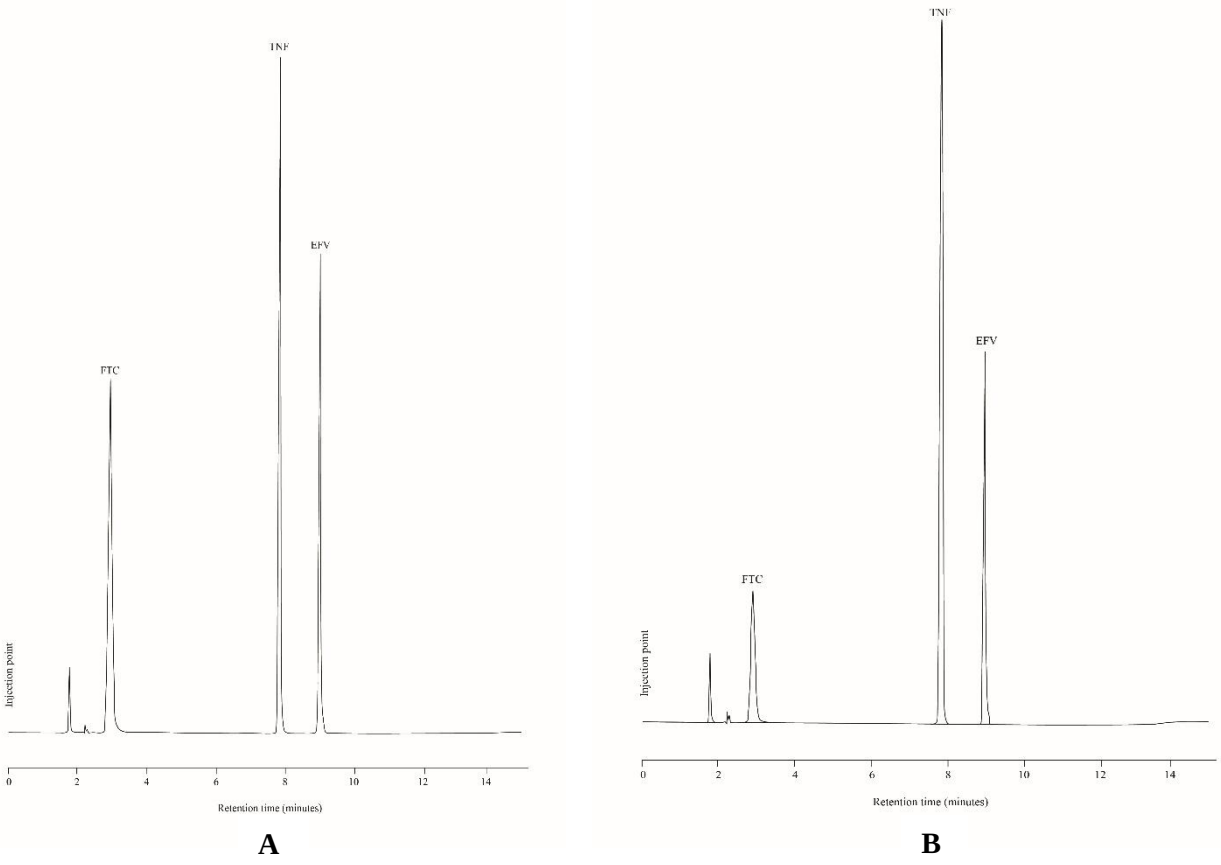


Figure 3.8 *Chromatogram of FTC, TNF and EFV (100 µg/ml) (A) and overlaid chromatograms (B) showing degradation of FTC, TNF and EFV following exposure to UV light at 245 nm for 12 hours*

3.4 BIOANALYTICAL METHOD DEVELOPMENT FOR ANALYSIS OF EFV, FTC AND TNF IN PLASMA

3.4.1 Overview

Bioequivalence studies involve the analysis of analytes in biological fluids such as plasma, serum or urine. Samples for analysis require preparation prior to analysis using a validated analytical method. Ideally sample preparation should be simple, selective, sensitive, rapid and cost-effective. Solid phase extraction (SPE) is an example of a sample preparation approach that offers such flexibility [214]. SPE is versatile with wide application and is used for the clean-up of sample matrices, isolation of analyte(s) of interest, trace enrichment and exchange of solvent medium [215, 216]. The principles of SPE are similar to those of LC, where the sorbent bed in SPE can be compared to the stationary phase in LC [216]. In SPE a sample matrix (aqueous) containing the analyte(s) is passed through the sorbent bed and the analytes then partition between the liquid phase and the sorbent bed [214, 216]. SPE can be applied using different sorbents and formats (i.e. how the sorbent is contained) to achieve the best sample preparation approach for a specific matrix or analytical method. The choice and format of sorbent is crucial for the accurate quantitation and high quality analysis of samples, and selection of the best-suited sorbent, depends on the intended application of the analytical method and is important as it dictates the selectivity, sensitivity and retention capacity of the sample clean-up method [214–217].

The most commonly used format used for SPE involves the use of cartridges. The cartridges are made of polypropylene or glass that contains the sorbent bed and are available in different sizes to accommodate different sample volumes. Frits are placed above and below the sorbent bed and are made of polyethylene, stainless steel or Teflon and other SPE formats include discs, pipette tips and 96-well plates for off-line analysis where the SPE method is separate from the LC method. Small volume columns are also available for on-line analysis where SPE may be an integral part of the LC method and system [214, 217].

The choice of sorbent and format depends on the intended application of the analytical method and selection of the best-suited sorbent and format is important for adequate selectivity, sensitivity and retention capacity in addition to accurate quantitative and qualitative determination of the analytes of interest [215–217]. Prior to selection of the sorbent one must take into account the physico-chemical properties of the analyte(s) of interest, the nature of the sample matrix from which the analytes are to be isolated and volume and the level of detection required. The primary interactions in SPE are the same as those reported for LC and includes hydrophobic, hydrogen bonding and ion-exchange interactions [217].

In one approach for SPE the analyte(s) are retained on the sorbent bed and interfering compounds are eluted in a wash step after which the analytes are subsequently eluted using a relatively high proportion of organic solvent in an elution step. Alternatively interfering compounds can be retained on the sorbent bed while the analytes are eluted either during loading or the wash step. Low retention capacities of the sorbent bed may result in analytes, particular polar analytes, being lost in breakthrough solvent volumes that are not retained on the SPE cartridge [214].

3.4.2 Silica–based sorbents

Silica and silica-bonded RP sorbents are the most commonly used sorbents, in particular C_{18} and C_8 for SPE applications. Bonded phase silica sorbents include C_1, C_2, CH, Ph, and CN backbones [214, 217] and organic solvents typically interact with the sorbent bed via hydrophobic interactions. The main disadvantage associated with these types of sorbent is the presence of residual silanol groups which negatively affect the reproducibility of extraction of polar analytes, in particular basic compounds, as they are retained through hydrophilic interactions [214, 217]. By altering the sorbent bed to pure silica, alumina, magnesium silicate or diatomaceous earth, normal phase principles can then be applied to the extraction of polar molecules. However hydrogen bonding sites are sometimes gradually damaged when using aqueous solvents, resulting in poor extraction reproducibility [214]. In addition recovery is also affected by the limited sorption capacity of some compound, analyte displacement or plugging of sorbent pores by components of the sample matrix. Silica-based sorbents are stable between pH 2 and 8 and non-end-capped C_{18} sorbents are better for the retention of polar molecules when compared to end-capped columns. However the extraction of polar analytes from

large volumes of a sample matrix is often difficult when a C_{18} column is used. As a rule of thumb, analytes with a partition coefficient of < 1.5 are poorly extracted unless the sample volumes are low [217]. The retention of ionised analytes in biological fluids can be achieved using ion-exchange SPE, and in such cases the pH used for the process is critical as this parameter dictates the ionisation of the analytes to be isolated [214].

3.4.3 Polymer –based sorbents

Polymer-based sorbent beds have a higher retention capacity than silica-based beds and are stable across the entire pH range. The hydrophilic-lipophilic copolymer of *N*-vinylpyrrolidone and divinylbenzene is commercially available as Oasis® HLB (Waters® Corporation, Milford, USA) and is capable of retaining a wide variety of analytes of different physico-chemical properties. The sorbent is water-wettable due to the presence of *N*-vinylpyrrolidone which is hydrophilic, thus eliminating the need for any conditioning and equilibration steps resulting in a simple, rapid and economic sample preparation approach prior to analyses. The lipophilic component of the product, divinylbenzene, imparts hydrophobic characteristics to the SPE sorbent that facilitates RP extraction of non-polar analytes. In addition the efficiency of the SPE is not affected by drying of the sorbent bed [214]. The Oasis® HLB 1 ml cartridges are ideal for analysis of EFV, FTC and TNF in plasma as only a small amount of plasma sample may be required to achieve the necessary sensitivity and analytical outcome. The high retention capacity of Oasis® HLB for a wide range of analytes is advantageous as EFV, FTC and TNF exhibit large differences in their solubility and physico-chemical properties, and the use of this relatively new sorbent for bio-analysis of these compounds has been reported [185]. The method uses mass spectroscopy, which is more selective than UV detection.

3.4.4 Sensitivity of RP-HPLC gradient method

Plasma analysis requires a higher level of sensitivity than that for QC analysis as drug concentrations are expected to be in the ng/ml range. The sensitivity of the RP-HPLC gradient method was investigated by injecting solutions of 10-100 ng/ml EFV, FTC and TNF. FTC was readily detected in this concentration range, however interfering peaks were observed at the retention time of TNF and EFV and consequently the source of the interfering peaks was investigated.

3.4.4.1 Ghost peaks and band compression

A major drawback of gradient RP-HPLC methods of analysis is the fairly common appearance of 'ghost peaks'. Ghost peaks are sometimes referred to as artefacts, system or pseudo peaks and are usually due to the presence of contamination. These peaks may overlap with the analytes of interest and make quantitation, particularly at lower concentrations, almost impossible. Possible sources of the contamination are widespread and originate from air bubbles, a dirty needle tip or dirty sample vials. Other common sources of contamination include inorganic impurities in water, organic solvents, improper or contaminated reagents used to prepare buffers, compounds that leach out of plastic containers, dirty glassware and even microbial growth [218–220]. The ghost peaks were observed

only when a high level of sensitivity was required for the analysis of plasma samples containing EFV, FTC and TNF. These peaks co-eluted primarily when the strength of the mobile phase strength was increased by using increasing proportions of acetonitrile up to a maximum level. The quantitation of TNF and EFV was not possible at the levels required for plasma analysis using the equipment and method available and therefore further investigation into the source of the ghost peaks was undertaken.

3.4.4.2 Water as a source of contamination

Water and aqueous solvents are prone to bacterial growth, which may be the source of the interfering peaks observed. The water from our and another laboratory was assessed and the same interfering peaks were present in both samples. Preparation of a new buffer using higher grade reagents also revealed no improvement and the ghost peaks were still obvious. Flushing the separation module of the HPLC system was then performed using a solution of isopropyl alcohol, HPLC-grade water, methanol and acetonitrile in equal proportions as this is the solution that is recommended by the manufacturer to remove any contaminants including bacteria that may have accumulated in the solvent lines of the separation module [219]. The lines were flushed individually for 1-2 hours and this exercise too revealed no improvement when blank mobile phase was injected. Other sources of contamination investigated included the glassware, sample vials and organic solvent, yet interfering peaks were always present when the strength of the mobile phase was increased to a maximum. These findings may be explained by a phenomenon called band compression and analytes or contaminants present in the mobile phase appear as bands once injected onto the stationary phase [221]. When using gradient HPLC a band of analytes or contaminants is present at the head of the column and as the organic solvent concentration is lower at the front of the band and molecules that comprise the front of the band are more strongly retained than when a higher concentration of organic solvent is present such as that that occurs at the back of the band. Consequently strongly retained molecules move along the stationary phase as the concentration of the organic increases to a point to facilitate their movement via longitudinal diffusion. This compression mechanism is heavily reliant on longitudinal diffusion, and the effects can be reduced by using columns with smaller particles and by decreasing the steepness of the gradient, although for these studies reducing the steepness of the gradient had no effect. The compressed bands appear as sharp peaks when band compression is adequate and inadequately compressed bands will not be symmetrical [219, 221]. The presence of inadequately compressed bands made the quantitation of TNF and EFV impossible at low concentrations. The source of contamination was not identified and so this gradient RP-HPLC method using UV detection was not considered suitable for the analysis of FTC, TNF and EFV in plasma. An example of a chromatogram obtained when only mobile phase containing no actives was injected is depicted in Figure 3.9, and the presence of interfering peaks or compressed bands can be seen from approximately 8 to 10.2 min.

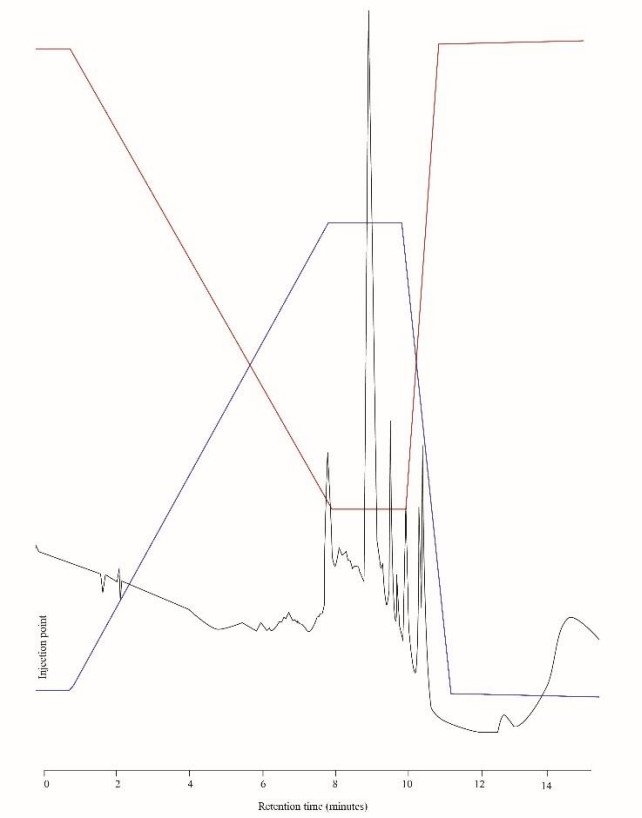


Figure 3.9 *Chromatogram showing interfering peaks following injection of mobile phase*

3.5 CONCLUSIONS

A RP-HPLC gradient method for the simultaneous analysis of FTC, TNF and EFV has been developed and validated. The correlation coefficient for the calibration curve for each analyte was > 0.999, indicating that the calibration curve is linear over the concentration ranges of 0.4-40 µg/ml, 0.6-60 µg/ml and 12-120 µg/ml for FTC, TNF and EFV, respectively. The equation of the best-fit least squares linear regression lines for FTC, TNF and EFV were y = 0.0191x+0.0007, y = 0.0163x+0.0116 and y = 0.01x+0.016, respectively. The analytical method is accurate as the y-intercept was < 2 % for each analyte and the % RSD indicated that the method was precise in terms of intra-assay and inter-day precision. Reproducibility was not assessed as intermediate precision had been demonstrated and the method was to be used by one analyst using the same equipment in one laboratory. The accuracy of the method was assessed using commercially available tablets. At 80, 100 and 120 % of the label claim the % recovery for FTC was 84, 84, and 83 %, for TNF was 79.5, 79.8 and 79 % and for EFV was 88, 87.9 and 86.2 %. The % RSD for all analytes at each concentration level was < 2 %, and therefore suitable accuracy was achieved for this assay.

The stability-indicating nature of the RP-HPLC method was established after exposing samples of all API to acid, alkali, peroxide, light and heat. Forced degradation studies on each analyte alone permitted the identification of degradation products specific to that analyte and when the individual chromatograms for each API were overlaid for evaluation purposes possible interferences could be

identified. The only degradation product formed, and that may affect the quantitation of another analyte, was degradation product TK that was produced following exposure of TDF to 100 °C dry heat for 12 hours. This degradation product may not be formed under conventional storage and working conditions but its presence may affect the quantitation of EFV if it does form. If this degradation product were to form when testing the stability of formulations containing FTC, TNF and EFV, then the same forced degradation conditions may be used to assess the individual raw material and analytes and the amount of EFV present or that has degraded could be determined. The method is therefore precise, accurate and specific for the quantitative analysis of FTC, TNF and EFV alone or in combination. This method is simple and the only one reported using this stationary phase and for which all ARV elute under 10 min.

The validated RP-HPLC gradient method is not suitable for the analysis of FTC, TNF and EFV in plasma due to band compression and the presence of interfering compounds at the times at which TNF and EFV elute. A column with smaller particles could be attempted to reduce the effect of band compression. Decreasing the gradient steepness will decrease the separation power of the method as observed from studies described in Chapter 2, Figure 2.6. The use of a mass spectrometer for detection would be beneficial as it would have not exhibit band compression usually observed with UV detection and it is a selective detection system.

CHAPTER FOUR
APPLICATION OF THE GRADIENT RP-HPLC METHOD OF ANALYSIS

4.1 INTRODUCTION

4.1.1 Overview

Dissolution is the process in which molecules of an API from a pharmaceutical formulation dissolve and move into solution in a dissolution medium [222, 223]. Drug absorption following administration of solid oral dosage forms initially requires the dosage form in which the API is delivered to disintegrate, after which dissolution of the API into the surrounding medium must occur as a prerequisite for absorption from the GIT [52, 223]. The rate of dissolution may affect the rate of absorption for some classes of compound and bioavailability is a function of dissolution and absorption on which effective drug therapy is dependent. Therefore the physico-chemical properties of a molecule and the manner in which it is formulated contributes significantly to the rates of dissolution and absorption, and consequently the bioavailability which may impact therapeutic outcome [223, 224]. Dissolution studies are used to characterise the *in vitro* rate and extent of release of an API from oral dosage forms during drug product development and to ensure the ongoing quality of existing products through routine quality assessment of the rate and extent of release from formulations. Dissolution test results can be used as a tool to predict *in vivo* performance if an *in vitro-in vivo* correlation (IVIVC) has been established [225, 226] and if one exists the dissolution test for quality is more valuable, however it is not always possible to establish an IVIVC for all therapeutic molecules [52]. Dissolution studies can also be used to assess the rate of release of poorly soluble API and the impact of formulation composition on the rate of release of that API. The rate and extent of API release from a formulation is usually evaluated against acceptance criteria published in various pharmacopoeias or guidelines. In comparative dissolution studies, mathematical models are also used to describe dissolution profiles and/or compare API release from two or more formulations [222]. Bio-waivers or permission to avoid bioavailability or bioequivalence studies are granted when two products contain a highly soluble and permeable API and are considered similar at the outset, obviating the need for expensive and/or extensive bioequivalence studies in humans, and are waived according to relevant regulatory guidelines [224]. It is desirable to be granted permission to proceed with licensing with a biowaiver as it is cost-effective and not excessively time-consuming to generate appropriate data for the market authorisation application [226, 227].

The BCS can be used to predict IVIVC using data from dissolution studies [52]. There are four BCS classes and FTC, EFV and TNF are classified as BCS class 1, class 2 and class 3 (§1.2.4, Chapter one) compounds respectively. BCS class 1 and 3 molecules are characterised by high aqueous solubility but differ in terms of membrane permeability. Class 1 molecules exhibit high membrane permeability whereas class 3 molecules exhibit low membrane permeability [224, 228]. BCS class 1 and 3 compounds do not exhibit dissolution rate limited bioavailability however, the rapid dissolution

exhibited by immediate-release dosage forms containing class 1 and/or 3 molecules may result in scenarios from which it is difficult to predict absorption [52, 223]. The absorption of BCS class 1 and 3 molecules is, in part, limited by gastric emptying and class 3 molecules may exhibit limited absorption due to poor membrane permeability, however establishing an IVIVC may still be possible [52, 229]. The development of controlled release tablet formulations to deliver class 3 molecules may also prove challenging [224]. BCS class 2 molecules are characterised by low aqueous solubility and high membrane permeability and for these compounds, dissolution may be the rate limiting step for absorption and subsequently bioavailability, often resulting in highly variable bioavailability. Therefore the enhancement of dissolution for class 2 molecules may facilitate the establishment of an IVIVC [224]. Dissolution testing and profile development in dissolution media of different pH is recommended for drugs in this BCS class [52]. BCS class 4 molecules are characterised by low aqueous solubility and poor membrane permeability, and pose significant challenges for formulation scientists when developing oral dosage forms [52] and all regulatory authorities do not grant bio-waivers for BCS class 4 molecules [46].

The most simple and convenient method of drug delivery is the oral route, and the majority of solid oral dosage forms include tablets, capsules and powders [223, 224]. API release from different dosage forms occurs at different rates, and by way of example, coated tablets release API at a slower rate than capsules and liquid oral dosage forms [223, 230]. Immediate-release tablets should disintegrate rapidly into granules, followed by disaggregation into powder particles so as to facilitate the release of API [223]. The excipients used in a formulation can and often do affect the rate of dissolution of an API from the dosage form [229]. Excipients used in tableting fulfil primary and secondary roles and fall into broad performance categories including disintegrants, diluents, binders, anti-frictional agents and surfactants [223], and many excipients fulfil more than one role.

The type and amount of disintegrant, diluents and other excipients used when manufacturing tablets can and often does affect tablet disintegration, which in turn affects the ultimate dissolution rate of an API from a tablet. The role of disintegrating agents is to facilitate the breakup of tablets into granules and powder particles. Water-soluble diluents will ensure faster disintegration of dosage forms in an aqueous environment [223].

Microcrystalline cellulose is a water insoluble but wetable diluent that facilitates disintegration by permitting water uptake into the table core through capillary formation [223, 231]. Binders are added to a formulation to ensure that cohesive forces within a blend and after compaction are sufficient to keep the granules and tablets intact. Addition of excess binder can result in the manufacture of hard tablets that do not release adequate amounts of the API contained in the dosage form. Excessive amounts of hydrophobic lubricants such as magnesium stearate can and often do slow the rate of

disintegration of tablets as they reduce the hydrophilicity of the tablet core. Surfactants are added to formulations in which poorly water soluble API are included so as to improve the dissolution rate of such compounds by reducing the interfacial tension between the tablet surface, API and aqueous dissolution medium [223]. Other factors that affect the dissolution rate of an API include volume, temperature, pH and agitation rate of the dissolution medium, and the physico-chemical properties of the API such as particle size, size distribution, polymorphism, specific surface area and the quality of the raw materials used to manufacture the formulation are also of importance [223, 232].

4.1.2 Dissolution apparatus

Apparatus for dissolution testing must meet specific performance criteria, specifications and standards [223]. In general the specifications for equipment used for performance of dissolution testing are described in USP general chapter <711> [233] or in other pharmacopoeias. Official pharmacopoeias differ slightly in terms of their published specifications. Ultimately the choice of apparatus should be appropriate for the dosage form and API being tested and should as far as possible mimic *in vivo* conditions. USP apparatus 1 and 2 are the most commonly used apparatus for testing API release from solid oral dosage forms [222, 223]. USP apparatus 2 or the rotating paddle apparatus has better agitation capabilities than is possible using USP apparatus 1 or the rotating basket apparatus, and therefore USP apparatus 2 is more appropriate for testing the release characteristics of sparingly soluble API than USP apparatus 1. The paddle blade is covered with an inert coating material and the paddle is attached to a stainless steel shaft such that it is located 25 mm above the inside surface of the dissolution vessel [223]. Sinkers may be used if the dosage form to be tested floats as capsules may do, however sinkers may not be necessary when testing tablets but are useful for preventing film-coated tablets from adhering to the bottom of the dissolution vessel. Any differences in dissolution results should be attributed to modifications in test formulations and should not be a consequence of equipment and/or apparatus-related variability. In addition all results should be reproducible and through standardisation and verification of dissolution equipment, reproducibility can be assured [223]. The specifications for USP apparatus 2 are summarised in Table 4.1. The performance of equipment is verified by calibration on a six-monthly basis in our laboratory to ensure that the equipment does not contribute to discrepancies in dissolution test results.

Table 4.1 *Summary of specifications for USP apparatus 2 [233]*

Component Apparatus 2	Dimensions mm
Vessel	
Height	160-175
Internal diameter	98-106
Paddle	
Shaft diameter without coating	9.4-10.1
Blade	
Upper chord	74.0-75.0
Lower chord	42.0 ± 1.0
Height	19.0 ± 0.5
Radius of disk	42.0 ± 1.0
Radius upper corners	1.2
Thickness	4.0 ± 1.0

4.1.3 Dissolution media

The choice of dissolution medium is important as in order for dissolution studies to be meaningful the medium selected should as far as possible mimic *in vivo* dissolution conditions [222, 223]. To compensate for the low volume of dissolution fluid, sink conditions must be used and therefore the volume of the dissolution medium must be at least three times the volume required to produce a saturated solution of the API under investigation [222]. The pH, temperature and volume of the dissolution medium should be carefully considered and the addition of sodium lauryl sulphate (SLS), a preferred surfactant for dissolution testing, may be necessary when testing the dissolution behaviour of poorly water-soluble drugs. The pH, temperature and volume of the dissolution medium must resemble *in vivo* conditions as far as possible to obtain meaningful data, and the amount of SLS used in a dissolution test must be justified in the context of the test being developed and/or used [222, 223]. A 0.1 M HCl dissolution medium is considered appropriate to simulate gastric acid conditions for dissolution testing as the pH of this fluid is 1.2. Buffers of pH 4.5 and pH 6.8 are considered suitable for the simulation of different conditions in the small intestine. Simulated gastric fluid is a practical and suitable dissolution medium for testing BCS class 1 molecules as these are highly soluble and permeable compounds. If formulated in immediate-release dosage forms that disintegrate rapidly in gastric fluids, these molecules will exhibit close to 100 % bioavailability following oral administration provided they are not susceptible to degradation in acidic media or are extensively metabolised by hepatic first pass metabolism. BCS class 2 molecules that are low solubility molecules often require the addition of a surfactant to the dissolution medium to improve aqueous solubility, whereas BCS class 3 molecules require testing in simple aqueous based dissolution media [224]. The pH and transit times in and through different parts of the gastrointestinal tract are listed in Table 4.2. These data are useful for establishing an appropriate dissolution method for test formulation evaluation.

Table 4.2 *Summary of pH and transit times in the gastrointestinal tract [223]*

Location	pH	Transit time Min
Stomach	1.0-2.0	90
Duodenum	4.0 - 5.5	30-40
Jejunum	5.5 - 7.0	90-120
Ileum	7.0 - 7.5	300-420

4.1.4 Acceptance criteria

The FDA, other official pharmacopoeias and ICH provide information about performing *in vitro* dissolution studies and the acceptance criteria for data generated from those studies [64, 223, 233–235]. The acceptance criteria for immediate-release tablets are described in the General Test Chapter titled *Dissolution* <711> (Table 4.3) in the USP [233]. The general approach is to use the term Q to refer to the amount or quantity of API that has been released in relation to the label claim and the value for Q is specific to an API in the USP. For immediate-release tablets the acceptance criteria state that an average of 12 units must not releases less than (NLT) the specified value of Q in the monograph for that API in that dosage form [233] and no single unit must yield a result of Q-15 % [223, 233]. According to the individual monographs for EFV and TNF tablets and FTC capsules in the USP, Q is 80 % [236–238]. The International Pharmacopoeia draft monograph for EFV, FTC and TNF tablets states that the amount released from each tablet must not be < 80 % of the label claim for each ARV however time was not specified. A further six tablets may be tested if one tablet yields a result < 80 % and the average API released from the 12 tablets must not be < 75 % and no tablet may yield a result < 60 % [239]. The guidelines for dissolution testing for immediate-release products containing BCS class 1 or 3 molecules state that a single point dissolution test is sufficient if NLT 85 % (Q=80 %) is released in ≤ 60 min when used for routine quality control testing. BCS Class 2 molecules require a two-point dissolution test and the first sample must be taken at 15 min and the second at a later time point when undertaking quality control tests. The recommended operating speed for USP apparatus 1 and 2 is 50-100 and 50-75 rpm, respectively [52]. New draft guidance for industry on dissolution testing for BCS class 1 and 3 molecules also specifies a single point dissolution test for use in the quality control of immediate-release formulations. BCS class 1 molecules must release NLT 80 % of the label claim in at 30 min whereas BCS 3 three molecules should release NLT 80 % in 15 min [228]. FTC and TNF are classified as BCS class 1 and 3 [50, 51] molecules, whereas EFV is a BCS class 2 molecule that requires 80 % API to be released in 30 min [46, 53]. Rapidly dissolving immediate-release formulations are designed to release NLT 85 % of label claim when placed in 0.1 M HCl, pH 4.5 and 6.8 buffers (without enzymes) within 30 min or less when using USP Apparatus 1 or 2 operated at 100 rpm and 50 rpm, respectively [240]. The stages of dissolution testing required for immediate-release products and the corresponding acceptance criteria as well as the number of units to be tested are listed in Table 4.3. In stage one, six units are tested and an acceptable test result is obtained when each unit has reached NLT Q+ 5 %. In stage two testing, an additional six units are tested and the average of 12 units must be ≥ Q and no unit may

release < Q-15 %. An additional twelve units are tested in stage three testing, and the average of 24 units must be ≥ Q, with no more than two units releasing < Q-15 % and no unit releasing < Q-25 %.

Table 4.3 *USP general test chapter dissolution <711> acceptance criteria for immediate-release products [232]*

Stage	Number of units tested	Acceptance criteria
S 1	6	Each unit NLT Q + 5 %
S 2	6	An additional 6 units are tested and the average release from 12 units (S1 and S2) must be ≥ Q with no unit releasing < Q-15 %
S 3	12	An additional 12 units are tested and the average release from 24 units (S1+ S2 + S3) must be ≥ Q with no > than 2 units releasing < Q-15 % and no unit releasing < Q-25 %

To properly characterise the similarity or difference between the release of API from two products, dissolution profiles are plotted for comparative purposes and the percent released must be measured at more than one specified time point so as to generate data suitable to plot dissolution or drug release profiles.

4.1.5 Dissolution profile comparisons

A dissolution profile represents the *in vitro* release of molecules from a dosage form into a dissolution test medium. During testing samples of the dissolution medium are taken at specified times and analysed to determine the concentration of API in the fluid. Dissolution medium is either replaced or not. The concentration data are reduced to permit a calculation of the cumulative percent API released over time [226]. The dissolution profiles of two products may be assessed in terms of their overall similarity or difference, using model independent approaches which include the use of the difference and similarity factors. The difference factor (f1) is used to calculate the percent difference in rate of release between two dissolution profiles, whereas the similarity factor (f2) is used to assess the similarity between two profiles. This has been used frequently in our laboratory [241].

The difference factor is a measure of the relative error between two dissolution profiles and can be calculated using Equation 4.1 [52, 242].

$$f1 = \left\{\frac{[\sum_{i=1}^{P} |R-T|]}{[\sum_{i=1}^{P} R]}\right\} \qquad \textbf{\textit{Equation 4.1}}$$

Where,

f1 = difference factor
R = percent released for the reference formulation at P time points
T = percent released for the test formulation at P time points
P = number of time points

The similarity factor is the logarithmic reciprocal square root transformation of the sum of squared error for two sets of data [52, 226] and is calculated using Equation 4.2.

$$f2 = 50 \log\left\{\left[1 + \left(\frac{1}{P}\right)\sum_{i=1}^{P}(R - T)^2\right]^{\frac{1}{2}} \times 100\right\} \qquad \textit{Equation 4.2}$$

Where,

f2 = similarity factor
R = percent released for the reference formulation at P time points
T = percent released for the test formulation at P time points
P = number of time points

Dissolution profile comparisons are useful only when three or more samples are available [242]. To establish the similarity and difference factors a minimum of 12 dosage units of both the test and reference products must be tested. The mean percent released is calculated at each time point for all 12 units and the dissolution profiles are considered different when the value for f1 is close to zero, and are similar when the value for f2 is close to 100. In general f2 values between 50 and 100 and f1 values between 0 and 15 are considered sufficient to prove equivalence between two dissolution profiles [52, 226, 243]. Dissolution tests should be conducted under the same conditions *viz.* using the same dissolution media and sample times and only one measurement should be made after 85 % dissolution of the API has occurred [52]. The % RSD should be < 20 % for early samples and < 10 % for all other sampling times [5].

4.2 EXPERIMENTAL

A summary of the suggested approach for dissolution testing of FTC, TNF and EFV is summarised in Table 4.4 and these data was used as a guide for developing a dissolution test method for these studies. Atripla®, Truvada® and Aspen Efavirenz® are all formulated as immediate-release tablets. Atripla® (MSD Pty Ltd, Johannesburg, South Africa) was used as the test product and Truvada® (TNF) and FTC) (Aspen Pharmacare Ltd, and Gilead Sciences, Inc, Port Elizabeth, South Africa) and Aspen Efavirenz® (Aspen Pharmacare Ltd, Johannesburg, South Africa) were used as the reference products. All reference products were placed in the same dissolution vessel and tested simultaneously to simulate therapeutic dosing of a FDC or the clinical use of the individual dosage forms. USP apparatus 2 dissolution studies were performed using a Hanson Research SR8 plus Dissolution Test Station (Chatsworth, USA) fitted with an Autoplus™ Maximizer Syringe Fraction Collector (Chatsworth, USA) and AutoPlus™ Multifill™ (Chatsworth, USA). Dissolution tests were performed in 900 ml of dissolution fluid at three different pH representing the pH of the environment at different points along the GIT and included 0.1 M HCl (pH = 1.2) and buffers of pH = 4.5 and 6.8 [223]. The dissolution media and apparatus were equilibrated to 37 ± 0.5 °C, after which the tablets were dropped into the vessels and the agitation rate set at 100 rpm as recommended [244]. The USP also recommends the addition of 2.0 % w/v SLS to the dissolution medium to improve the dissolution rate of EFV from tablets, however the addition of 0.5 % w/v and 1.0 % w/v has been reported [225, 232, 236, 244]. Aliquots (3 ml) were withdrawn at 10, 20, 30, 45, 90 and 120 min, and automatically

filtered using a 0.45 µm Durapore® HVLP in-line filter membranes (Merck Millipore Ltd, Cork, Ireland), and the volume was replaced with fresh dissolution medium [244]. One millilitre aliquots of the filtered sample were diluted with 9 ml of 60:40 % v/v of water-acetonitrile and analysed using the validated stability-indicating RP-HPLC method reported in Chapter 3 of this thesis.

Table 4.4 *Summary of suggested dissolution methods for in-vitro dissolution testing of EFV, FTC and TNF tablets*

Drug	Dosage form	USP apparatus	Speed rpm	Volume ml	Sample times	Ref.
EFV/FTC/TNF	Tablet	2	100	1000	10, 20, 30, 45	[228]
EFV	Tablet	2	50	1000	10, 15, 30, 45, 60	[228]
TNF	Tablet	2	50	900	10, 20, 30, 45,	[228]
FTC/TNF	Tablet	2	50	900	5, 10, 15, 30, 45	[228, 245, 246]

4.3 RESULTS

The average cumulative percent EFV released with and without SLS (1.0 % w/v) is summarised in Table 4.5 and the dissolution profiles are depicted in Figure 4.1. It is clear that the release of EFV is slow when no SLS was used in the dissolution medium due to the low aqueous solubility of the compound, and at 120 min approximately only 10 % EFV had been released. Following the addition of 1.0 % w/v SLS the solubility of EFV was enhanced and > 80 % release was observed within 30 min.

Table 4.5 *Average cumulative release of EFV (n=12) when no SLS and 1.0 % SLS added to 0.1 M HCl dissolution media*

	No SLS			1.0 % SLS		
Time min	Mean amount releases mg	Cumulative % released	Standard deviation	Mean amount releases mg	Cumulative % released	Standard deviation
10	11.2	1.9	0.000533	286.6	47.8	0.015612
20	48.5	8.1	0.000636	462.4	77.6	0.005500
30	49.8	8.3	0.001033	481.3	80.2	0.008164
45	50.8	8.5	0.001117	502.4	83.7	0.007422
90	62.3	10.4	0.004931	523.9	87.3	0.015682
120	66.6	11.1	0.008484	526.6	87.8	0.010175

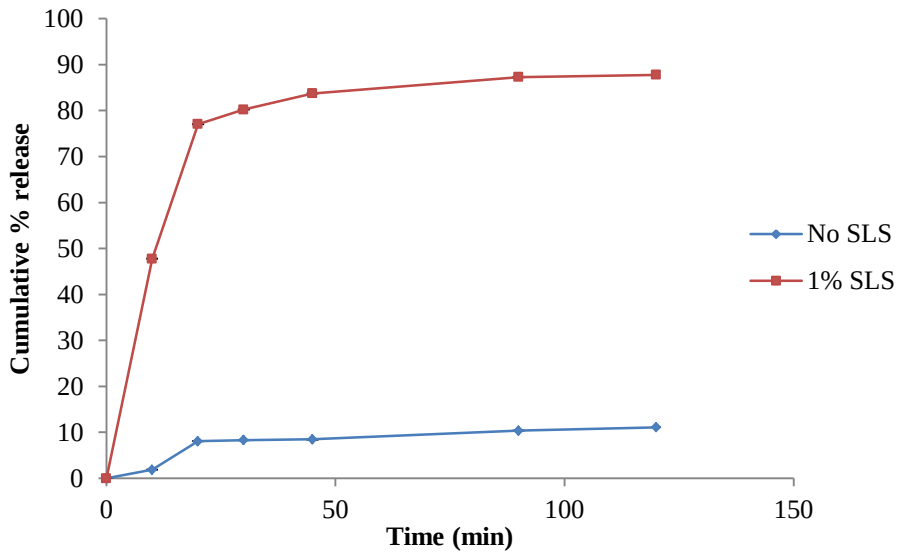


Figure 4.1 *Dissolution profile of mean (n=12) cumulative % EFV released with 1.0 % w/v and without SLS in a 0.1M HCl dissolution fluid*

Two products may be considered similar if the values for Q achieved within the specified time frames are met. For the release of FTC from different dosage forms to be considered similar the percent FTC released must be NLT 80 % after 30 min of testing as FTC is a BCS class 1 compound, and if achieved the test and reference products can be considered similar. The cumulative % FTC released from the test formulation was approximately 50 % after 10 min, and it is evident that the release from the test product is slower in all three media, which may be due to the different excipient used in the formulation. The method of formulation is not specified and therefore it is only possible to speculate as to the cause of difference in release of FTC. Common excipients in the tablet core in the test and reference products include croscarmellose sodium, magnesium stearate and microcyrystalline cellulose. Croscarmellose sodium is a super-disintegrant [247] that aids disintegration of tablets into smaller particles for subsequent rapid dissolution in the aqueous fluids that surround the dosage form. The mode of addition of a disintegrant may affect disintegration time [248]. The test product contains hydroxylpropyl cellulose and SLS and the reference product contains lactose monohydrate pre-gelatinised starch [247]. The % FTC released was at least 80 % from both formulations within 30 min in all media tested. These data are summarised in Table 4.6.

Table 4.6 *Mean cumulative percent FTC released for test and reference products*

Cumulative % released												
	0.1 M HCl				pH 4.5				pH 6.8			
Time Min	**Ref**	**SD**	**Test**	**SD**	**Ref**	**SD**	**Test**	**SD**	**Ref**	**SD**	**Test**	**SD**
10	74.5	0.0069	54.4	0.0113	88.6	0.0083	52.8	0.0081	84.6	0.0147	54.0	0.0149
20	90.6	0.0051	86.3	0.0049	90.4	0.0059	89.3	0.0081	90.6	0.0022	91.2	0.0072
30	91.9	0.0055	86.5	0.0042	92.5	0.0159	89.3	0.0058	89.9	0.0029	91.6	0.0048
45	91.7	0.0047	86.5	0.0063	90.9	0.0067	86.3	0.0085	89.7	0.0038	91.9	0.0078
90	92.2	0.0055	86.4	0.0094	92.2	0.0048	88.4	0.0079	92.5	0.0030	91.4	0.0069
120	91.1	0.004	86.9	0.0068	91.4	0.0064	87.9	0.0037	92.7	0.0031	91.9	0.0131

The dissolution profiles (n=12) for FTC release from test and reference products in 0.1 M HCl are depicted in Figure 4.2. The test product appears to release less FTC than the reference product. Standard deviations are not visible on the curve as the method is precise. In both cases Q has been reached.

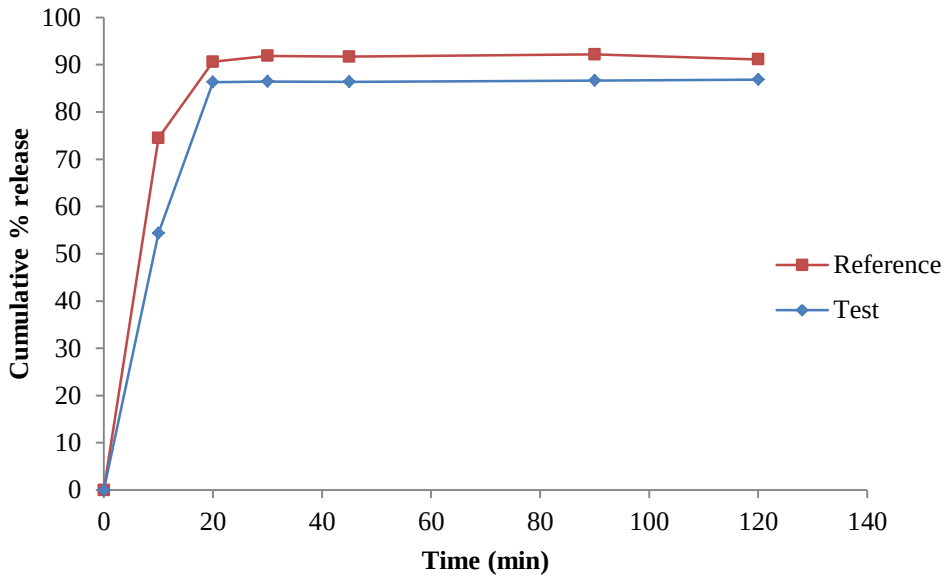


Figure 4.2 *Dissolution profile of mean (n=12) cumulative % FTC released from test and reference products in a 0.1 M HCl dissolution medium*

The dissolution curves (n=12) for FTC release from test and reference products in a dissolution medium of pH 4.5 is depicted in Figure 4.3 and the test product appears to release less FTC than the reference product at this pH. Standard deviations are not visible on the curves as the method is precise. In both cases Q has been reached.

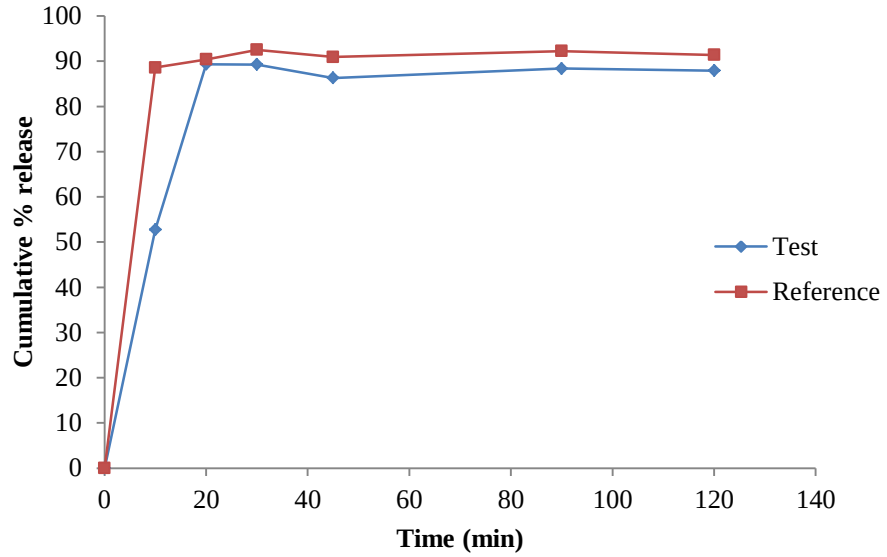


Figure 4.3 *Dissolution profile of mean (n=12) cumulative % FTC released from test and reference products in a pH 4.5 dissolution medium*

The dissolution curves (*n*=12) for FTC release from test and reference products in a buffer of pH 6.8 is depicted in Figure 4.4 and once again the test product appears to release less FTC than the reference product and the standard deviations are not visible on the curve as the method is precise. In both cases Q has been reached.

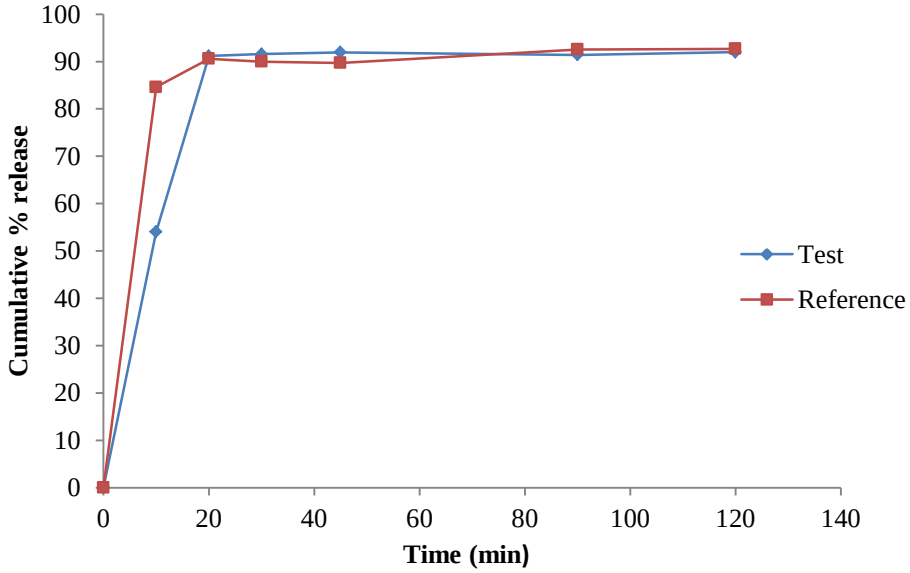


Figure 4.4 *Dissolution profile of mean (n=12) cumulative % FTC released from test and reference products in a pH 6.8 dissolution medium*

For TNF release from different dosage forms to be considered similar, the percent TNF released for this BCS class 3 molecule should not NLT 80 % at 15 min. In these experiments the samples times did not include a 15 min time point for ease of comparison for all three experiments. However, the percent TNF released after 20 min is > 85 % for all products in all dissolution media tested. Results

are summarised in Table 4.7, however since the 15 min time point was not included one cannot infer similarity at that time point. In addition the test formulation released only approximately 50 % TNF at 10 min, which could be due to the different excipients used to manufacture the test and reference products and that may affect the disintegration and/or performance of the test product. Disintegration tests may be performed on FTC and TNF as USP disintegration tests may be used in lieu of dissolution testing, provided that a Q = 80 % is achieved in 15 min for theses BCS class one and three molecules [228, 250]. Complete disintegration within five min using USP apparatus and 0.01 M HCl dissolution media will permit disintegration to be used as an alternate method to test release and stability of products containing these compounds.

Table 4.7 *Mean cumulative percent TNF released from test and reference products*

	Cumulative % released											
	0.1 M HCl				**pH 4.5**				**pH 6.8**			
Time Min	**Ref**	**SD**	**Test**	**SD**	**Ref**	**SD**	**Test**	**SD**	**Ref**	**SD**	**Test**	**SD**
10	74.3	0.0109	53.8	0.0152	86.9	0.0089	50.4	0.0097	82.9	0.0209	52.9	0.0204
20	90.5	0.0072	87.0	0.0068	91.7	0.0081	86.2	0.0108	91.3	0.0035	90.8	0.0097
30	90.9	0.0072	85.3	0.0067	93.9	0.0233	87.2	0.0088	90.7	0.0036	91.4	0.0069
45	90.9	0.0065	85.3	0.0083	92.6	0.0096	85.1	0.0119	90.4	0.0049	91.2	0.0106
90	90.2	0.0073	84.6	0.0122	94.3	0.0081	87.9	0.0106	93.1	0.0037	90.5	0.0088
120	88.5	0.0064	84.2	0.0087	93.4	0.0091	88.1	0.0051	93.1	0.0044	91.4	0.0163

The dissolution profiles (n=12) for TNF release from test and reference products in a dissolution medium of 0.1 M HCl is depicted in Figure 4.5 and the test product appears to release less TNF than the reference product. Standard deviations are not visible on the curve as the method is precise.

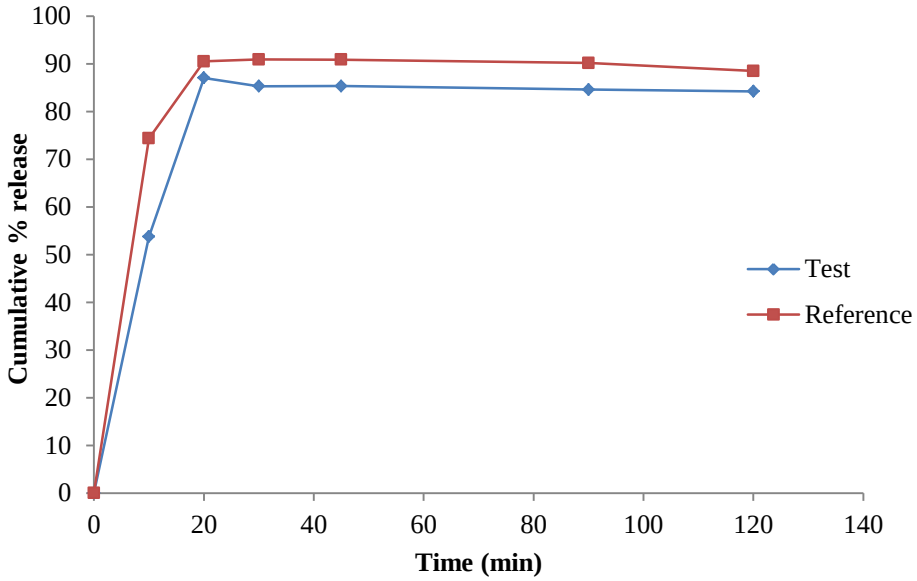


Figure 4.5 *Dissolution profile of mean (n=12) cumulative % TNF released from test and reference products in a 0.1 M HCl dissolution medium*

The dissolution curves (*n*=12) for TNF release from test and reference products in a buffer of pH 4.5 is depicted in Figure 4.6. The test product appears to release less TNF than the reference product and the standard deviations are not visible on the curve as the method is precise.

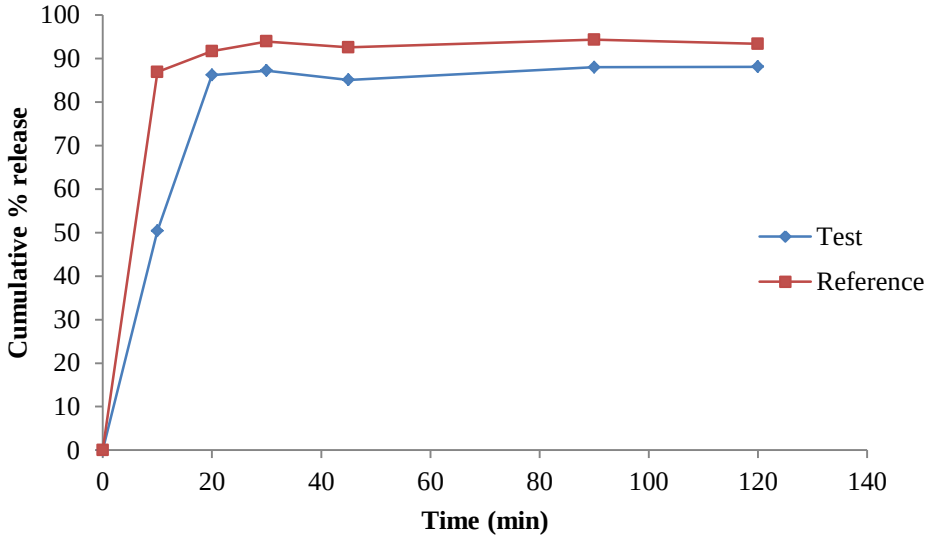


Figure 4.6 *Dissolution profile of mean (n=12) cumulative % TNF released from test and reference products in a pH 4.5 dissolution medium*

The dissolution curves (*n*=12) for TNF release from test and reference products in a buffer of pH 6.8 is depicted in Figure 4.7. The test and reference products appear to release similar amounts of TNF and the standard deviations are not visible on the curves as the method is precise.

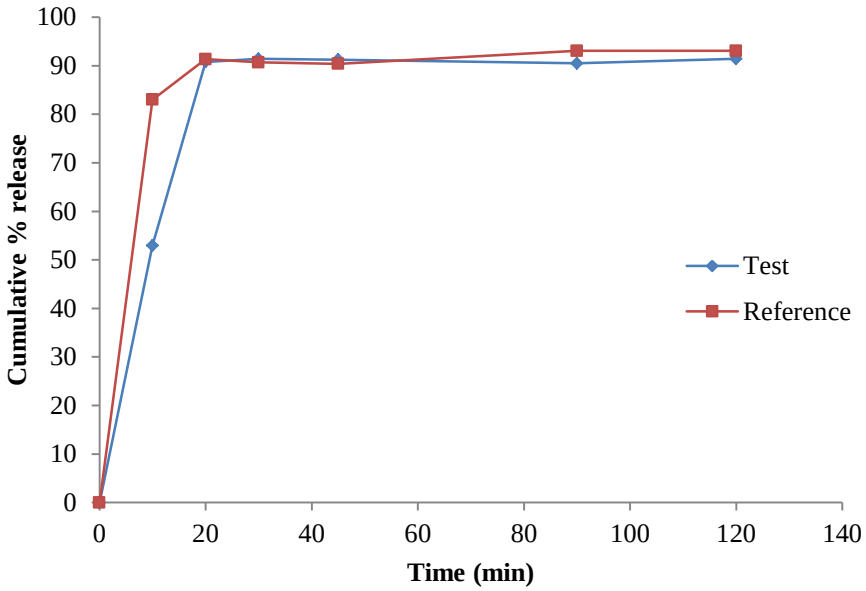


Figure 4.7 *Dissolution profile of mean (n=12) cumulative % TNF released from test and reference products in a pH 6.8 dissolution medium*

The percent EFV released in 30 min satisfied the requisite specification only for the value for Q for the test formulation and for which the percent EFV released in 0.1 M. pH 4.5 and pH 6.8 buffers was 80.2 %, 83.% and 86.4 %, respectively. The data for all studies are summarised in Table 4.8. Once again it appears as though the test formulation had a slower dissolution rate at 10 min than that of the reference product. The list of excipients used in the Aspen Efavirenz® formulation is proprietary on the Republic of South Africa and therefore it is not possible to attribute the dissolution results to any formulation effects. The reference product for EFV is formulated and produced to deliver EFV only, whereas the test product is formulated to release all ARV. In addition the use of a higher % SLS in the dissolution media may have resulted in a higher percent EFV released from the test and reference products, however this may have masked the apparent discriminatory nature of the dissolution method used in these studies.

Table 4.8 *Mean cumulative percent EFV released from test and reference products*

	Cumulative % released											
	0.1 M HCl				pH 4.5				pH 6.8			
Time Min	**Ref**	**SD**	**Test**	**SD**	**Ref**	**SD**	**Test**	**SD**	**Ref**	**SD**	**Test**	**SD**
10	28.1	0.0096	47.8	0.0156	52.6	0.0259	45.6	0.0102	52.7	0.0285	47.1	0.0416
20	51.9	0.0089	77.1	0.0055	70.2	0.0062	75.6	0.0154	72.2	0.0079	71.7	0.0801
30	60.8	0.0073	80.2	0.0081	78.6	0.0225	83.7	0.0108	78.9	0.0052	86.4	0.0098
45	68.3	0.0071	83.7	0.0074	83.1	0.0094	84.0	0.0204	83.3	0.0083	89.2	0.0103
90	78.3	0.0059	87.3	0.0157	90.3	0.0079	88.9	0.0079	90.8	0.0073	91.1	0.0096
120	80.5	0.0087	87.8	0.0102	90.9	0.0068	88.8	0.0011	92.3	0.0092	93.4	0.0246

The dissolution curves (n=12) for EFV from test and reference products in a dissolution medium of 0.1 M HCl is depicted in Figure 4.8 and it appears that the reference product releases substantially less EFV than the test product. Standard deviations are not visible on the curves as the method is precise.

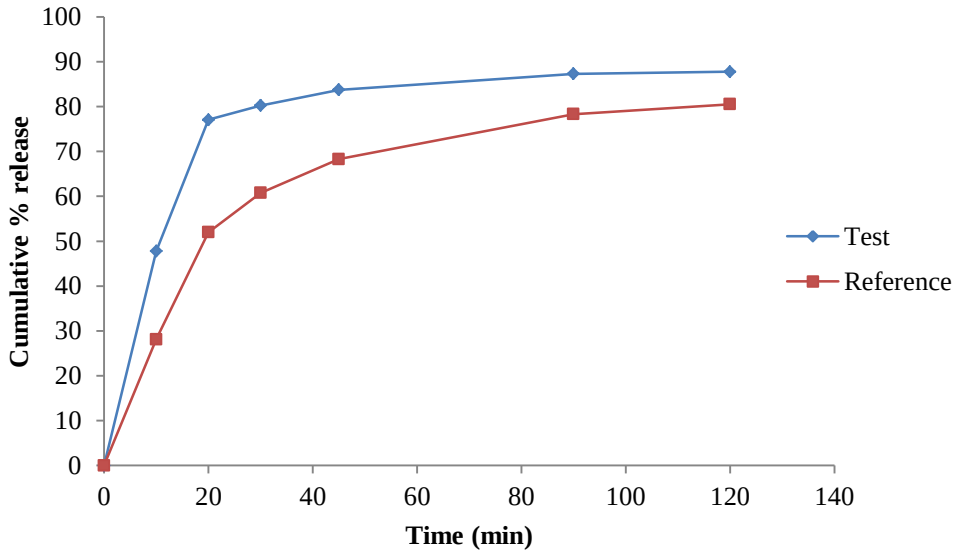


Figure 4.8 *Dissolution profile of mean (n=12) cumulative % EFV released from test and reference products in a 0.1 M HCl dissolution medium*

The dissolution curves (n=12) for EFV from the test and reference products in a buffer of pH 4.5 are depicted in Figure 4.9 and the test and reference products appear to release similar amounts of EFV. Standard deviations are not visible on the curves as the method is precise.

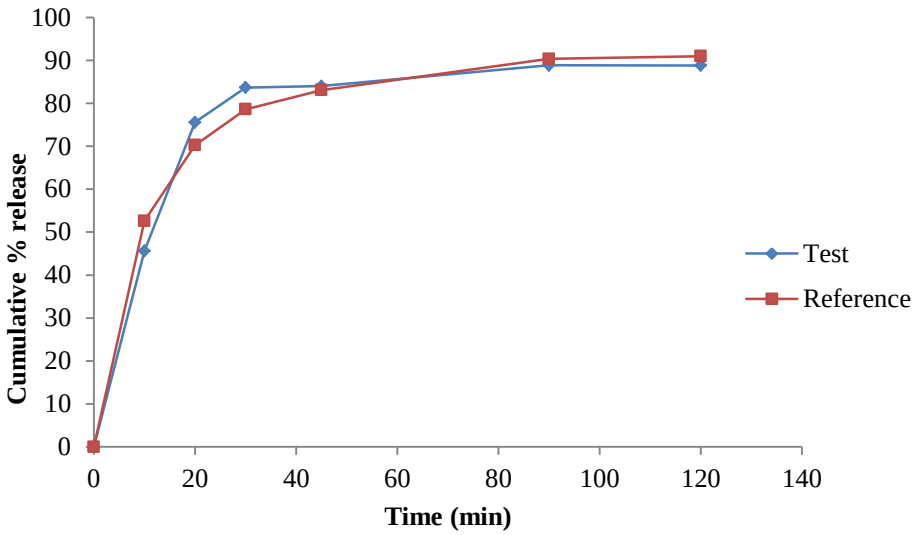


Figure 4.9 *Dissolution profile of mean (n=12) cumulative % EFV released from test and reference products in a pH 4.5 dissolution medium*

The dissolution curves (*n*=12) for EFV from the test and reference products in a buffer of pH 6.8 are depicted in Figure 4.10 and the test and reference products appear to release similar amounts of EFV. Standard deviations are not visible on the curves as the method is precise.

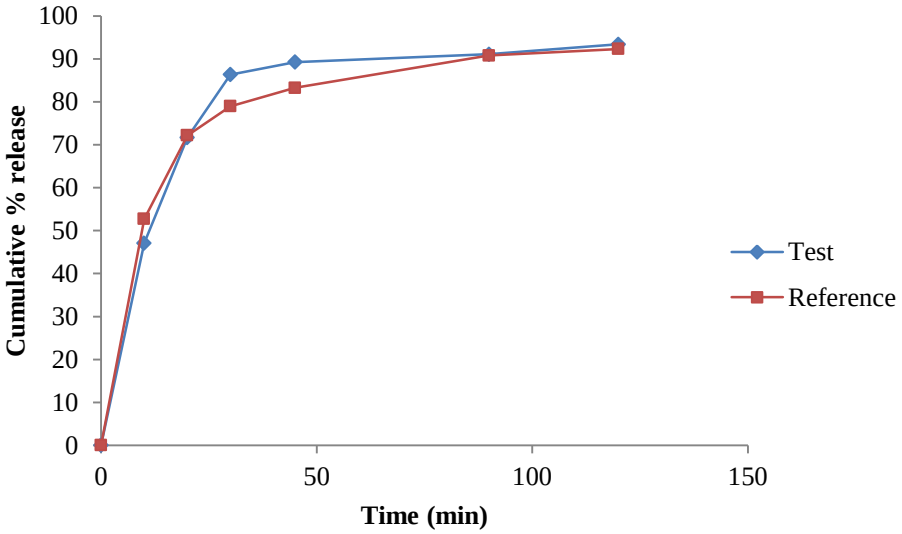


Figure 4.10 *Dissolution profile of mean (n=12) cumulative % EFV released from test and reference products in a pH 6.8 dissolution medium*

To compare the dissolution profiles using a model-independent statistical model in which the difference and similarity factors, f1 and f2 are calculated was performed so as to evaluate and compare the release of FTC, TNF and EFV from the test (FDC) and reference formulations. The

resultant data are summarised in Table 4.9, and the shaded blocks reveal values for the factors that fail to meet the acceptance criteria for f1 and f2.

Table 4.9 *f1 and f2 factors for dissolution profile comparison of FTC, TNF and EFV release from test and reference products*

	0.1 M		pH 4.5		pH 6.8	
	f1	**f2**	**f1**	**f2**	**f1**	**f2**
FTC	8.4	51.2	9.5	41.2	6.8	45.0
TDF	8.6	50.9	12.3	39.8	6.7	45.4
EFV	26.0	38.9	4.4	66.6	4.7	67.8

The mean amount of API released from the test and reference products was used when calculating the f1 and f2 factors and according to the acceptance criteria for immediate-release formulations, the release of FTC can be considered similar from both products as > 80 % was released within 30 min of commencing the test. However it is evident that only the dissolution profiles for FTC in 0.1 M HCl were similar when using the difference and similarity factors. The f2 values for the percent FTC released in buffers of pH 4.5 and 6.8 were < 50, thereby indicating that the profiles are not similar. This could be a result of the differences in formulation composition and performance, and where for example a difference in initial disintegration may have resulted in different dissolution profiles. In addition the method of manufacture in unknown and the production may have an effect on release. The reference product is formulated and includes two molecules, whereas the test product contains all three molecules. The percent TNF released was high from both formulations but as TNF is a BCS class 3 molecule it is expected that NLT 80 % of TNF should be released in 15 min. However in buffers of pH 4.5 and 6.8 the dissolution profiles of the test and reference product failed to meet the requirement for similarity as the values for f2 were < 50. EFV failed to meet the dissolution criteria for similarity in 0.1M HCl only. In the acidic medium the release was particularly slow and only reached 80 % for the reference product at 120 min which may be a consequence of the low solubility of EFV in acidic media.

4.4 CONCLUSIONS

The performance of quality control testing on solid oral dosage forms containing ARV agents, including dissolution testing, is very important. Inadequate or slow release of ARV from formulations and may result in sub-therapeutic levels of the compounds in patients that may lead to treatment failure or promote the emergence of resistance. While bio-waivers are desirable since they are a cost effective and efficient approach to obtain regulatory approval for dosage forms when compared to bioequivalence studies, however the cost of treatment failure when treating this global epidemic is much greater. The RP-HPLC method developed and reported in earlier chapters of this thesis was successfully used in dissolution testing of commercially available dosage forms. The release of FTC in all dissolution media met the criteria for acceptance of immediate-release formulations containing BCS class 1 molecules. The release of TNF in all dissolution media was > 80 % but another

dissolution test in which a 15 min time point is included may confirm if TNF release from these dosage forms would indeed qualify for a bio-waiver. The addition of Aspen Efavirenz® and Truvada® to the same dissolution vessel when performing dissolution testing of the reference products does not appear to have limited the release of EFV and EFV will not qualify for a bio-waiver. The formulation of test and references products seems to have a significant effect on the release of these molecules, however this dissolution method developed and used in these studies reveals discrimination between the test and reference products. The co-formulation of EFV with FTC and TNF would require bioequivalence testing in conjunction with pharmacokinetic studies to quantitate the ARV in biological fluids and therefore the value of dissolution testing is for quality control purposes only in this case.

CHAPTER FIVE

CONCLUSIONS

The use of a FDC tablet containing EFV, FTC and TNF for the treatment of HIV-1-infected people is the simplest form of ART available [6] and is particularly useful in a South African context where a large proportion of the population is affected by HIV and the reduced pill burden may promote adherence to therapy in low health literate populations [6, 11, 12, 18]. The use of a FDC is recommended as first-line therapy and wide-spread use of this approach in the future requires the development of simple and rapid validated analytical methods for the simultaneous analysis of such combinations to ensure rapid product release from manufacturers to meet the increasing demand for ARV therapy. Furthermore the development of an analytical method with more than one application for testing of such compounds in combination has not yet been investigated or reported.

Cyano-based stationary phases exhibit intermediate polarity and can be used for NP- and RP-HPLC applications [186, 187, 189]. The use of a cyano column for gradient HPLC analysis of FTC, TNF and EFV has been reported [183], however the long run time reported in that method makes it time consuming, wasteful in respect of solvent use and unnecessary. The development of a simple and rapid RP-HPLC method using a cyano stationary phase was therefore investigated. Initially an isocratic separation was evaluated and the type of organic solvent, buffer characteristics, mobile phase composition and flow rate were manipulated to assess the impact of these parameter changes on the retention times of FTC, TNF and EFV. Although easy to perform with a resultant high degree of predictability and reproducibility, an isocratic separation was not suitable for the simultaneous analysis of FTC, TNF and EFV.

Isocratic separations are characterised by low peak capacity and the separation of analytes that vary significantly in terms of polarity becomes extremely difficult. FTC, a weakly basic hydrophilic molecule with a pKa of 2.6 is unionised at two or more pH units around the pKa. Therefore the compound will largely be unionised at a pH of 4 and higher. FTC elutes first due to its high polarity and affinity for the mobile phase and elutes very close to the void, indicating that it is almost an un-retained compound despite being unionised at the pH used in these studies. This short analysis time may also be problematic if the method were to be applied to the analysis of FTC in plasma or in stability studies. The presence of residual proteins and salts in a sample even following sample preparation using SPE may interfere with the quantitation of FTC since these residual proteins and salts elute close to the void. The pKa of TNF is 3.76 and it is a weakly basic and hydrophilic molecule but is less polar than FTC, whereas EFV is a weakly acidic hydrophobic molecule that is present in the unionised form at two or more pH units below its pKa of 10.2. EFV has the highest affinity for the CN stationary phase and thus is retained for longer on the column. Manipulation of the buffer concentration and pH within the recommended ranges for use with the stationary phase had no

significant effect on the retention times of FTC, TNF and EFV and the use of a mobile phase with a high proportion of buffer resulted in an even longer delay in the elution of EFV and a loss in analytical efficiency. When the proportion of the aqueous component of the mobile phase was increased to 95 % v/v EFV and TNF did not elute from the column after 60 min. However under these conditions the retention time of FTC increased with a resulting good peak shape and adequate separation efficiency. Cleary a compromise in the mobile phase composition was required to effect an adequate separation of all three compounds. Furthermore a slow flow rate did not appear to prolong the retention time of FTC sufficiently however it did result in a decrease in column efficiency and an increase in the void time and flow rate manipulation is therefore not an ideal approach to control the retention time of analytes. Efficiency and retention time should not be the only criteria taken into consideration when using a slower flow rate, as the number of column plates was still > 2000 plates for FTC, which indicates the column and method is adequate to facilitate an efficient separation. Ultimately a buffer concentration of 0.03 M of pH 4 was used to maintain peak shape and ensure method reproducibility. However despite some advantages and possible benefits of using an isocratic method for the analysis of the ARV in the FDC, it was decided to look at alternative approaches to HPLC analysis.

A gradient RP separation method was then investigated in an attempt to overcome the challenges experienced with developing an isocratic separation for FTC, TNF and EFV. Gradient elution is a powerful alternative to isocratic method of analysis since the mobile phase composition can be changed over the course of a chromatographic separation. The use of gradient elution results in enhanced peak capacity and resolution. Initial gradient conditions were set at 95 % v/v 0.03M phosphate buffer of pH 4. The buffer content was maintained at 95 % v/v for one min to ensure that FTC was retained for at least 2.9 min. An adequate gradient steepness was achieved by increasing the acetonitrile content linearly from 5 % v/v to 70 % v/v and a steep gradient improved the resolution between TNF and EFV through increasing the acetonitrile content from 1 to 8 min. Maintenance of the acetonitrile content at 70 % v/v for two min resulted in the elution of TNF and EFV in under 10 min, which resulted in an efficient analytical separation with a relatively short analysis time. However a 15 min run time was required to allow for column re-equilibration prior to the next injection to maintain the reproducibility of response between sample injections. Using the optimised gradient conditions the retention times for FTC, TNF and EFV were 2.9 min, 7.9 min and 9.1 min respectively. The retention factors for FTC, TNF and EFV are 0.7, 3.5 and 4.2 and higher efficiencies and better peak shape for TNF and EFV were achieved under gradient conditions than was observed for the isocratic separation.

ZD was used as an IS due to the relatively short retention time, similar wavelength of maximum absorption and the proximity of the FTC peak to the ZD. It was also suspected that there may be

challenges with the quantitation of FTC when this method was applied to the analysis of biological samples and that the use of ZD in that instance may enhance the reproducibility of the method.

The RP-HPLC gradient method was then validated according to ICH guidelines. Correlation coefficients > 0.999 were obtained for each assessment of linearity for each analyte indicating that the quantitation of FTC, TNF and EFV was linear within the ranges 0.4-40 µg/ml, 0.6-60 µg/ml and 12-120 µg/ml, respectively. The method also demonstrated adequate precision and accuracy and was successfully applied to the analysis of commercially available tablets containing these ARV compounds.

Forced degradation studies enable the identification of degradation products and can be used to demonstrate the stability-indicating power of a RP-HPLC method. Exposure of each analyte to harsh hydrolytic, oxidative and photolytic degradation conditions demonstrated that the validated RP-HPLC gradient method is indeed stability-indicting and is selective for the quantitative analysis FTC, TNF and EFV in dosage forms and during dissolution testing.

Low rates and amount of API released from solid oral dosage forms may result in sub-therapeutic levels of the API which could lead to treatment failure or the emergence of resistance to these ARV. The use of dissolution testing as a tool for routine quality control of solid oral dosage forms is important and dissolution testing was undertaken using tests media of 0.1 M HCl and buffers of pH = 4.5 and 6.8. The release of FTC in all dissolution media from commercially available Atripla® and Truvada® met the criteria for IR formulations that contain BCS class 1 molecules. The release of TNF and EFV did not meet the relevant criteria. The use of the difference and similarity factors to assess dissolution curve similarity indicated that the products were not similar in all dissolution media, and this is probably due to differences in formulation or method of manufacture about which we can only speculate, since definite knowledge of the formulations and method of manufacture are not public knowledge. The addition of Aspen Efavirenz® and Truvada® to the same dissolution test vessel when performing dissolution testing on the reference products did not appear to have limited the release of EFV, however bioequivalence testing in conjunction with pharmacokinetic analysis of drug concentrations in biological fluids are recommended. It is evident that the validated gradient RP-HPLC method is suitable for application to the assay of dosage forms and *in-vitro* dissolution studies.

When analytes in a mobile phase with a high aqueous content are injected onto an analytical column the analytes concentrate at the head of the column. Analytes partition from the stationary phase into the mobile phase as the strength of the mobile phase increases. This is also true for contaminants, and this shortcoming was observed when the method was evaluated for use in the analysis of FTC, TNF and EFV in plasma. Furthermore the method lacks the necessary sensitivity to analyse the low concentrations expected when testing plasma samples. The source of the contamination was not identified despite a systematic investigation into the possible source of contaminants, thus the method

is not suitable for use in the analysis of FTC, TNF and EFV in plasma. As an alternative to UV detection, the HPLC system may be coupled with a mass spectrometer as the detector is more selective and sensitive than UV detectors [185], and therefore the development of a method using this mode of detection should be considered .

The formulation of multiple molecules into a single dosage form is not a new concept and is likely to become the norm for treating complex and difficult diseases. The combination of FTC, TNF and EFV is an example of a complex dosage form for which an analytical separation for simultaneous analysis is necessary, and it is likely that more FDC tablets will be available in the future. It is important to have knowledge about the physico-chemical properties of the molecules being formulated as this is vital to develop an appropriate method of analysis. Where possible a method analysis for all API in a FDC that is applicable to pharmacokinetic, quality control and raw material testing for all API simultaneously would reduce analytical costs and improve analytical efficiency.

An analytical method using gradient RP-HPLC hase been successfully developed and validated to simultaneously analyse EFV, FTC and TNF using a 0.03 M, pH 4 phosphate buffer and acetonitile as the mobile phase. A relatively short run time of 15 min was achieved with a flow rate of 1.0 ml/min. Buffer composition was set at 95 % from 0-1 min, then from 1-8 min the buffer composition was decreased from 95 % to 30 % and held for 2 min. At 10-11 min the composition of buffer was again increased to 95 % and maintained until 15 min. FTC, TNF and EFV were monitored at 260 nm and retetention times of FTC, TNF and EFV at 2.9, 7.9 and 9.1 min respectively.

REFERENCES

[1] F. Maggiolo, "Efavirenz: a decade of clinical experience in the treatment of HIV," *J. Antimicrob. Chemother.*, vol. 64, no. 5, pp. 910–928, 2009.

[2] P. R. Harrigan, R. S. Hogg, W. W. Y. Dong, B. Yip, B. Wynhoven, J. Woodward, C. J. Brumme, Z. L. Brumme, T. Mo, C. S. Alexander, and J. S. G. Montaner, "Predictors of HIV drug-resistance mutations in a large antiretroviral-naive cohort initiating triple antiretroviral therapy," *J. Infect. Dis.*, vol. 191, no. 3, pp. 339–347, 2005.

[3] J. M. Molina, F. Ferchal, C. Rancinan, F. Raffi, W. Rozenbaum, D. Sereni, P. Morlat, V. Journot, J. M. Decazes, and G. Chêne, "Once-daily combination therapy with emtricitabine, didanosine, and efavirenz in human immunodeficiency virus-infected patients," *J. Infect. Dis.*, vol. 182, no. 2, pp. 599–602, 2000.

[4] J. E. Gallant, S. Staszewski, A. L. Pozniak, E. DeJesus, J. M. A. H. Suleiman, M. D. Miller, D. F. Coakley, B. Lu, J. J. Toole, A. K. Cheng, and 903 Study Group, "Efficacy and safety of tenofovir DF vs stavudine in combination therapy in antiretroviral-naive patients: a 3-year randomized trial," *JAMA*, vol. 292, no. 2, pp. 191–201, 2004.

[5] V. D. Lima, P. R. Harrigan, M. Sénécal, B. Yip, E. Druyts, R. S. Hogg, and J. S. G. Montaner, "Epidemiology of antiretroviral multiclass resistance," *Am. J. Epidemiol.*, vol. 172, no. 4, pp. 460–468, 2010.

[6] J. M. Llibre and B. Clotet, "Once-daily single-tablet regimens: a long and winding road to excellence in antiretroviral treatment," *AIDS Rev.*, vol. 14, no. 3, pp. 168–178, 2012.

[7] I. A. Echenique and J. D. Rich, "EFV/FTC/TDF-associated hepatotoxicity: a case report and review," *AIDS Patient Care STDs*, vol. 27, no. 9, pp. 493–497, 2013.

[8] Aspen Pharmacare, "TribussTM," 2011. [Online]. Available: http://www.sahivsoc.org/upload/documents/news_partner_TRIBUSS.pdf. [Accessed: 07-Feb-2014].

[9] T. Zamisa, "HIV patients revel in the one pill a day age," 2014. [Online]. Available: http://www.health-e.org.za/2014/06/24/hiv-patients-revel-one-pill-day-age/.

[10] European Medicines Agency, "Summary of product characteristics: Viread 245 mg film-coated tablets," 2007. [Online]. Available: http://ec.europa.eu/health/documents/community-register/2010/2010090686009/anx_86009_en.pdf.

[11] E. D. Deeks and C. M. Perry, "Efavirenz/emtricitabine/tenofovir disoproxil fumarate single-tablet regimen (Atripla®): a review of its use in the management of HIV infection," *Drugs*, vol. 70, no. 17, pp. 2315–2338, 2010.

[12] M. Takahashi, Y. Kudaka, N. Okumura, A. Hirano, K. Banno, and T. Kaneda, “Determination of plasma tenofovir concentrations using a conventional LC-MS method,” *Biol. Pharm. Bull.*, vol. 30, no. 9, pp. 1784–1786, 2007.

[13] S. Purser, P. R. Moore, S. Swallow, and V. Gouverneur, “Fluorine in medicinal chemistry,” *Chem. Soc. Rev.*, vol. 37, no. 2, pp. 320–330, 2008.

[14] S. Sathigari, G. Chadha, Y.-H. P. Lee, N. Wright, D. L. Parsons, V. K. Rangari, O. Fasina, and R. J. Babu, “Physicochemical characterization of efavirenz-cyclodextrin inclusion complexes,” *AAPS PharmSciTech*, vol. 10, no. 1, pp. 81–87, 2009.

[15] S. D. Young, S. F. Britcher, L. O. Tran, L. S. Payne, W. C. Lumma, T. A. Lyle, J. R. Huff, P. S. Anderson, D. B. Olsen, and S. S. Carroll, “L-743, 726 (DMP-266): a novel, highly potent nonnucleoside inhibitor of the human immunodeficiency virus type 1 reverse transcriptase,” *Antimicrob. Agents Chemother.*, vol. 39, no. 12, pp. 2602–2605, 1995.

[16] Gilead Sciences and Bristol-Myers Squibb LLC, “Product Information ATRIPLA® (tenofovir disoproxil fumarate, emtricitabine and efavirenz) tablets,” 2009. [Online]. Available: http://www.ashm.org.au/images/prescriber/atripla_pi_v2%20_08oct09_.pdf.

[17] N. Ford, L. Mofenson, K. Kranzer, L. Medu, L. Frigati, E. J. Mills, and A. Calmy, “Safety of efavirenz in first-trimester of pregnancy: a systematic review and meta-analysis of outcomes from observational cohorts,” *AIDS Lond. Engl.*, vol. 24, no. 10, pp. 1461–1470, 2010.

[18] Department of Health, Republic of South Africa, “National consolidated guidelines for the prevention of mother-to-child transmission of HIV (PMTCT) and the management of HIV in children, adolescents and adults.” 2014.

[19] Gilead Sciences Inc, “Product information: SUSTIVA® oral capsules.” 2013.

[20] N. N. Bumpus, U. M. Kent, and P. F. Hollenberg, “Metabolism of efavirenz and 8-hydroxyefavirenz by P450 2B6 leads to inactivation by two distinct mechanisms,” *J. Pharmacol. Exp. Ther.*, vol. 318, no. 1, pp. 345–351, 2006.

[21] E. T. Ogburn, D. R. Jones, A. R. Masters, C. Xu, Y. Guo, and Z. Desta, “Efavirenz primary and secondary metabolism in vitro and in vivo: identification of novel metabolic pathways and cytochrome P450 2A6 as the principal catalyst of efavirenz 7-hydroxylation,” *Drug Metab. Dispos. Biol. Fate Chem.*, vol. 38, no. 7, pp. 1218–1229, 2010.

[22] V. Michaud, E. Ogburn, N. Thong, A. O. Aregbe, T. C. Quigg, D. A. Flockhart, and Z. Desta, “Induction of CYP2C19 and CYP3A activity following repeated administration of efavirenz in healthy volunteers,” *Clin. Pharmacol. Ther.*, vol. 91, no. 3, pp. 475–482, 2012.

[23] P. D. Hamarapurkar and A. N. Parate, “HPLC method for the determination of emtricitabine and related degradation substances,” *J. Chromatogr. Sci.*, vol. 51, no. 5, pp. 419–424, 2013.

[24] Division of Clinical Pharmacology Faculty of Health Sciences University of Cape Town, *South African Medicines Formulary*, 9th ed. 2010.

[25] K. Susmitha, M. Thirumalachary, T. C. Singh, and G. Venkateshwarlu, “Extractive spectrophotometric determination of tenofovir disoproxil fumarate using acidic triphenylmethane dyes,” *Int. Sch. Res. Not.*, vol. 2014, p. e856760, 2014.

[26] L. Menéndez-Arias, “Mechanisms of resistance to nucleoside analogue inhibitors of HIV-1 reverse transcriptase,” *Virus Res.*, vol. 134, no. 1–2, pp. 124–146, 2008.

[27] A. Chen and D. W. Dowdy, “Clinical Effectiveness and Cost-Effectiveness of HIV Pre-Exposure Prophylaxis in Men Who Have Sex with Men: Risk Calculators for Real-World Decision-Making,” *PLoS ONE*, vol. 9, no. 10, p. e108742, 2014.

[28] Medicines Control Council, “Press release: Medicines Control Council approves fixed-dose combination of tenofovir disoproxyl fumarate and emtricitabine for pre-exposure prophylaxis of HIV.” Department of Health, 2015.

[29] V. Varghese, E. Wang, F. Babrzadeh, M. H. Bachmann, R. Shahriar, T. Liu, S. J. M. Mappala, B. Gharizadeh, W. J. Fessel, D. Katzenstein, S. Kassaye, and R. W. Shafer, “Nucleic acid template and the risk of a PCR-Induced HIV-1 drug resistance mutation,” *PLoS ONE*, vol. 5, no. 6, p. e10992, 2010.

[30] S. Boucher, P. Recordon-Pinson, J. M. Ragnaud, M. Dupon, H. Fleury, and B. Masquelier, “HIV-1 reverse transcriptase (RT) genotypic patterns and treatment characteristics associated with the K65R RT mutation,” *HIV Med.*, vol. 7, no. 5, pp. 294–298, 2006.

[31] Food and Drug Administration, “Full prescribing information, ATRIPLA® (efavirenz/emtricitabine/tenofovir disoproxil fumarate) tablets.” Gilead Sciences, 2013.

[32] J. E. Gallant, M. A. Parish, J. C. Keruly, and R. D. Moore, “Changes in renal function associated with tenofovir disoproxil fumarate treatment, compared with nucleoside reverse-transcriptase inhibitor treatment,” *Clin. Infect. Dis. Off. Publ. Infect. Dis. Soc. Am.*, vol. 40, no. 8, pp. 1194–1198, 2005.

[33] T. Chapman, J. McGavin, and S. Noble, “Tenofovir disoproxil fumarate,” *Drugs*, vol. 63, no. 15, pp. 1597–1608, 2003.

[34] S. K. Gupta, A. M. Anderson, R. Ebrahimi, T. Fralich, H. Graham, V. Scharen-Guivel, J. F. Flaherty, C. Fortin, R. C. Kalayjian, A. Rachlis, and C. M. Wyatt, “Fanconi syndrome accompanied by renal function decline with tenofovir disoproxil fumarate: a prospective, case-control study of predictors and resolution in HIV-infected patients,” *PLoS ONE*, vol. 9, no. 3, p. e92717, 2014.

[35] L. Calza, F. Trapani, C. Salvadori, E. Magistrelli, R. Manfredi, V. Colangeli, M. A. Di Bari, M. Borderi, and P. Viale, “Incidence of renal toxicity in HIV-infected, antiretroviral-naïve patients starting tenofovir/emtricitabine associated with efavirenz, atazanavir/ritonavir, or lopinavir/ritonavir,” *Scand. J. Infect. Dis.*, vol. 45, no. 2, pp. 147–154, 2013.

[36] S. G. Deeks, P. Barditch-Crovo, P. S. Lietman, F. Hwang, K. C. Cundy, J. F. Rooney, N. S. Hellmann, S. Safrin, and J. O. Kahn, “Safety, pharmacokinetics, and antiretroviral activity of

intravenous 9-[2-(R)-(Phosphonomethoxy)propyl]adenine, a novel anti-human immunodeficiency virus (HIV) therapy, in HIV-infected adults," *Antimicrob. Agents Chemother.*, vol. 42, no. 9, pp. 2380–2384, 1998.

[37] European Medicines Agency, "Summary of product characteristics: Atripla 600 mg/200 mg/245 mg film-coated tablets," 2012. [Online]. Available: http://www.ema.europa.eu/docs/en_GB/document_library/EPAR_-_Product_Information/human/000797/WC500028102.pdf. [Accessed: 07-Feb-2014].

[38] World Health Organisation, *Efavirenz monograph*. 2005.

[39] AIDS Info, "Efavirenz," 2014. [Online]. Available: http://aidsinfo.nih.gov/drugs/269/efavirenz/0/professional. [Accessed: 07-Apr-2014].

[40] World Health Organisation, *Emtricitabine monograph*, 4th ed. The International Pharmacopoiea, 2009.

[41] AIDS Info, "Emtricitabine," 2014. [Online]. Available: http://aidsinfo.nih.gov/drugs/208/emtricitabine/0/professional. [Accessed: 07-Feb-2014].

[42] National Center for Biotechnology Information, "Emtricitabine," 2005. [Online]. Available: http://pubchem.ncbi.nlm.nih.gov/compound/Emtricitabine. [Accessed: 22-Oct-2014].

[43] World Health Organisation, *Tenofovir monograph*. 2010.

[44] AIDS Info, "Tenofovir disoproxil fumarate," 2013. [Online]. Available: http://aidsinfo.nih.gov/drugs/290/tenofovir-disoproxil-fumarate/0/professional. [Accessed: 07-Apr-2014].

[45] Food and Drug Administration, "Full prescribing information, COMPLERA® (emtricitabine/rilpivirine/tenofovir disoproxil fumarate)." Gilead Sciences, 2014.

[46] R. Cristofoletti, A. Nair, B. Abrahamsson, D. W. Groot, S. Kopp, P. Langguth, J. E. Polli, V. P. Shah, and J. B. Dressman, "Biowaiver monographs for immediate release solid oral dosage forms: efavirenz," *J. Pharm. Sci.*, vol. 102, no. 2, pp. 318–329, 2013.

[47] S. R. Rabel, M. Patel, S. Sun, and M. B. Maurin, "Electronic and resonance effects on the ionization of structural analogues of efavirenz," *AAPS PharmSci*, vol. 3, no. 4, p. E28, 2001.

[48] AIDS Info, "Emtricitabine/tenofovir disoproxil fumarate," 2015. [Online]. Available: http://aidsinfo.nih.gov/drugs/406/truvada/0/professional. [Accessed: 03-Sep-2015].

[49] R. I. Mahato and A. S. Narang, *Pharmaceutical dosage forms and drug delivery*, 2nd ed. CRC Press, 2011.

[50] K. Valkó, "Application of high-performance liquid chromatography based measurements of lipophilicity to model biological distribution," *J. Chromatogr. A*, vol. 1037, no. 1–2, pp. 299–310, 2004.

[51] T. Takagi, C. Ramachandran, M. Bermejo, S. Yamashita, L. X. Yu, and G. L. Amidon, "A Provisional Biopharmaceutical Classification of the Top 200 Oral Drug Products in the United States, Great Britain, Spain, and Japan," *Mol. Pharm.*, vol. 3, no. 6, pp. 631–643, 2006.

[52] Food and Drug Administration, “Guidance for industry: dissolution testing of immediate release solid oral dosage forms.” Centre for Drug Evaluation and Research (CDER), 1997.

[53] A. A. Mathias, J. Hinkle, M. Menning, J. Hui, S. Kaul, B. P. Kearney, and Efavirenz/Emtricitabine/Tenofovir Disoproxil Fumarate Single-Tablet Regimen Development Team, “Bioequivalence of efavirenz/emtricitabine/tenofovir disoproxil fumarate single-tablet regimen,” *J. Acquir. Immune Defic. Syndr. 1999*, vol. 46, no. 2, pp. 167–173, 2007.

[54] World Health Organisation, “General notes on biopharmaceutics classification system (BCS)-based biowaiver applications,” 2011. [Online]. Available: http://apps.who.int/prequal/info_applicants/BE/BW_general_2011November.pdf. [Accessed: 22-Oct-2014].

[55] World Health Organisation, “Tenofovir disoproxil fumarate WHOPAR part 6,” 2010. [Online]. Available: http://apps.who.int/prequal/whopar/whoparproducts/HA423part6v1.pdf. [Accessed: 22-Oct-2014].

[56] Drug Bank, “Efavirenz,” 2013. [Online]. Available: http://www.drugbank.ca/drugs/db00625. [Accessed: 07-Apr-2014].

[57] Drug Bank, “Emtricitabine,” 2013. [Online]. Available: http://www.drugbank.ca/drugs/DB00879. [Accessed: 07-Apr-2014].

[58] Drug Bank, “Tenofovir,” 2013. [Online]. Available: http://www.drugbank.ca/drugs/DB00300. [Accessed: 07-Apr-2014].

[59] S. A. Ghorpade, M. S. Sali, A. H. Kategaonkar, D. M. Patel, V. P. Choudhari, and B. S. Kuchekar, “Simultaneous determination of emtricitabine and tenofovir by area under curve and dual wavelength spectrophotometric method,” *J. Chil. Chem. Soc.*, vol. 55, no. 1, 2010.

[60] V. Viswanath, P. Shanmugasundaram, and V. Ravichandiran, “UV-spectrophotometric absorbance correction method for the simultaneous estimation of tenofovir disoproxil fumerate and emtricitabine in combined ablet dosage form,” vol. 5, no. 3, pp. 1179–1185, 2013.

[61] B. Lavanya, P. Hariprasad, A. Venkatapraveen, and D. P. Lakshmi, “Method development and validation of combined tablet dosage form of emtricitabine and tenofovir disoproxil fumarate by ultraviolet spectroscopy,” *IRJP*, vol. 3, no. 12, pp. 104–108, 2012.

[62] S. Mogatle and I. Kanfer, “Rapid method for the quantitative determination of efavirenz in human plasma,” *J. Pharm. Biomed. Anal.*, vol. 49, no. 5, pp. 1308–1312, 2009.

[63] S. Hansen, S. Pedersen-Bjergaard, and K. Rasmussen, *Introduction to pharmaceutical chemical analysis*. John Wiley & Sons, 2011.

[64] World Health Organisation, “The International Pharmacopoeia 5th ed,” 2015. [Online]. Available: http://apps.who.int/phint/en/p/docf/.

[65] N. Sluis-Cremer and G. Tachedjian, “Mechanisms of inhibition of HIV replication by non-nucleoside reverse transcriptase inhibitors,” *Virus Res.*, vol. 134, no. 1–2, pp. 147–156, 2008.

[66] A. Ranise, A. Spallarossa, S. Cesarini, F. Bondavalli, S. Schenone, O. Bruno, G. Menozzi, P. Fossa, L. Mosti, M. La Colla, G. Sanna, M. Murreddu, G. Collu, B. Busonera, M. E. Marongiu, A. Pani, P. La Colla, and R. Loddo, "Structure-based design, parallel synthesis, structure-activity relationship, and molecular modeling studies of thiocarbamates, new potent non-nucleoside HIV-1 reverse transcriptase inhibitor isosteres of phenethylthiazolylthiourea derivatives," *J. Med. Chem.*, vol. 48, no. 11, pp. 3858–3873, 2005.

[67] J. Ren and D. K. Stammers, "Structural basis for drug resistance mechanisms for non-nucleoside inhibitors of HIV reverse transcriptase," *Virus Res.*, vol. 134, no. 1–2, pp. 157–170, 2008.

[68] D. Ellis, K. L. Kuhen, B. Anaclerio, B. Wu, K. Wolff, H. Yin, B. Bursulaya, J. Caldwell, D. Karanewsky, and Y. He, "Design, synthesis, and biological evaluations of novel quinolones as HIV-1 non-nucleoside reverse transcriptase inhibitors," *Bioorg. Med. Chem. Lett.*, vol. 16, no. 16, pp. 4246–4251, 2006.

[69] M. Patel, R. J. McHugh, B. C. Cordova, R. M. Klabe, L. T. Bacheler, S. Erickson-Viitanen, and J. D. Rodgers, "Synthesis and evaluation of novel quinolinones as HIV-1 reverse transcriptase inhibitors," *Bioorg. Med. Chem. Lett.*, vol. 11, no. 14, pp. 1943–1945, Jul. 2001.

[70] R. Vyas, W. J. Zahurancik, and Z. Suo, "Structural basis for the binding and incorporation of nucleotide analogs with L-stereochemistry by human DNA polymerase λ," *Proc. Natl. Acad. Sci. U. S. A.*, vol. 111, no. 30, pp. E3033–3042, 2014.

[71] P. Chearskul, C. Rongkavilit, H. Al-Tatari, and B. Asmar, "New antiretroviral drugs in clinical use," *Indian J. Pediatr.*, vol. 73, no. 4, pp. 335–341, 2006.

[72] V. Gaur, R. Vyas, J. D. Fowler, G. Efthimiopoulos, J. Y. Feng, and Z. Suo, "Structural and kinetic insights into binding and incorporation of L-nucleotide analogs by a Y-family DNA polymerase," *Nucleic Acids Res.*, vol. 42, no. 15, pp. 9984–9995, 2014.

[73] P. Liu, A. Sharon, and C. K. Chu, "Fluorinated nucleosides: synthesis and biological implication," *J. Fluor. Chem.*, vol. 129, no. 9, pp. 743–766, 2008.

[74] A. S. Ray, R. F. Schinazi, E. Murakami, A. Basavapathruni, J. Shi, S. M. Zorca, C. K. Chu, and K. S. Anderson, "Probing the mechanistic consequences of 5-fluorine substitution on cytidine nucleotide analogue incorporation by HIV-1 reverse transcriptase," *Antivir. Chem. Chemother.*, vol. 14, no. 3, pp. 115–125, 2003.

[75] A. Klug, "The discovery of the DNA double helix: historical perspective," *J. Mol. Biol.*, no. 335, pp. 3–26, 2004.

[76] E. D. Clercq and A. Holý, "Acyclic nucleoside phosphonates: a key class of antiviral drugs," *Nat. Rev. Drug Discov.*, vol. 4, no. 11, pp. 928–940, 2005.

[77] K. Y. Hostetler, "Alkoxyalkyl prodrugs of acyclic nucleoside phosphonates enhance oral antiviral activity and reduce toxicity: current state of the art," *Antiviral Res.*, vol. 82, no. 2, pp. A84–98, 2009.

[78] A. Holý, “Antiviral acyclic nucleoside phosphonates structure activity studies,” *Antiviral Res.*, vol. 71, no. 2–3, pp. 248–253, 2006.

[79] J. C. M. William A Lee, “Perspectives on the development of acyclic nucleotide analogs as antiviral drugs,” *Antiviral Res.*, vol. 71, no. 2–3, pp. 254–9, 2006.

[80] E. De Clercq, “Antiviral drug discovery and development: where chemistry meets with biomedicine,” *Antiviral Res.*, vol. 67, no. 2, pp. 56–75, 2005.

[81] D. Jochmans, “Novel HIV-1 reverse transcriptase inhibitors,” *Virus Res.*, vol. 134, no. 1–2, pp. 171–185, 2008.

[82] P. Hamrapurkar, P. Patil, M. Phale, N. Shah, and S. Pawar, “Optimization and validation of RP-HPLC stability-indicating method for determination of efavirenz and its degradation products,” *Int. J. Appl. Sci. Eng.*, vol. 8, no. 2, pp. 155–165, 2010.

[83] T. Gadkari, P. Chandrachood, A. Ruikar, S. Tele, N. Deshpande, J. Salvekar, and Sonawane S, “Validated stability indicating LC-PDA-MS method to investigate pH rate profile and degradation kinetics of efavirenz and identification of hydrolysis product by LCMS,” vol. 2, no. 1, 2010.

[84] R. Kumar, M. Sharma, and G. R. Verma, “Stability indicating analytical method development and validation of efavirenz quantification by high performance liquid chromatographic technique,” *J. Chem.*, vol. 8, no. 4, pp. 1498–1503, 2011.

[85] P. S. Devrukhakar, R. Borkar, N. Shastri, and K. V. Surendranath, “A validated stability-indicating RP-HPLC method for the simultaneous determination of tenofovir, emtricitabine, and a efavirenz and statistical approach to determine the effect of variables,” *Int. Sch. Res. Not.*, vol. 2013, p. e878295, 2013.

[86] M. B. Maurin, S. M. Rowe, K. Blom, and M. E. Pierce, “Kinetics and mechanism of hydrolysis of efavirenz,” *Pharm. Res.*, vol. 19, no. 4, pp. 517–521, 2002.

[87] C. Dykes, K. Fox, A. Lloyd, M. Chiulli, E. Morse, and L. M. Demeter, “Impact of clinical reverse transcriptase sequences on the replication capacity of HIV-1 drug-resistant mutants,” *Virology*, vol. 285, no. 2, pp. 193–203, 2001.

[88] J. Cohen, “Raising the limits,” *Science*, vol. 296, no. 5577, pp. 2322–2322, 2002.

[89] V. A. Braz, L. A. Holladay, and M. D. Barkley, “Efavirenz binding to HIV-1 reverse transcriptase monomers and dimers,” *Biochemistry (Mosc.)*, vol. 49, no. 3, pp. 601–610, 2010.

[90] K. Das, P. J. Lewi, S. H. Hughes, and E. Arnold, “Crystallography and the design of anti-AIDS drugs: conformational flexibility and positional adaptability are important in the design of non-nucleoside HIV-1 reverse transcriptase inhibitors,” *Prog. Biophys. Mol. Biol.*, vol. 88, no. 2, pp. 209–231, 2005.

[91] J. Y. Feng, J. K. Ly, F. Myrick, D. Goodman, K. L. White, E. S. Svarovskaia, K. Borroto-Esoda, and M. D. Miller, “The triple combination of tenofovir, emtricitabine and efavirenz

shows synergistic anti-HIV-1 activity in vitro: a mechanism of action study," *Retrovirology*, vol. 6, p. 44, 2009.

[92] P. Srivastava, G. S. Moorthy, R. Gross, and J. S. Barrett, "A sensitive and selective liquid chromatography/tandem mass spectrometry method for quantitative analysis of efavirenz in human plasma," *PLoS ONE*, vol. 8, no. 6, p. e63305, 2013.

[93] South African Electronic Package Inserts, "ASPEN EFAVIRENZ 600 mg (tablets)," 2008. .

[94] Gilead Sciences Inc, "Product Information: ATRIPLA(TM) oral tablets." Gilead Sciences Inc, 2006.

[95] J.-M. Molina, P. Cahn, B. Grinsztejn, A. Lazzarin, A. Mills, M. Saag, K. Supparatpinyo, S. Walmsley, H. Crauwels, L. T. Rimsky, S. Vanveggel, K. Boven, and ECHO study group, "Rilpivirine versus efavirenz with tenofovir and emtricitabine in treatment-naive adults infected with HIV-1 (ECHO): a phase 3 randomised double-blind active-controlled trial," *Lancet*, vol. 378, no. 9787, pp. 238–246, 2011.

[96] Department of Health, South Africa, "The South African antiretroviral treatment guidelines," 2013. [Online]. Available: http://www.kznhealth.gov.za/medicine/2013_art_guidelines.pdf.

[97] Gilead Sciences Inc, "Product Information: TRUVADA(TM)." Gilead Sciences Inc, 2004.

[98] A. Soni and Thakral, "Simultaneous estimation of tenofovir and emtricitabine in human plasma using HPLC after protein precipitation extraction," *J. Anal. Bioanal. Tech.*, vol. 04, no. 03, 2013.

[99] South African Electronic Package Insert, "TRUVADA® tablets," 2007. [Online]. Available: http://home.intekom.com/pharm/aspen-p/truvada.html. [Accessed: 11-Feb-2014].

[100] South African Electronic Package Insert, "VIREAD® tablets," 2008. [Online]. Available: http://home.intekom.com/pharm/aspen-p/viread.html.

[101] P. Hynes, A. Urbina, A. McMeeking, L. Barisoni, and R. Rabenou, "Acute renal failure after initiation of tenofovir disoproxil fumarate," *Ren. Fail.*, vol. 29, no. 8, pp. 1063–1066, 2007.

[102] J. E. Frampton and C. M. Perry, "Emtricitabine: a review of its use in the management of HIV infection," *Drugs*, vol. 65, no. 10, pp. 1427–1448, 2005.

[103] P. L. Anderson, J. J. Kiser, E. M. Gardner, J. E. Rower, A. Meditz, and R. M. Grant, "Pharmacological considerations for tenofovir and emtricitabine to prevent HIV infection," *J. Antimicrob. Chemother.*, vol. 66, no. 2, pp. 240–250, 2011.

[104] Food and Drug Administration, "Full prescribing information , EMTRIVA® (emtricitabine)." Gilead Sciences, 2012.

[105] T. Hawkins, "Understanding and managing the adverse effects of antiretroviral therapy," *Antiviral Res.*, vol. 85, no. 1, pp. 201–209, 2010.

[106] T. Isaacs, M. R. Ngwanya, S. Dlamini, and R. J. Lehloenya, "Annular erythema and photosensitivity as manifestations of efavirenz-induced cutaneous reactions: a review of five consecutive cases," *J. Antimicrob. Chemother.*, vol. 68, no. 12, pp. 2871–2874, 2013.

[107] A. Carr and D. A. Cooper, “Adverse effects of antiretroviral therapy,” *Lancet*, vol. 356, no. 9239, pp. 1423–1430, 2000.

[108] J. M. Leung, J. G. O’Brien, H. K. Wong, and D. L. Winslow, “Efavirenz-induced hypersensitivity reaction manifesting in rash and hepatitis in a Latino male,” *Ann. Pharmacother.*, vol. 42, no. 3, pp. 425–429, 2008.

[109] G. Pistone, A. Pistone, D. Sorbello, E. Viviano, and M. R. Bongiorno, “Cutaneous adverse reactions to highly antiretroviral therapy in HIV-positive patients,” *Case Rep. Dermatol.*, vol. 6, no. 2, pp. 145–149, 2014.

[110] F. S. Sarfo, M. A. Sarfo, B. Norman, R. Phillips, and D. Chadwick, “Incidence and determinants of nevirapine and efavirenz-related skin rashes in West Africans: nevirapine’s epitaph?,” *PLoS ONE*, vol. 9, no. 4, p. e94854, 2014.

[111] A. Rotunda, R. J. Hirsch, N. Scheinfeld, and J. M. Weinberg, “Severe cutaneous reactions associated with the use of human immunodeficiency virus medications,” *Acta Derm. Venereol.*, vol. 83, no. 1, pp. 1–9, 2003.

[112] J.-C. Zhang and Y.-T. Sun, “Efavirenz-induced exfoliative dermatitis,” *Scand. J. Infect. Dis.*, vol. 45, no. 1, pp. 70–72, 2013.

[113] V. Falcó, D. Rodríguez, E. Ribera, E. Martínez, J. M. Miró, P. Domingo, R. Diazaraque, J. R. Arribas, J. J. González-García, F. Montero, L. Sánchez, and A. Pahissa, “Severe nucleoside-associated lactic acidosis in human immunodeficiency virus-infected patients: report of 12 cases and review of the literature,” *Clin. Infect. Dis. Off. Publ. Infect. Dis. Soc. Am.*, vol. 34, no. 6, pp. 838–846, 2002.

[114] Y. Guo and H. B. Fung, “Fatal lactic acidosis associated with coadministration of didanosine and tenofovir disoproxil fumarate,” *Pharmacotherapy*, vol. 24, no. 8, pp. 1089–1094, 2004.

[115] M. D. Murphy, M. O’Hearn, and S. Chou, “Fatal lactic acidosis and acute renal failure after addition of tenofovir to an antiretroviral regimen containing didanosine,” *Clin. Infect. Dis. Off. Publ. Infect. Dis. Soc. Am.*, vol. 36, no. 8, pp. 1082–1085, 2003.

[116] Sulkowski MS, Thomas DL, Chaisson RE, and Moore RD, “Hepatotoxicity associated with antiretroviral therapy in adults infected with human immunodeficiency virus and the role of hepatitis c or b virus infection,” *JAMA*, vol. 283, no. 1, pp. 74–80, 2000.

[117] M. Núñez and V. Soriano, “Hepatotoxicity of antiretrovirals: incidence, mechanisms and management,” *Drug Saf.*, vol. 28, no. 1, pp. 53–66, 2005.

[118] H. H. Hirsch, G. Kaufmann, P. Sendi, and M. Battegay, “Immune reconstitution in HIV-infected patients,” *Clin. Infect. Dis. Off. Publ. Infect. Dis. Soc. Am.*, vol. 38, no. 8, pp. 1159–1166, 2004.

[119] Y. C. Manabe, J. D. Campbell, E. Sydnor, and R. D. Moore, “Immune reconstitution inflammatory syndrome: risk factors and treatment implications,” *J. Acquir. Immune Defic. Syndr. 1999*, vol. 46, no. 4, pp. 456–462, 2007.

[120] A. Drake, A. Mijch, and J. Sasadeusz, “Immune reconstitution hepatitis in HIV and hepatitis B coinfection, despite lamivudine therapy as part of HAART,” *Clin. Infect. Dis. Off. Publ. Infect. Dis. Soc. Am.*, vol. 39, no. 1, pp. 129–132, 2004.

[121] T. Puzantian, “Central nervous system adverse effects with efavirenz: case report and review,” *Pharmacotherapy*, vol. 22, no. 7, pp. 930–933, 2002.

[122] P. Lochet, H. Peyrière, A. Lotthé, J. M. Mauboussin, B. Delmas, and J. Reynes, “Long-term assessment of neuropsychiatric adverse reactions associated with efavirenz,” *HIV Med.*, vol. 4, no. 1, pp. 62–66, 2003.

[123] J. A. Muñoz-Moreno, C. R. Fumaz, M. J. Ferrer, M. González-García, J. Moltó, E. Negredo, and B. Clotet, “Neuropsychiatric symptoms associated with efavirenz: prevalence, correlates, and management. a neurobehavioral review,” *AIDS Rev.*, vol. 11, no. 2, pp. 103–109, 2009.

[124] F. Gutiérrez, A. Navarro, S. Padilla, R. Antón, M. Masiá, J. Borrás, and A. Martín-Hidalgo, “Prediction of neuropsychiatric adverse events associated with long-term efavirenz therapy, using plasma drug level monitoring,” *Clin. Infect. Dis. Off. Publ. Infect. Dis. Soc. Am.*, vol. 41, no. 11, pp. 1648–1653, 2005.

[125] A. M. Hall, “Update on tenofovir toxicity in the kidney,” *Pediatr. Nephrol. Berl. Ger.*, vol. 28, no. 7, pp. 1011–1023, 2013.

[126] M. E. Haverkort, B. W. van der Spek, P. Lips, W. A. Slieker, R. ter Heine, A. D. Huitema, and W. Bronsveld, “Tenofovir-induced Fanconi syndrome and osteomalacia in two HIV-infected patients: role of intracellular tenofovir diphosphate levels and review of the literature,” *Scand. J. Infect. Dis.*, vol. 43, no. 10, pp. 821–826, 2011.

[127] K. Klassen, A. R. Martineau, R. J. Wilkinson, G. Cooke, A. P. Courtney, and M. Hickson, “The effect of tenofovir on vitamin D metabolism in HIV-infected adults is dependent on sex and ethnicity,” *PLoS ONE*, vol. 7, no. 9, p. e44845, 2012.

[128] G. A. McComsey, D. Kitch, E. S. Daar, C. Tierney, N. C. Jahed, P. Tebas, L. Myers, K. Melbourne, B. Ha, and P. E. Sax, “Bone mineral density and fractures in antiretroviral-naive persons randomized to receive abacavir-lamivudine or tenofovir disoproxil fumarate-emtricitabine along with efavirenz or atazanavir-ritonavir: Aids Clinical Trials Group A5224s, a substudy of ACTG A5202,” *J. Infect. Dis.*, vol. 203, no. 12, pp. 1791–1801, 2011.

[129] H. Haskelberg, N. Pocock, J. Amin, P. R. Ebeling, S. Emery, A. Carr, and STEAL study investigators, “Hip structural parameters over 96 weeks in HIV-infected adults switching treatment to tenofovir-emtricitabine or abacavir-lamivudine,” *PLoS ONE*, vol. 9, no. 4, p. e94858, 2014.

[130] E. Negredo, P. Domingo, N. Pérez-Álvarez, M. Gutiérrez, G. Mateo, J. Puig, R. Escrig, P. Echeverría, A. Bonjoch, and B. Clotet, “Improvement in bone mineral density after switching from tenofovir to abacavir in HIV-1-infected patients with low bone mineral density: two-

centre randomized pilot study (OsteoTDF study)," *J. Antimicrob. Chemother.*, vol. 69, no. 12, pp. 3368–3371, 2014.

[131] A. Carr and J. Hoy, "Low bone mineral density with tenofovir: does statistically significant mean clinically significant?," *Clin. Infect. Dis.*, vol. 51, no. 8, pp. 973–975, 2010.

[132] T. A. Rasmussen, D. Jensen, M. Tolstrup, U. S. Nielsen, E. J. Erlandsen, H. Birn, L. Østergaard, B. L. Langdahl, and A. L. Laursen, "Comparison of bone and renal effects in HIV-infected adults switching to abacavir or tenofovir based therapy in a randomized trial," *PLoS ONE*, vol. 7, no. 3, p. e32445, 2012.

[133] H. Haskelberg, J. F. Hoy, J. Amin, P. R. Ebeling, S. Emery, A. Carr, and STEAL Study Group, "Changes in bone turnover and bone loss in HIV-infected patients changing treatment to tenofovir-emtricitabine or abacavir-lamivudine," *PLoS ONE*, vol. 7, no. 6, p. e38377, 2012.

[134] C. Gervasoni, P. Meraviglia, S. Landonio, S. Baldelli, S. Fucile, L. Castagnoli, E. Clementi, A. Riva, M. Galli, G. Rizzardini, and D. Cattaneo, "Low body weight in females is a risk factor for increased tenofovir exposure and drug-related adverse events," *PLoS ONE*, vol. 8, no. 12, p. e80242, 2013.

[135] V. Amorosa and P. Tebas, "Bone disease and HIV infection," *Clin. Infect. Dis. Off. Publ. Infect. Dis. Soc. Am.*, vol. 42, no. 1, pp. 108–114, 2006.

[136] E. Dailly, O. Tribut, P. Tattevin, C. Arvieux, P. Perré, F. Raffi, and P. Jolliet, "Influence of tenofovir, nevirapine and efavirenz on ritonavir-boosted atazanavir pharmacokinetics in HIV-infected patients," *Eur. J. Clin. Pharmacol.*, vol. 62, no. 7, pp. 523–526, 2006.

[137] L. I. Malaty and J. J. Kuper, "Drug interactions of HIV protease inhibitors," *Drug Saf.*, vol. 20, no. 2, pp. 147–169, 1999.

[138] E. Dailly, C. Allavena, F. Raffi, and P. Jolliet, "Pharmacokinetic evidence for the induction of lopinavir metabolism by efavirenz," *Br. J. Clin. Pharmacol.*, vol. 60, no. 1, pp. 32–34, 2005.

[139] J. C. Adkins and S. Noble, "Efavirenz," *Drugs*, vol. 56, no. 6, pp. 1055–1064; discussion 1065–1066, 1998.

[140] K. J. Wilby, E. D. Greanya, J.-A. E. Ford, E. M. Yoshida, and N. Partovi, "A review of drug interactions with boceprevir and telaprevir: implications for HIV and transplant patients," *Ann. Hepatol.*, vol. 11, no. 2, pp. 179–185, 2012.

[141] L. Dickinson, S. Khoo, and D. Back, "Pharmacokinetics and drug-drug interactions of antiretrovirals: an update," *Antiviral Res.*, vol. 85, no. 1, pp. 176–189, 2010.

[142] R. Wanke, S. G. Harjivan, S. A. Pereira, M. M. Marques, and A. M. M. Antunes, "The role of competitive binding to human serum albumin on efavirenz-warfarin interaction: a nuclear magnetic resonance study," *Int. J. Antimicrob. Agents*, vol. 42, no. 5, pp. 443–446, 2013.

[143] S. Bonora, M. Lanzafame, A. D'Avolio, L. Trentini, E. Lattuada, E. Concia, and G. Di Perri, "Drug interactions between warfarin and efavirenz or lopinavir-ritonavir in clinical treatment," *Clin. Infect. Dis. Off. Publ. Infect. Dis. Soc. Am.*, vol. 46, no. 1, pp. 146–147, 2008.

[144] S. M. Robertson, F. Maldarelli, V. Natarajan, E. Formentini, R. M. Alfaro, and S. R. Penzak, "Efavirenz induces CYP2B6-mediated hydroxylation of bupropion in healthy subjects," *J. Acquir. Immune Defic. Syndr. 1999*, vol. 49, no. 5, pp. 513–519, 2008.

[145] S. Sriwiriyajan, W. Mahatthanatrakul, W. Ridtitid, and S. Jaruratanasirikul, "Effect of efavirenz on the pharmacokinetics of ketoconazole in HIV-infected patients," *Eur. J. Clin. Pharmacol.*, vol. 63, no. 5, pp. 479–483, 2007.

[146] H. L. Koo, R. J. Hamill, and R. A. Andrade, "Drug-drug interaction between itraconazole and efavirenz in a patient with AIDS and disseminated histoplasmosis," *Clin. Infect. Dis. Off. Publ. Infect. Dis. Soc. Am.*, vol. 45, no. 6, pp. e77–79, 2007.

[147] H. Peyriere, C. Eiden, J.-C. Macia, and J. Reynes, "Antihypertensive drugs in patients treated with antiretrovirals," *Ann. Pharmacother.*, vol. 46, no. 5, pp. 703–709, 2012.

[148] J. S. Berns and N. Kasbekar, "Highly active antiretroviral therapy and the kidney: an update on antiretroviral medications for nephrologists," *Clin. J. Am. Soc. Nephrol.*, vol. 1, no. 1, pp. 117–129, 2006.

[149] J. G. Gerber, S. L. Rosenkranz, C. J. Fichtenbaum, J. M. Vega, A. Yang, B. L. Alston, S. W. Brobst, Y. Segal, J. A. Aberg, and AIDS Clinical Trials Group A5108 Team, "Effect of efavirenz on the pharmacokinetics of simvastatin, atorvastatin, and pravastatin: results of AIDS clinical trials group 5108 study," *J. Acquir. Immune Defic. Syndr. 1999*, vol. 39, no. 3, pp. 307–312, 2005.

[150] J. A. Robinson, R. Jamshidi, and A. E. Burke, "Contraception for the HIV-positive woman: a review of interactions between hormonal contraception and antiretroviral therapy," *Infect. Dis. Obstet. Gynecol.*, vol. 2012, p. e890160, 2012.

[151] H. Sevinsky, T. Eley, A. Persson, D. Garner, C. Yones, R. Nettles, K. Krantz, R. Bertz, and J. Zhang, "The effect of efavirenz on the pharmacokinetics of an oral contraceptive containing ethinyl estradiol and norgestimate in healthy HIV-negative women," *Antivir. Ther.*, vol. 16, no. 2, pp. 149–156, 2011.

[152] C. B. Eap, T. Buclin, and P. Baumann, "Interindividual variability of the clinical pharmacokinetics of methadone: implications for the treatment of opioid dependence," *Clin. Pharmacokinet.*, vol. 41, no. 14, pp. 1153–1193, 2002.

[153] A. Pelet, B. Favrat, M. Cavassini, C. B. Eap, J. Besson, and M. Monnat, "Usefulness of methadone plasma concentration measurement in patients receiving nevirapine or efavirenz," *Am. J. Drug Alcohol Abuse*, vol. 37, no. 4, pp. 264–268, Jul. 2011.

[154] H. McIlleron, G. Meintjes, W. J. Burman, and G. Maartens, "Complications of antiretroviral therapy in patients with tuberculosis: drug interactions, toxicity, and immune reconstitution inflammatory syndrome," *J. Infect. Dis.*, vol. 196 Suppl 1, pp. S63–75, 2007.

[155] P. Byakika-Kibwika, M. Lamorde, H. Mayanja-Kizza, S. Khoo, C. Merry, and Van geertruyden Jean-Pierre, "Artemether-lumefantrine combination therapy for treatment of

uncomplicated malaria: the potential for complex interactions with antiretroviral drugs in HIV-infected individuals," *Malar. Res. Treat.*, vol. 2011, p. e703730, 2011.

[156] G. L. Birbeck, J. A. French, E. Perucca, D. M. Simpson, H. Fraimow, J. M. George, J. F. Okulicz, D. B. Clifford, H. Hachad, R. H. Levy, Quality Standards Subcommittee Of The American Academy Of Neurology, and Ad Hoc Task Force Of The Commission On Therapeutic Strategies Of The International League Against Epilepsy, "Antiepileptic drug selection for people with HIV/AIDS: evidence-based guidelines from the ILAE and AAN," *Epilepsia*, vol. 53, no. 1, pp. 207–214, 2012.

[157] P. Ji, B. Damle, J. Xie, S. E. Unger, D. M. Grasela, and S. Kaul, "Pharmacokinetic interaction between efavirenz and carbamazepine after multiple-dose administration in healthy subjects," *J. Clin. Pharmacol.*, vol. 48, no. 8, pp. 948–956, 2008.

[158] S. M. Robertson, S. R. Penzak, J. Lane, A. K. Pau, and J. M. Mican, "A potentially significant interaction between efavirenz and phenytoin: a case report and review of the literature," *Clin. Infect. Dis. Off. Publ. Infect. Dis. Soc. Am.*, vol. 41, no. 2, pp. e15–18, 2005.

[159] M. Mirochnick, B. M. Best, A. M. Stek, E. V. Capparelli, C. Hu, S. K. Burchett, S. S. Rossi, E. Hawkins, M. Basar, E. Smith, J. S. Read, and IMPAACT 1026s Study Team, "Atazanavir pharmacokinetics with and without tenofovir during pregnancy," *J. Acquir. Immune Defic. Syndr. 1999*, vol. 56, no. 5, pp. 412–419, 2011.

[160] M.-Y. Tung, S. Mandalia, M. Bower, B. Gazzard, and M. Nelson, "The durability of virological success of tenofovir and didanosine dosed at either 400 or 250 mg once daily," *HIV Med.*, vol. 6, no. 3, pp. 151–154, 2005.

[161] A. Pruvost, E. Negredo, H. Benech, F. Theodoro, J. Puig, E. Grau, E. García, J. Moltó, J. Grassi, and B. Clotet, "Measurement of intracellular didanosine and tenofovir phosphorylated metabolites and possible interaction of the two drugs in human immunodeficiency virus-infected patients," *Antimicrob. Agents Chemother.*, vol. 49, no. 5, pp. 1907–1914, 2005.

[162] J. N. Blanchard, M. Wohlfeiler, A. Canas, K. King, and J. T. Lonergan, "Pancreatitis treated with didanosine and tenofovir disoproxil fumarate," *Clin. Infect. Dis.*, vol. 37, no. 5, pp. e57–e62, 2003.

[163] P. Barreiro and V. Soriano, "Suboptimal CD4 gains in HIV-infected patients receiving didanosine plus tenofovir," *J. Antimicrob. Chemother.*, vol. 57, no. 5, pp. 806–809, 2006.

[164] E. Negredo, A. Bonjoch, R. Paredes, J. Puig, and B. Clotet, "Compromised immunologic recovery in treatment-experienced patients with HIV infection receiving both tenofovir disoproxil fumarate and didanosine in the TORO studies," *Clin. Infect. Dis. Off. Publ. Infect. Dis. Soc. Am.*, vol. 41, no. 6, pp. 901–905, 2005.

[165] C. E. Koval, C. Dykes, J. Wang, and L. M. Demeter, "Relative replication fitness of efavirenz-resistant mutants of HIV-1: correlation with frequency during clinical therapy and evidence of

compensation for the reduced fitness of K103N + L100I by the nucleoside resistance mutation L74V," *Virology*, vol. 353, no. 1, pp. 184–192, 2006.

[166] H. J. Ribaudo, D. W. Haas, C. Tierney, R. B. Kim, G. R. Wilkinson, R. M. Gulick, D. B. Clifford, C. Marzolini, C. V. Fletcher, K. T. Tashima, D. R. Kuritzkes, E. P. Acosta, and Adult AIDS Clinical Trials Group Study, "Pharmacogenetics of plasma efavirenz exposure after treatment discontinuation: an adult AIDS clinical trials group study," *Clin. Infect. Dis. Off. Publ. Infect. Dis. Soc. Am.*, vol. 42, no. 3, pp. 401–407, 2006.

[167] J. Lindberg, S. Sigurdsson, S. Löwgren, H. O. Andersson, C. Sahlberg, R. Noréen, K. Fridborg, H. Zhang, and T. Unge, "Structural basis for the inhibitory efficacy of efavirenz (DMP-266), MSC194 and PNU142721 towards the HIV-1 RT K103N mutant," *Eur. J. Biochem. FEBS*, vol. 269, no. 6, pp. 1670–1677, 2002.

[168] J. Ren, J. Milton, K. L. Weaver, S. A. Short, D. I. Stuart, and D. K. Stammers, "Structural basis for the resilience of efavirenz (DMP-266) to drug resistance mutations in HIV-1 reverse transcriptase," *Struct. Lond. Engl. 1993*, vol. 8, no. 10, pp. 1089–1094, 2000.

[169] V. Svicher, C. Alteri, A. Artese, F. Forbici, M. M. Santoro, D. Schols, K. Van Laethem, S. Alcaro, G. Costa, C. Tommasi, M. Zaccarelli, P. Narciso, A. Antinori, F. Ceccherini-Silberstein, J. Balzarini, and C. F. Perno, "Different evolution of genotypic resistance profiles to emtricitabine versus lamivudine in tenofovir-containing regimens," *J. Acquir. Immune Defic. Syndr. 1999*, vol. 55, no. 3, pp. 336–344, 2010.

[170] A. G. Marcelin, C. Charpentier, M. Wirden, R. Landman, M. A. Valantin, A. Simon, C. Katlama, P. Yeni, D. Descamps, C. Aubron-Olivier, and V. Calvez, "Resistance profiles of emtricitabine and lamivudine in tenofovir-containing regimens," *J. Antimicrob. Chemother.*, vol. 67, no. 6, pp. 1475–1478, 2012.

[171] C. Csajka, C. Marzolini, K. Fattinger, L. A. Décosterd, J. Fellay, A. Telenti, J. Biollaz, and T. Buclin, "Population pharmacokinetics and effects of efavirenz in patients with human immunodeficiency virus infection," *Clin. Pharmacol. Ther.*, vol. 73, no. 1, pp. 20–30, 2003.

[172] L. H. Wang, J. Begley, R. L. St Claire, J. Harris, C. Wakeford, and F. S. Rousseau, "Pharmacokinetic and pharmacodynamic characteristics of emtricitabine support its once daily dosing for the treatment of HIV infection," *AIDS Res. Hum. Retroviruses*, vol. 20, no. 11, pp. 1173–1182, 2004.

[173] M. R. Blum, G. E. Chittick, J. A. Begley, and J. Zong, "Steady-state pharmacokinetics of emtricitabine and tenofovir disoproxil fumarate administered alone and in combination in healthy volunteers," *J. Clin. Pharmacol.*, vol. 47, no. 6, pp. 751–759, 2007.

[174] K. Cundy, S. Safrin, and R. Coleman, "Oral PMPA prodrug: relationship between clinical pharmacokinetics, safety and anti-HIV activity (abstract)." 12th World AIDS Conference, 1998.

[175] E. Valade, J.-M. Tréluyer, N. Bouazza, J. Ghosn, F. Foissac, S. Benaboud, F. Fauchet, J.-P. Viard, S. Urien, and D. Hirt, "Population pharmacokinetics of emtricitabine in HIV-1-infected adult patients," *Antimicrob. Agents Chemother.*, vol. 58, no. 4, pp. 2256–2261, 2014.

[176] V. Jullien, J.-M. Tréluyer, E. Rey, P. Jaffray, A. Krivine, L. Moachon, A. Lillo-Le Louet, A. Lescoat, N. Dupin, D. Salmon, G. Pons, and S. Urien, "Population pharmacokinetics of tenofovir in human immunodeficiency virus-infected patients taking highly active antiretroviral therapy," *Antimicrob. Agents Chemother.*, vol. 49, no. 8, pp. 3361–3366, 2005.

[177] A. E. Mutlib, H. Chen, G. A. Nemeth, J. A. Markwalder, S. P. Seitz, L. S. Gan, and D. D. Christ, "Identification and characterization of efavirenz metabolites by liquid chromatography/mass spectrometry and high field NMR: species differences in the metabolism of efavirenz," *Drug Metab. Dispos. Biol. Fate Chem.*, vol. 27, no. 11, pp. 1319–1333, 1999.

[178] J.-M. Molina, G. Peytavin, S. Perusat, C. Lascoux-Combes, D. Sereni, W. Rozenbaum, and G. Chene, "Pharmacokinetics of emtricitabine, didanosine and efavirenz administered once-daily for the treatment of HIV-infected adults (pharmacokinetic substudy of the ANRS 091 trial)," *HIV Med.*, vol. 5, no. 2, pp. 99–104, 2004.

[179] A. Jackson, G. Moyle, V. Watson, J. Tjia, A. Ammara, D. Back, M. Mohabeer, B. Gazzard, and M. Boffito, "Tenofovir, emtricitabine intracellular and plasma, and efavirenz plasma concentration decay following drug intake cessation: implications for HIV treatment and prevention," *J. Acquir. Immune Defic. Syndr. 1999*, vol. 62, no. 3, pp. 275–281, 2013.

[180] N. A. Raju, J. V. Rao, K. V. Prakash, K. Mukkanti, and K. Srinivasu, "Simultaneous estimation of tenofovir disoproxil, emtricitabine and efavirenz in tablet dosage form by RP-HPLC," *Orient. J. Chem.*, vol. 24, no. 2, pp. 645–650, 2008.

[181] V. K. Rao, K. K. Kumar, V. Raveendrahnath T, L. Madhuri P, and K. Sasikanth, "RP-HPLC method for the simultaneous estimation of emtricitabine, tenofovir and efavirenz in pharmaceutical dosage form," *JGTPS.*, vol. 2, no. 2, pp. 177–189, 2011.

[182] S. Nadig, J. T. Jacob, I. Bhat, and K. V, "A stability indicating RP-HPLC method for the simultaneous estimation of emtricitabine, tenofovir disoproxil fumarate and efavirenz in pharamceutical dosage forms," *Int. J. Res. Pharm. Sci.*, vol. 4, no. 2, pp. 391–396, 2013.

[183] A. Ramaswamy and A. S. Arul Gnana Dhas, "Development and validation of analytical method for quantitation of Emtricitabine, Tenofovir, Efavirenz based on HPLC," *Arab. J. Chem.*, 2014.

[184] P. Varma D and A. L. Rao, "Stability-Indicating RP-HPLC method for the simultaneous estimation of efavirenz, tenofovir and emtricitabine in pharmaceutical formulations," *IJPP.*, vol. 1, no. 1, 2014.

[185] R. Nirogi, G. Bhyrapuneni, V. Kandikere, K. Mudigonda, P. Komarneni, R. Aleti, and K. Mukkanti, "Simultaneous quantification of a non-nucleoside reverse transcriptase inhibitor efavirenz, a nucleoside reverse transcriptase inhibitor emtricitabine and a nucleotide reverse

transcriptase inhibitor tenofovir in plasma by liquid chromatography positive ion electrospray tandem mass spectrometry," *Biomed. Chromatogr. BMC*, vol. 23, no. 4, pp. 371–381, 2009.

[186] L. Ohannesian and A. Streeter, *Handbook of pharmaceutical analysis*. CRC Press, 2001.

[187] J. A. Adamovics, *Chromatographic analysis of pharmaceuticals*, 2nd ed. CRC Press, 1996.

[188] I. W. Wainer, *Liquid chromatography in pharmaceutical development: an introduction*. Aster Pub. Corp., 1985.

[189] Y. V. Kazakevich and R. LoBrutto, *HPLC for pharmaceutical scientists*. USA: John Wiley & Sons, Inc., 2007.

[190] "Chromatographic Columns," *chemwiki.ucdavis.edu*. [Online]. Available: http://chemwiki.ucdavis.edu/Analytical_Chemistry/Instrumental_Analysis/Chromatography/Chromatographic_Columns. [Accessed: 16-Aug-2015].

[191] C. Kaushal and B. Srivastava, "A process of method development: A chromatographic approach," *J. Chem. Pharm. Res.*, vol. 2, no. 2, pp. 519–545, 2010.

[192] J. G. Dorsey and K. A. Dill, "The molecular mechanism of retention in reversed-phase liquid chromatography," *Chem. Rev.*, vol. 89, no. 2, pp. 331–346, 1989.

[193] L. R. Snyder, J. W. Dolan, and P. W. Carr, "The hydrophobic-subtraction model of reversed-phase column selectivity," *J. Chromatogr. A*, vol. 1060, no. 1–2, pp. 77–116, 2004.

[194] D. H. Marchand, K. Croes, J. W. Dolan, and L. R. Snyder, "Column selectivity in reversed-phase liquid chromatography: cyanopropyl columns," *J. Chromatogr. A*, vol. 1062, no. 1, pp. 57–64, 2005.

[195] "HPLC column selection," *LC-GC N. Am.*, vol. 31, no. 5, pp. 430–430, 2013.

[196] Food and Drug Administration, "Reviewer guidance: validation of chromatographic methods." Center for Drug Evaluation and Research (CDER), 1994.

[197] J. C. Arsenault and P. D. McDonald, *Beginners guide to liquid chromatography*. Waters Corporation, 2009.

[198] "High performance liquid chromatography," *chemwiki.ucdavis.edu*. [Online]. Available: http://chemwiki.ucdavis.edu/Analytical_Chemistry/Instrumental_Analysis/Chromatography/High_performance_liquid_chromatography. [Accessed: 16-Aug-2015].

[199] M. Swartz, "Hplc detectors: a brief review," *J. Liq. Chromatogr. Relat. Technol.*, vol. 33, no. 9–12, pp. 1130–1150, 2010.

[200] G. Aymard, M. Legrand, N. Trichereau, and B. Diquet, "Determination of twelve antiretroviral agents in human plasma sample using reversed-phase high-performance liquid chromatography," *J. Chromatogr. B. Biomed. Sci. App.*, vol. 744, no. 2, pp. 227–240, 2000.

[201] O. McPolin, *Validation of Analytical Methods for Pharmaceutical Analysis*. Lulu.com, 2009.

[202] H. Bridwell, V. Dhingra, D. Peckman, J. Roark, and T. Lehman, "Perspectives on method validation: importance of adequate method validation," *Qual. Assur. J.*, vol. 13, no. 3–4, pp. 72–77, 2010.

[203] The European Medicines Agency, “ICH Topic Q2 (R1), validation of analytical procedures: text and methodology [CPMP/ICH/381/95].” 1995.

[204] Food and Drug Administration, “Analytical procedures and methods validation for drugs and biologics, guidance for industry.” Center for Drug Evaluation and Research (CDER), Center for Biologics Evaluation and Research (CBER), 2015.

[205] The United States Pharmacopeial Convention, “Validation of compendial procedures <1225>,” in *The United States Pharmacopeia and National Formulary*, vol. USP 38-NF 33, Rockville, MD: The United States Pharmacopeial Convention. Inc, 2015, pp. 1445–1450.

[206] M. Kazusaki, S. Ueda, N. Takeuchi, and Y. Ohgami, “Validation of analytical procedures by high−performance liquid chromatography for pharmaceutical analysis,” vol. 33, no. 2, pp. 65–73, 2012.

[207] G. A. Shabir, “Validation of high-performance liquid chromatography methods for pharmaceutical analysis: Understanding the differences and similarities between validation requirements of the US Food and Drug Administration, the US Pharmacopeia and the International Conference on Harmonization,” *J. Chromatogr. A*, vol. 987, no. 1–2, pp. 57–66, 2003.

[208] T. C. Paino and A. D. Moore, “Determination of the LOD and LOQ of an HPLC method using four different techniques,” *Pharm. Technol.*, vol. 23, no. 10, pp. 86–90, 1999.

[209] International Conference of Harmonisation, “Guidance for industry: stability testing of new drug substances and products (revision 2), ICH guidelines, Q1A(R2).” 2003.

[210] Food and Drug Administration, “Guidance for industry: INDs for phase II and III studies-chemistry, manufacturing, and controls information.” Centre for Drug Evaluation and Research (CDER), 2003.

[211] H. Brummer, “How to approach a forced degradation study,” *Life Sci. Technol. Bull.*, no. 31, pp. 1–4, 2011.

[212] International Conference of Harmonisation, “Stability testing: photostability testing of new drug substances and products, Q1B.” 1996.

[213] M. Blessy, R. D. Patel, P. N. Prajapati, and Y. K. Agrawal, “Development of forced degradation and stability indicating studies of drugs—A review,” *J. Pharm. Anal.*, vol. 4, no. 3, pp. 159–165, 2014.

[214] B. Buszewski and M. Szultka, “Past, present, and future of solid phase extraction: a review,” *Crit. Rev. Anal. Chem.*, vol. 42, no. 3, pp. 198–213, 2012.

[215] W. Bi, J. Zhou, and K. H. Row, “Solid phase extraction of three phenolic acids from Saliconia Herbacea L. by different ionic liquid-based silicas,” *J. Liq. Chromatogr. Relat. Technol.*, vol. 35, no. 5, pp. 723–736, 2012.

[216] C. F. Poole, “New trends in solid-phase extraction,” *TrAC Trends Anal. Chem.*, vol. 22, no. 6, pp. 362–373, 2003.

[217] M.-C. Hennion, “Solid-phase extraction: method development, sorbents, and coupling with liquid chromatography,” *J. Chromatogr. A*, vol. 856, no. 1–2, pp. 3–54, 1999.

[218] J. Dolan W., S. Sadikin, D. D. Zhang, R. Inloes, and S. Redkar, “Ghost peak investigation in a reversed-phase gradient LC system,” 2011. [Online]. Available: http://www.chromatographyonline.com/ghost-peak-investigation-reversed-phase-gradient-lc-system?id=&pageID=1&sk=&date=. [Accessed: 04-Nov-2015].

[219] S. Williams, “Ghost peaks in reversed-phase gradient HPLC: a review and update,” *J. Chromatogr. A*, vol. 1052, no. 1–2, pp. 1–11, 2004.

[220] G. R. Strasser and I. Váradi, “Investigations of artifact peaks in sensitive high-performance liquid chromatography methods,” *J. Chromatogr. A*, vol. 869, no. 1–2, pp. 85–90, 2000.

[221] U. Neue D., D. Marchand H., and L. R. Snyder, “Peak compression in reveresed-phase gradient elution,” *J. Chromatogr. A*, vol. 1111, pp. 32–39, 2006.

[222] C. K. Brown, H. P. Chokshi, B. Nickerson, R. A. Reed, B. R. Rohrs, and P. A. Shah, “Acceptable analytical practices for dissolution testing of poorly soluble compounds,” *Pharm. Technol.*, vol. 28, no. 12, pp. 56–65, 2004.

[223] A. Palmieri III, *Dissolution theory, methodology, and testing*, 1st ed. Dissolution Technologies, 2007.

[224] A. Kapoor, R. Sharma, P. Sharma, and P. Gupta, “BCS classification system: benchmark for solubility and permeability,” *Indo Am. J. Pharm. Res.*, vol. 4, no. 4, pp. 2233–2238, 2014.

[225] V. Y. Raju, G. Sunitha, A. K. Pal, A. Haripriya, N. Sirisha, and P. A. Kumar, “Validated stability indicating analytical method and in-vitro dissolution studies of efavirenz formulation by RP-HPLC,” *Int. J. Pharm. Pharm. Sci.*, vol. 4, no. 5, pp. 572–576, 2012.

[226] J. Zuo, Y. Gao, N. Bou-Chacra, and R. Löbenberg, “Evaluation of the DDSolver Software Applications,” *BioMed Res. Int.*, vol. 2014, 2014.

[227] I. Kanfer and L. Shargel, *Generic drug product development: bioequivalence issues*, vol. 180. New York: Informa healthcare, 2007.

[228] Food and Drug Administration, “Draft guidance for industry: dissolution testing and specification criteria for immediate-release solid oral dosage forms containing biopharmaceutics classification.” 2015.

[229] C.-L. Cheng, L. X. Yu, H.-L. Lee, C.-Y. Yang, C.-S. Lue, and C.-H. Chou, “Biowaiver extension potential to BCS Class III high solubility-low permeability drugs: bridging evidence for metformin immediate-release tablet,” *Eur. J. Pharm. Sci.*, vol. 22, no. 2004, pp. 297–304, 2004.

[230] U. V. Banakar, *Pharmaceutical dissolution testing*, vol. 49. New York, USA: Informa healthcare, 2010.

[231] G. S. Banker and C. T. Rhodes, Eds., *Modern pharmaceutics*, 4th ed., vol. 121. Marcel Dekker, Inc., 2002.

[232] E. Costa Pinto, L. Mendes Cabral, and V. Pereira de Sousa, “Development of a discriminative intrinsic dissolution method for efavirenz,” *Dissolution Technol.*, vol. 2014, pp. 31–40, 2014.

[233] The United States Pharmacopeial Convention, “Dissolution <711>,” in *The United States Pharmacopeia and National Formulary*, vol. USP 38-NF 33, Rockville, MD: The United States Pharmacopeial Convention. Inc, 2015, p. 486.

[234] International Conference of Harmonisation, “Specifications: test procedures and acceptance criteria for new drug substances and new drug products: chemical substances Q6A.” 1999.

[235] European Medicines Agency, “Guideline on the investigation of bioequivalence.” Commitee for medical products for human use (CHMP), 2010.

[236] The United States Pharmacopeial Convention, *Efavirenz monogragh*, vol. USP 38-NF 33. Rockville, MD: The United States Pharmacopieal Covention. Inc, 2015.

[237] The United States Pharmacopeia, “Tenofovir disoproxil fumarate: authorised USP monograph version 1,” 2011. [Online]. Available: http://www.usp.org/sites/default/files/usp_pdf/EN/USPNF/pendingStandards/m3429_authorized.pdf. [Accessed: 11-Dec-2015].

[238] The United States Pharmacopeia, “Emtricitabine: authorised USP monograph version 1,” 2012. [Online]. Available: http://www.usp.org/sites/default/files/usp_pdf/EN/USPNF/pendingStandards/m3546.pdf. [Accessed: 11-Dec-2015].

[239] World Health Organisation, *Draft monograph for the international pharmacopoeia: efavirenz, emtricitabine and tenofovir tablets*. 2011.

[240] S. K. Niazi, *Handbook of bioequivalence testing*, vol. 171. New York, USA: Informa healthcare, 2007.

[241] A. F. B. Fauzee, S. M. Khamanga, and R. B. Walker, “The impact of manufacturing variables on in vitro release of clobetasol 17-propionate from pilot scale cream formulations,” *Drug Dev. Ind. Pharm.*, vol. 40, no. 12, pp. 1683–1692, Dec. 2014.

[242] S. Dash, P. N. Murthy, L. Nath, and P. Chowdhury, “Kinetic modeling on drug release from controlled drug delivery systems,” *Acta Pol. Pharm.-Drug Res.*, vol. 67, no. 3, pp. 217–223, 2010.

[243] Z. Gao, T. W. Moore, A. P. Smith, W. H. Doub, and B. J. Westenberger, “Studies of variability in dissolution testing with USP apparatus 2,” *J. Pharm. Sci.*, vol. 96, no. 7, pp. 1794–1801, Jul. 2007.

[244] Food and Drug Administration, “Dissolution methods,” 2015. [Online]. Available: http://www.accessdata.fda.gov/scripts/cder/dissolution/dsp_SearchResults_Dissolutions.cfm?PrintAll=1. [Accessed: 07-Nov-2015].

[245] V. P. Choudhari, S. Ingale, S. R. Gite, D. D. Tajane, V. G. Modak, and A. Ambekar, “Spectrophotometric simultaneous determination of tenofovir disoproxil fumarate and

emtricitabine in combined tablet dosage form by ratio derivative, first order derivative and absorbance corrected methods and its application to dissolution study," *Pharm. Methods*, vol. 2, no. 1, pp. 47–52, 2011.

[246] K. Jayapalu, H. Malipeddi, and A. Chinnasamy, "Chromatographic separation and in vitro dissolution assessment of tenofovir disoproxil fumarate, emtricitabine and nevirapine in a fixed dose combination of antiretrovirals," *JAPS*, vol. 4, no. 11, pp. 76–80, 2014.

[247] S. Mehta, T. De Beer, J. P. Remon, and C. Vervaet, "Effect of disintegrants on the properties of multiparticulate tablets comprising starch pellets and excipient granules," *Int. J. Pharm.*, vol. 422, no. 1–2, pp. 310–317, 2012.

[248] M. Nazmi, S. M. A. Islam, M. A. Bhuiyan, and M. S. Reza, "Effect of superdisintegrants and their mode of incorporation on disintegration time and release profile of carbamazepine from immediate release tablet," *JAPS*, vol. 3, no. 5, pp. 80–84, 2013.

[249] Food and Drug Administration, "Full prescribing information, TRUVADA® (emtricitabine/tenofovir disoproxil fumarate)." Gilead Sciences, 2013.

[250] The United States Pharmacopeial Convention, "Disintegration <701>," in *The United States Pharmacopeia and National Formulary*, vol. USP 38-NF 33, Rockville, MD: The United States Pharmacopieal Covention. Inc, 2015, p. 483.