The Development and Validation of a Direct LC-MS/MS Assay for the Determination of Tenofovir-diphosphate in Dried Blood Spots for the Analysis of Clinical Samples

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Contents

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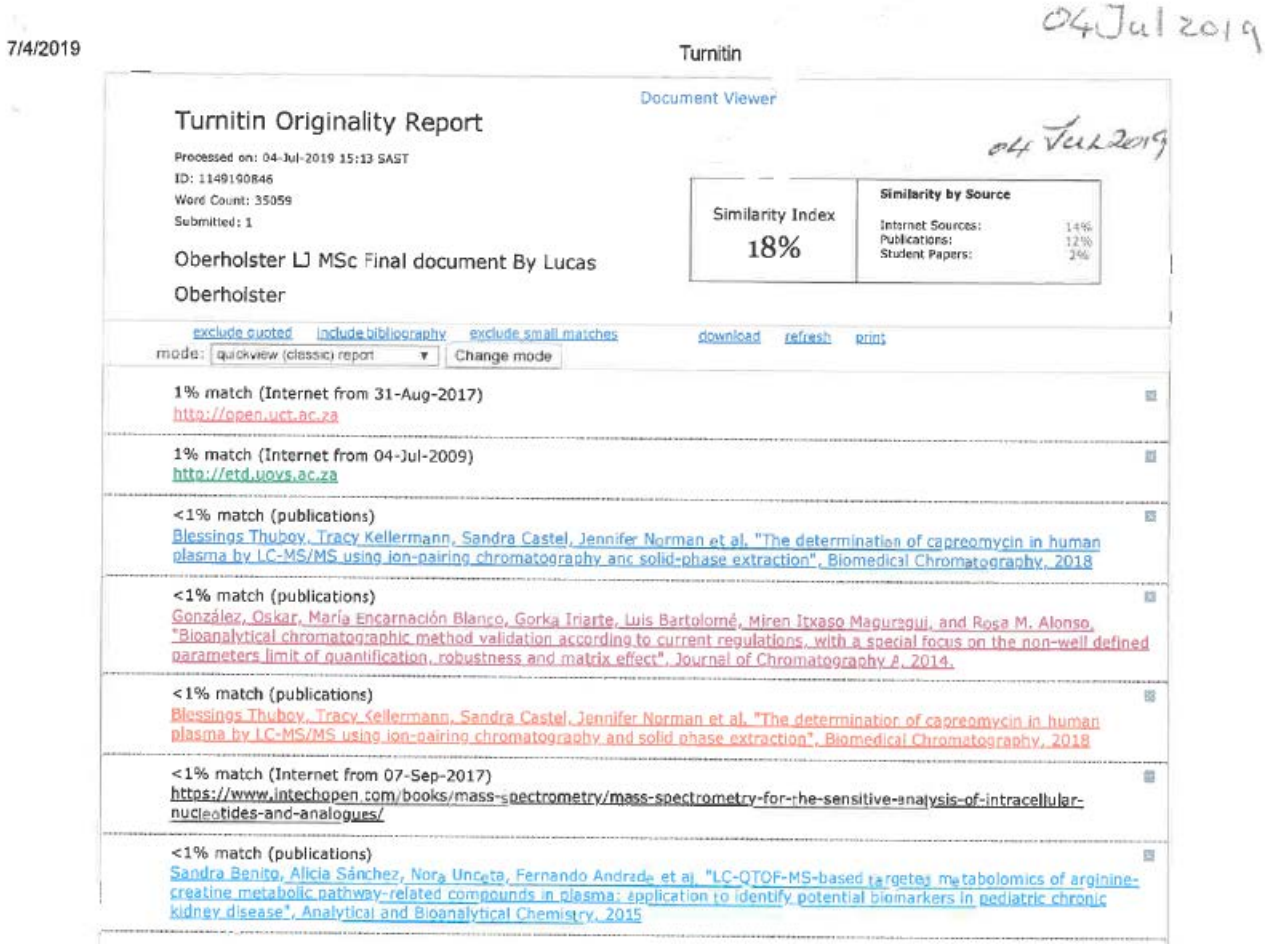

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Abstract

Tenofovir (TFV) and emtricitabine (FTC) are nucleoside reverse transcriptase inhibitors, often used in pre-exposure prophylaxis (PrEP) trials: where antiretroviral drugs are administered to high-risk, HIV-negative individuals to prevent HIV infection. Both drugs are safe when taken either daily or intermittently, which is ideal for PrEP regimens where adherence may not be high. The minimum number of doses estimated to confer high PrEP efficacy for a TFV/FTC regimen is four or more doses per week, resulting in a 95% lower risk of HIV acquisition. However, this is highly dependent on various host factors, of which adherence plays the largest role.

The aim of the project was to develop a novel sensitive, specific, and robust direct method for the measurement of adherence, utilising tenofovir-diphosphate (TFV-DP) in dry blood spots (DBS) through LC-MS/MS analysis, to replace the current costly and laborious indirect method currently used to elucidate adherence of patients. This indirect method faces challenges, due to the polar nature of TFV and its metabolites, leading to separation and retention issues. The existing method applied a technique which separated the parent drug from the metabolite and then back-converted all metabolites to the parent drug before analysing the samples on LC-MS/MS. The developed alternative method aimed to reduce the time taken for each assay and the associated cost of consumables.

TFV-DP is a highly polar compound and traditional reverse-phase chromatography has poor retention and separation capabilities when used to retain polar compounds, therefore alternative strategies were implemented. In this developed direct method, an anion exchange column was used along with a pH gradient, with the aim of improving separation and chromatography of TFV, TFV-DP, and tenofovir-monophosphate (TFV-MP). The method was optimised and validated using current U.S. Food and Drug Administration (FDA) and European Medical Agency (EMA) guidelines. The use of the anion exchange column resulted in a marked increase in retention time and allowed baseline separation of TFV, TFV-DP, and TFV-MP.

Determination of TFV-DP from DBS was performed using three 3 mm DBS punches per sample, which underwent an extraction procedure followed by high-performance liquid chromatography with tandem mass spectrometry detection on an AB Sciex Qtrap 5500 mass spectrometer. The transitions of the protonated precursor ions were monitored at m/z 448.0 and 452.9 to the product ions m/z 350.0 and 354.9 for TFV-DP and the deuterated TFV-DP internal standard, respectively. The method was validated over a range of 50–6400 fmol/punch for TFV-DP. The developed direct method had a lower limit of quantification (LLOQ) of 50 fmol/punch, which was higher than that of the indirect method; therefore, it had less sensitivity. The reduced sensitivity was acceptable, since the methods were meant for the measurement of adherence. The direct method had an ULOQ of 6400 fmol/punch, which was similar to that of the indirect method. The direct method also required significantly less on-bench sample processing and, therefore, was less time consuming and costly. To determine the suitability and accuracy of the direct method in comparison to the indirect method a comparative analysis was completed by analysing the same samples using both the indirect and direct method. The developed method met all the validation requirements and a strong correlation was observed between the results of the indirect and direct methods during the comparative analysis.

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Abbreviations

1,5-DMHA	1,5-Dimethylhexylamine
3TC	Lamivudine
ACN	Acetonitrile
AIDS	Acquired Immunodeficiency Syndrome
ALQ	Above the limit of quantitation
APCI	Atmospheric pressure chemical ionisation
APPI	atmospheric pressure photoionisation
ART	Antiretroviral therapy
ARV	Antiretrovirals
BLOQ	Below the limit of quantitation
CV(%)	Percentage covariance
%CV	Absolute coefficient of variation
$CD4^+$	Cluster of differentiation 4
dNTP's	Deoxynucleotide triphosphates
DBS	Dry blood spots
dFdC	Gemcitabine
dFdU	2-difluoro-2-deoxyuridine
DNA	Deoxyribonucleic acid
Dns-Cl	Dansyl chloride
DOT	Directly observed therapy
EFV	Efavirenz
EMA	European Medicines Agency
ESI	Electrospray ionisation

FDA	U.S. Food and Drug Administration
FDC	Fixed-dose combination
FTC	Emtricitabine
fmol	femtomole
GLP	Good lab practises
H_2O	Water
HIV	Human Immunodeficiency Virus
HPLC	High performance liquid chromatography
ISTD	Internal standard
ISW1	Internal standard working solution
LC-MS/MS	Liquid chromatography combined tandem mass spectrometry
LLE	Liquid-liquid extractions
LLOQ	Lower limit of quantitation
MEMS	Medication event monitoring system
MeOH	Methanol
mg	Milligram
ml	Millilitres
mRNA	Messenger ribonucleic acid
mM	MilliMolar
MRM	Multiple reaction monitoring
MTBE	Methyl tertiary-butyl ester
m/z	Mass-to-charge ratio
ng	Nanogram
NNRTI	Non-nucleoside reverse transcriptase inhibitors
NRTI	Nucleoside reverse transcriptase inhibitors
OPA	*o*-phthalaldehydes
PI	Protease inhibitors

PP	Protein precipitation
PBMCs	Peripheral blood mononuclear cells
pH	Power of Hydrogen
pKa	Acid dissociation constant at logarithmic scale
PrEP	Pre-exposure prophylaxis
QC	Quality control
RBC	Red blood cells
RP-HPLC	Reversed-phase high-performance liquid chromatography
RNA	Ribonucleic acid
SOP	Standard operating procedures
SPE	Solid phase extraction
SS	Stock solution 1
STD	Calibration standards
SYS	System suitability sample
TDF	Tenofovir disoproxil fumarate
TFV	Tenofovir
TFV-MP	Tenofovir-monophosphate
TFV-DP	Tenofovir-diphosphate
UCT	University of Cape Town
ULOQ	Upper limit of quantitation
µl	Microlitre
v/v	Volume to volume percentage
WHO	World health organisation
WS	Working solution

List of Figures

List of Tables

Chapter 1: Introduction

An estimated two-thirds of the 36.7 million people infected with the Human immunodeficiency virus (HIV), reside on the African continent [1]. Once infection has occurred, HIV destroys the hosts $CD4^+$ immune cells, leading to acquired immunodeficiency syndrome (AIDS) [2]. HIV/AIDS is wide spread throughout Sub-Saharan Africa and exacerbates various socioeconomic issues [3]. South Africa has some of the highest prevalence of HIV/AIDS in the world, with an estimated 7 million people living with HIV [4]. An estimated 115 000 HIV-related deaths occurred in South Africa in 2018, emphasizing the need for HIV research in Sub-Saharan countries [4].

1.1. Human Immunodeficiency Virus-1 and Virus Cycle

The HIV-1 virus consists of two copies of non-covalently linked, positive sense, single-stranded RNA molecules [5]. This is enclosed by a p24 viral protein conical capsid, situated within a viral envelope. The viral envelope is derived from the host cell membrane and contains viral glycoproteins; such as gp120 and gp41, which are imbedded in the viral envelope [2,6,7]. Once HIV-1 exposure occurs, the virus migrates to $CD4^+$ immune cells. This occurs due to the affinity of the HIV-1 viral envelope gp120/gp41 complex for CD4 antigen receptors on the surface of host $CD4^+$ immune cells [2,6]. Once the gp120 subunit of the viral envelope interacts with a CD antigen receptor, co-receptor binding takes place [2,7]. The co-receptor binding results in conformation changes in the HIV-1 envelope, eading to the exposure of the gp41 hydrophobic domain, allowing fusion of the envelope with the cellular membrane and the subsequent delivery of the viral core into the cytoplasm [2]. The uncoating of the viral core exposes Reverse transcriptase, a HIV enzyme, to deoxynucleotide triphosphates (dNTP's), resulting in reverse transcription and proviral DNA synthesis [2]. After reverse transcription is completed, the formation of a complex, consisting of viral and cellular components is formed, known as the viral pre-integration complex. This is transported to the cell's nucleus where the HIV-1 enzyme, Integrase, catalyses the integration of the viral genome with the human DNA [2,7]. This maintains the viral DNA in the infected host cell and allows transcription, thus the cell can express viral mRNA and viral RNA. Once transcription is initiated, the necessary viral constituents move to the inside of the plasma membrane and assemble to form an immature HIV-1 virus [2]. The newly formed immature virus buds off from the cell and releases protease to break down large HIV-1 protein chains. These broken down HIV protein chains, combine to form a mature HIV-1 virus [2].

The viral life cycle, discussed above (shown in Figure 1.1), can be subdivided into seven distinct stages, namely binding; fusion; reverse transcription; integration; replication; virus assembly/production; and budding. The HIV-1 lifecycle presents various potential opportunities for therapeutic intervention and six distinctclasses of antiretrovirals (ARVs) have been developed [2].

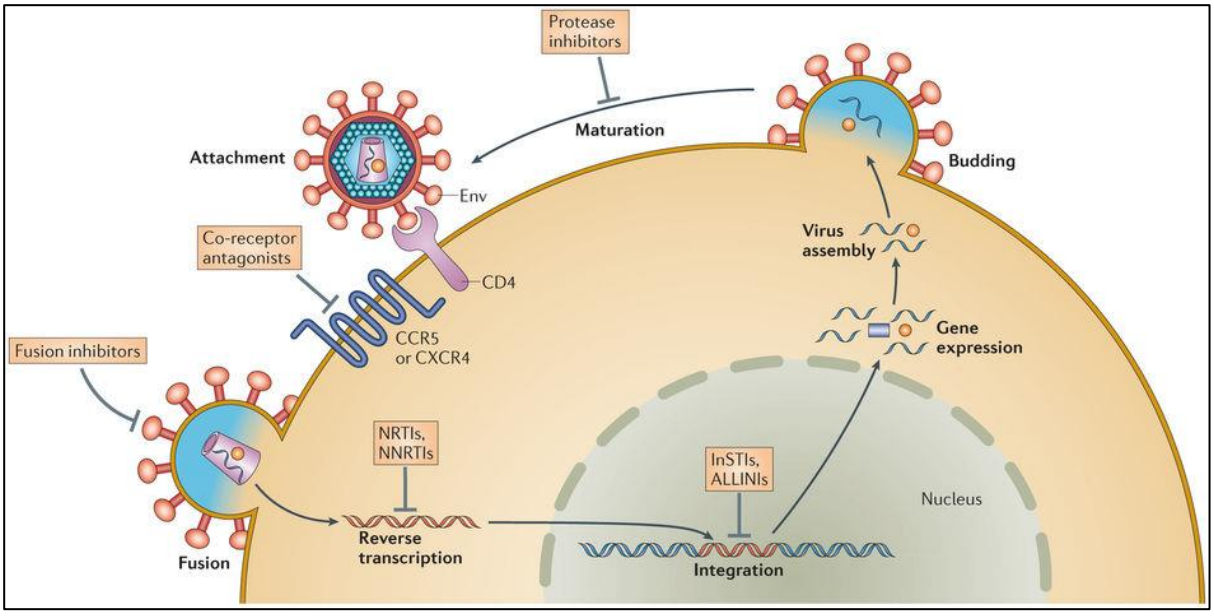


Figure 1.1: Each of the seven stages of the HIV-1 virus life cycle is shown above. The targeted sites for different classes of ARVs, are also shown [8].

1.2. Classes of Antiretrovirals

Each of the different ARV classes target a specific stage in the HIV-1 life cycle and can be subdivided as follows: nucleoside reverse transcriptase inhibitors (NRTIs), non-nucleoside reverse transcriptase inhibitors (NNRTIs), protease inhibitors (PIs), fusion inhibitors, integrase inhibitors, and co-receptor antagonists [2]. All the ARV classes, except co-receptor antagonists, are currently available in Southern Africa [9]. The most commonly used ARVs are NRTIs and NNRTIs and these account for half of all approved ARVs [2]. The 2017 Adult South African antiretroviral guidelines and World Health Organisation (WHO) recommend a regimen consisting of two NRTIs, co-administered with a NNRTI [9,10].

NRTIs are a class of ARVs used in the treatment and prevention of viral infections such as HIV; hepatitis viruses B and C; and the Human Herpes viruses. NRTIs target reverse transcription (Figure 1.1). These compounds are activated by phosphorylation carried out by cellular enzymes [7,11–13]. Once phosphorylation has occurred these compounds become analogues of the naturally occurring deoxynucleotides needed to synthesize viral DNA. NRTIs metabolites compete with the natural deoxynucleotides for incorporation into viral DNA during reverse transcription [7]. The NRTIs lack a 3′-hydroxyl group on the deoxyribose moiety, which is needed for binding of new deoxynucleotides, implying that the next 5′–3′ phosphodiester bond necessary to extend the DNA chain cannot form, resulting in viral DNA chain termination and the termination of viral replication within the host [8] (Figure 1.2). Proper treatment with NRTIs can prevent HIV infection and results in the suppression of the viral plasma levels below the limit of detection. This leads to an increase in the number of $CD4^+$ lymphocyte cells and is accompanied by the restoration of pathogen-specific immune function in HIV-1 infected patients. This subsequently leads to a significant decreases in HIV-associated morbidity and mortality [9,11,14–17].

1.3. Nucleoside Reverse Transcriptase Inhibitors *in vivo*

The chronic administration of NRTIs may lead to various adverse effects, due to the affinity of NRTIs for human DNA-polymerases, other than Reverse transcriptase [18]. Mitochondrial toxicity is considered as the most significant adverse effect of NRTIs. Other adverse effects include, myopathy, peripheral neuropathy, and hepatic steatosis with lactic acidosis, which can all be lethal conditions [18]. Toxicity can be decreased by optimising dosages and the use of drug regimens with high potency, good safety profiles and limited drug interactions. Low toxicity levels are paramount for both HIV patient treatment and pre-exposure prophylaxis (PrEP) regimens [11,19]. In PrEP regimens, antiretroviral drugs are administered to high-risk HIV-negative individuals, with the aim of preventing HIV infection should exposure occur [12,19,20].

The preferred HIV-1 first-line treatment is selected based on the antiretroviral therapy (ART) exhibiting favourable characteristics such as decreased toxicity; minimum drug-drug interactions; and increased simplicity and convenience [21]. Fixed-dose combinations (FDCs) allow once-daily regimens and any ART that can be prescribed in a FDC format is preferred due to its simplicity [10]. As stated earlier, according to the WHO guidelines, ART should consist of two NRTI and a NNRTI [10]. The recommended NRTI in first-line treatment consists of once-daily combinations of tenofovir disoproxil fumarate (TDF) with emtricitabine (FTC) or Lamivudine (3TC) [9]. Currently Efavirenz (EFV) is recommended as the preferred NNRTI co-administered in the first-line treatment [10]. The simplicity of once-daily regimens should help improve patient adherence. Truvada® was approved by the U.S. Food and Drug Administration (FDA) in 2012 and consists of TDF co-administered with FTC [22]. TDF is an oral prodrug of tenofovir (TFV) and requires initial diester hydrolysis to form TFV, which is an adenine-5′-monophosphate analogue [23]. Once absorbed, TFV is phosphorylated within the cell to the intermediate metabolite, tenofovir-monophosphate (TFV-MP), and ultimately, to the more abundant pharmacologically active metabolite, tenofovir-diphosphate (TFV-DP) [24]. It is often used in PrEP studies and has been shown to be effective in HIV treatment and prevention, although the efficacy is highly dependent on adherence [12].

Studies have demonstrated that TDF, in combination with FTC regimens, are safe when taken either daily or intermittently, which is ideal for PrEP, where adherence may not be as high. The minimum number of doses estimated to confer high PrEP efficacy for a TDF/FTC regimen is four or more per week, resulting in a 95% lower risk of HIV acquisition [19,25,26]. Adherence levels between 70–95% have been associated with clinical benefit in HIV patients, although higher levels are necessary for sustained viral suppression and the prevention of drug-resistant HIV-1 strains [27,28].

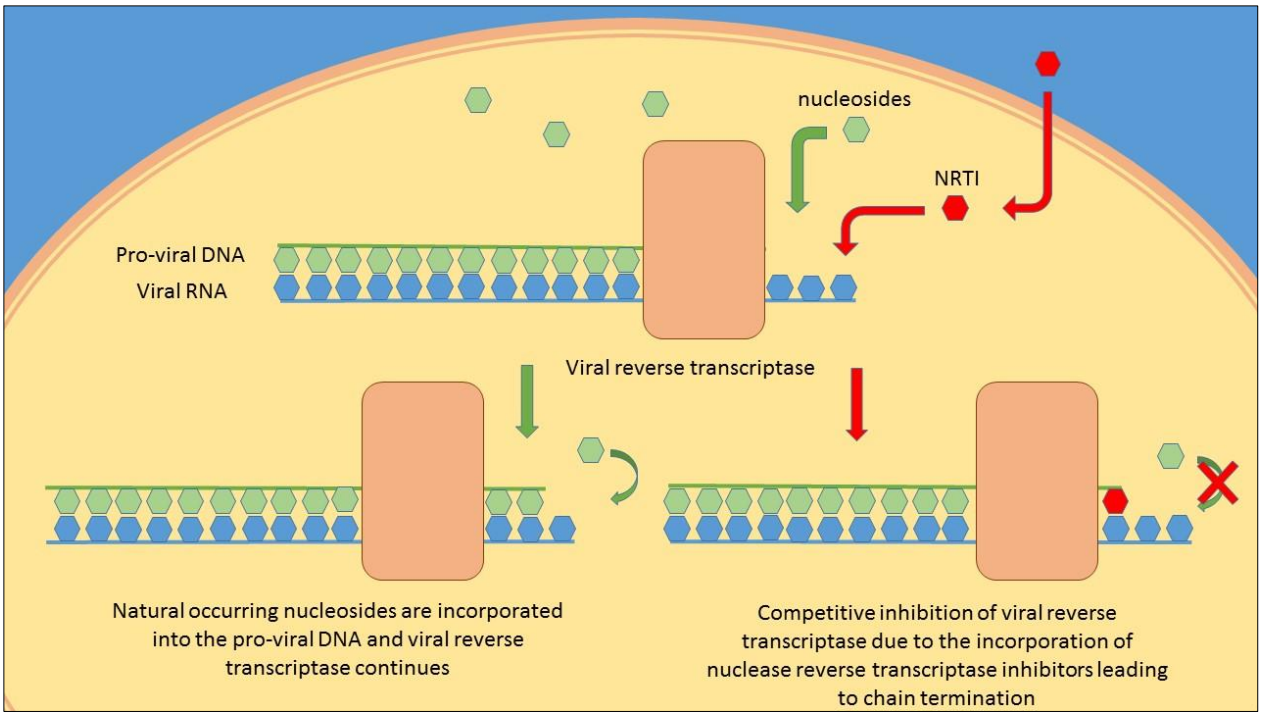


Figure 1.2: NRTIs mode of action. NRTIs act as competitive inhibitors of reverse transcriptase inhibitors. Adapted from Saayman *et al*. [8].

1.4. Measuring Adherence to Tenofovir

Adherence refers to the patient's compliance with the prescribed regimen. It is defined as the degree to which a patient correctly follows a prescribed treatment plan and abides by the prescribed dosage frequencies and times [29]. Adherence is a concern in any chronically administered treatment and measures are required to determine patient compliance. A recent study determined that the average nonadherence rate for patients is around 25%, indicting a serious need for compliance strategies [30].

Exact adherence measurements are often necessary for correct evaluation of therapeutic efficacy, to provide direct pharmacologic measures of drug exposure, since they have been shown to be paramount for trial interpretation in several studies [31,32]. Patient adherence is also essential when considering the development of drug resistance. Drug-resistant mutant strains of the HIV virus arise rapidly due to the short half-life of the virus and the error-prone process of reverse transcription [33]. These characteristics of the virus, in combination with poor adherence to ART, lead to HIV strains developing resistance to ARV regimens. These drug-resistant mutant strains can then be transmitted to drug-naïve patients leaving them with ineffective treatment options [33].

Various approaches have been developed as a means of measuring adherence, although self-reporting is often used to measure adherence in resource limited settings [29]. Self-reporting is a minimally invasive strategy and can easily be added to a clinical study; however, it is inaccurate and unreliable. Objective measures of patient compliance are far more accurate and are essential for the analysis of the efficacy of drug regimens [29]. Objective strategies include medication event monitoring systems (MEMSs), pharmacy refill data, directly observed therapy (DOT), and bioanalytical approaches (pharmacologic monitoring) [34]. Bioanalytical approaches include viral load and CD4 count, although measurement of drug concentrations in the patient's blood is still the best assessment of adherence. This provides an unbiased result; however, analytes with short half-lives will only allow the measurement of recent drug exposure. This does not allow the measurement of long-term drug exposure and, therefore, it may be difficult to determine the patient's adherence profile in its entirety [35].

Patient adherence can be determined by utilising compounds with substantial half-life differences to measure recent and long-term drug exposure. This will allow the elucidation of adherence; however, this is only possible for compounds where the parent, and its metabolites, have significant differences in their half-lives and pharmacokinetic characteristics [20,24,36].

TFV and TFV-DP are ideal candidates for this approach, since the TFV has a half-life of 15-hours and TFV-DP, the metabolite, has a 17-day half-life in red blood cells (RBCs). The exceptionally long half-life of TFV-DP leads to a 25-fold accumulation between first dose and steady state [20]. Therefore, TFV-DP exhibits characteristics ideal for monitoring dose exposure over time in RBCs. The long half-life of TFV-DP in RBCs can also be used to smooth out the pharmacokinetic curve so that it represents an average drug dose over time [11]. TFV has a short half-live (15 hours) and can be used as an indicator of recent drug exposure [20,24]. Additionally, the long TFV-DP half-life can also be used to inform whether recent clinician dosing masks remote non-adherence and will act as a cumulative adherence measure which provides a better representation of a subject's overall adherence. The long intracellular half-life of TFV-DP is favourable since it allows less frequent dosing; however, it could also be harmful, due to accumulation in higher-metabolising patients and the associated toxicity [12,37].

The significant difference in half-life of TFV and its metabolite, thus allows the measurement of both cumulative and recent drug adherence. This is useful, since recent adherence measures alone may lead to misclassification of adherence, due to patients becoming more adherent during the days preceding clinic and study visits [35].

The underlying principal for the measurement of adherence is the half-life of the drug. The half-life of compounds may differ depending on the absorbing cell type, resulting in accumulation of compounds. The half-life of TFV-DP in RBCs is significantly longer than those seen in peripheral blood mononuclear cells (PBMCs) (17 days and 6.25 days respectively). Since dried blood spots (DBSs) mostly consist of RBCs, it is a suitable matrix for TFV-DP testing [24]. DBSs offer a more versatile blood sampling method than traditional methods, this is due to the capability to measure both the intracellular metabolite and the parent drug [24].

1.5. Introduction to the Study

TFV is recommended as the preferred first-line ART in almost all HIV-1 treatment and chemoprevention [10]. This adds to the suitability of TFV in DBSs as a routine measure of adherence over time. Current TFV-DP quantification methods have various limitations relating to sensitivity, specificity and multiplex capabilities [13]. Extensive sample preparation steps are traditionally added to concentrate the product, resulting in improved selectivity of the method and an increase in the sensitivity of detection. These steps are necessary, but require intensive labour and manipulations, thus, the need exists for an alternative method to avoid these laborious steps [38–40]. The need for an inexpensive, simple, sensitive, and robust method for the determination of TFV and TFV-DP in DBS cannot be overstated, and this study serves to outline the development and validation of such a procedure. Therefore, the aim in the present study was to develop a sensitive, specific and robust multiplex method for the measurement of adherence to antiretroviral regimens, utilising TFV and TFV-DP in DBS using liquid chromatography combined tandem mass spectrometry (LC-MS/MS) analysis, to accomplish this, the following technical objectives were set:

- The development of a chromatography procedure that would allow sufficient baseline separation for TFV and its metabolites.
- Development of a simple and effective extraction method.
- The validation of the method according to current FDA and EMA standards.
- The completion of a correlation analysis, comparing results obtained from the current method and the newly developed method.

This Masters' thesis consists of six chapters, wherein the chapters will discuss each of the essential steps in the development and validation of this method. The introduction and background information is presented in Chapter 1. Chapter 2, the literature review, serves to discuss and investigate previous methods and their shortfalls, and alternative approaches - and the hurdles that may be encountered. Chapter 3 presents the method development process and covers three broad areas: namely the optimisation of chromatography; the development of an extraction method; and optimisation of sensitivity and selectivity on the mass spectrometer. Chapter 4 summarises the final developed method. Chapter 5 presents the validation process according to current FDA and EMA guidelines. Lastly, Chapter 6 summarises the findings in the study.

Thus far, the study produced the following outputs:

1. An oral presentation at the 4th Annual Antivirals conference in Sitges, Barcelona, Spain, 2016. Title "The Development and Validation of a Direct LC-MS/MS Assay for the Determination of Tenofovir, Emtricitabine and their metabolites in Dried Blood Spots for the Analysis of Clinical Samples."

2. A Poster presentation at the Annual South African Society for Basic and Clinical Pharmacology 2017 congress Title: The Development and Validation of a Direct LC-MS/MS Assay for the Determination of Tenofovir and its metabolites in Dried Blood Spots for the Analysis of Clinical Samples."

Chapter 2: Literature review

Pharmacologic monitoring of TFV and TFV-DP is essential for the elucidation of adherence which allows evaluation of regimen efficacy and, therefore, facilitates the prevention of antiretroviral resistance and HIV-related mortality and morbidity. To assess adherence, an accurate and robust bioanalytical method is required. This section considers existing analytical methods and investigates alternative methods.

2.1. Bioanalytical Method Development and Validation

LC-MS/MS has become the preferred bioanalytical tool, allowing the development of simple, high precision, automated analytical methods with improved accuracy, sensitivity, and selectivity [41–44]. Bioanalytical methods are used for the quantification of analytes and their metabolites in biological matrices. Such analytics play a vital role in the evaluation and interpretation of various studies, including bioavailability, bioequivalence, and toxicology studies [45]. Before a bioanalytical method can be used for the analysis of biological samples, the method must undergo a validation process. The method must demonstrate its suitability for its intended use and ability to produce reliable and reproducible results, hence, regulatory entities have issued guidelines to which methods must adhere [41,45,46].

Modern analytical instrumentation has led to improved selectivity and sensitivity. Of these analytical tools, LC-MS/MS is considered as the benchmark for qualitative and quantitative analysis [41,42,44]. However, limitations are encountered as a result of matrix effects and the associated loss of sensitivity of analytes in processed biological matrices [46]. This is often due to the complex nature of the background molecules in these samples. Biological matrices are comprised of various organic components, including salts, proteins, and small organic compounds, like lipids [41]. The choice of sampling matrix is analyte-specific and depends on various drug properties and the planned analytical approach.

2.2. Sample Collection and Storage Matrix

PBMCs are mostly used in studies with new NRTI, drug-drug interaction studies, and studies where new prescription strategies are developed [36]. Most therapeutic drug monitoring assays are traditionally performed by venous blood sampling and use either serum or plasma [47]. These approaches are often applied, although they are invasive and present various storage and transport issues. Dry blood spots (DBS) can be used as an effective alternative to traditional venous sampling methods and consists of whole blood, which contains erythrocytes, plasma, platelets, and interstitial fluid [48]. DBS only requires around 50–125 µl of whole blood and recent advances in bioanalytical analysis have allowed the measurement of analytes in DBS, with volumes ranging between 4–12 µl of spotted blood [49]. Plasma sampling, which is the matrix of choice for pharmacodynamics and pharmacokinetic studies, requires more than 4 ml of blood [49]. The smaller blood volumes required make DBS sampling more suitable for children, less invasive, and simpler in comparison to traditional venous sampling methods [47]. In addition to the small sampling volume, most analytes are also more stable when sampled and stored as DBS

cards [50]. The use of DBS also simplifies the transport and storage of the samples and this is accompanied by a decrease in cost and labour [24,47,49,51–53].

Capillary blood for DBS is sampled by means of a finger prick and this is usually carried out with a lancet. Patients will be able to perform the sampling on themselves after receiving adequate training and instruction, thus samples can be collected without the presence of a phlebotomist [49]. After the finger prick is made, the first drop is discarded, due to increased tissue fluid, and the second drop is spotted onto one of the pre-marked circles on a DBS card. The cards are subsequently left to dry at room temperature and stored [49]. Discs are punched out from the pre-marked circle and each disk represents a fixed volume of absorbed blood [49,54].

The logistical constraints accompanying sampling, storage and collection of traditional biological samples in community-based settings have significantly limited research and monitoring. Therefore, DBS is an ideal substitute to traditional sampling methods, since it provides a minimally invasive alternative, with simplified collection, storage and transport requirements; however, DBSs only provide small volumes, which require higher sensitivity in subsequent analysis. It also presents an issue when samples need to be reanalysed or if samples need to be used for more than one analysis [49]. Other disadvantages include the possible differences in analyte concentrations between capillary and venous blood samples, which may lead to discrepancies in results. There is also a risk of contaminating the filter paper if the same person who administers the dosing also collects the sample [49].

The effect of haematocrit on DBS assays, however, may be the greatest cause of variation in analysis results, since haematocrit has a significant effect on the viscosity of whole blood and is largely responsible for the extent to which sampled blood spreads on filter paper [55]. Haematocrit is the volume percentage of RBCs in whole blood and can directly influence the accuracy of a DBS bioanalytical assay. The extent of the haematocrit effect on the assay depends on the analyte's chemical and physical properties [56]. A study conducted by Zheng *et al.* analysed TFV and FTC in DBS and reported that samples with haematocrit values outside the range of 25–76%, did not meet the ±15% cut-off criteria required for accurate sample analysis, as set by the authors [47]. The authors also observed increased mass spectrometer response, of both TFV and FTC, with increasing haematocrit [47]. The analytes' recovery is also affected by haematocrit and higher recovery is often observed at low haematocrit values, whereas lower recovery is often observed at high haematocrit values [57].

The complications caused by varying haematocrit values can be avoided by analysing the DBS in its entirety, since this will eliminate any variation caused by non-homogenous spreading on the filter paper; however, using the whole DBS instead of punches will increase the importance of accurate volumetric application of blood to avoid variation, which makes sampling difficult for clinical sites [49]. The effect of haematocrit can also be decreased by standardising the calibration standard's haematocrit values closer to expected values; however, most DBS studies do not have any haematocrit information, making it impossible to compensate for varying haematocrit values [58]. Recent studies describe a method that utilises potassium as a measure to elucidate haematocrit of DBS samples directly. Potassium is predominantly present intracellularly at concentrations 35 fold compared to those observed in the extracellular fluid, thus it is ideal for estimating haematocrit [58]. However, this analytical approach is destructive to the sample and, therefore, is not ideal where sample volumes are limited. A non-destructive alternative has recently been developed and utilises noncontact diffuse reflectance spectroscopy to determine the

haematocrit of samples. The method has shown a significant correlation between the predicted haematocrit values and the true haematocrit values [59].

2.3. Analyte Stability

The correct storage and handling of clinical samples, standards, and quality controls (QCs) are essential for reproducibility of an assay. Stability should be calculated to determine adequate storage conditions and ensure reproducibility and reduce variation [46]. Stability is analyte and matrix specific and, therefore, this needs to be considered for sampling, storage and transport of samples.

A study done by Castillo-Mancilla *et al.* determined the stability of TFV-DP in DBS samples, by comparing samples stored at room temperature, 4^{0}C and -20^{0}C to samples stored at -80^{0}C. Acceptable stability was defined as any value within 15% of the -80^{0}C stored sample values [24]. No change (-0.3%) was observed for samples stored at -20^{0}C, although this was not the case after seven months of storage. Samples stored at 4^{0}C showed a % Difference of -6%; however, this was within the 15% acceptability range and was classified as stable. Samples stored at room temperature were not within the required 15% (-47%) and, thus, storage at this temperature does not allow acceptable sample stability [24]. This significant deviation was apparent after one week of storage. TFV-DP stability can also be determined by monitoring TFV concentrations, since TFV concentrations significantly increased in samples stored at room temperature. This is due to the degradation of TFV-DP back to its parent compound [47]. Thus, it is clear that TFV-DP DBS patient samples should be stored long term at -20^{0}C or lower to maintain integrity of samples [47].

2.4. Extraction

As previously mentioned, LC-MS/MS has become the preferred bioanalytical technique; however, LC-MS/MS is plagued by limitations as a result of the complex nature of biological matrices [41,46,60]. Biological samples contain background compounds, like salts, proteins, lipids, lipoproteins, and various other contaminants, which should be removed from samples prior to injection [60]. The complexity of biological matrix constituents differs for each individual matrix, thus each matrix faces unique challenges requiring different approaches to be implemented [41]. For example, plasma contains high concentrations of phospholipids, while whole blood needs to be lysed before analysis, and urine contains high levels of salts that need to be removed before injection into the mass spectrometer [61].

Biological matrix effects can result in significant variation in LC-MS/MS analysis, due to ion suppression or enhancement, and the non-volatile nature of some matrix constituents [61]. This occurs regardless of the use of electrospray ionisation (ESI) or atmospheric pressure chemical ionisation (APCI) [41,61]. However, matrix effects can be reduced during the sample extraction process, when it succeeds in isolating and concentrating the analyte of interest, and also removes matrix components from the processed sample [41]. Such extraction methods may require additional steps, depending on the selected biological matrix. Therefore, when selecting an extraction

approach, the chemical characteristics of the analytes of interest and the biological matrix must be taken into account [45].

There are three core extraction approaches, namely protein precipitation (PP), liquid-liquid extraction (LLE), and solid phase extraction (SPE) [41]. PP is the least laborious approach and has widely been used in bioanalytical methods [41]. PP removes contaminants through the denaturation of proteins [41]. This is often achieved by adding organic solvents to the biological matrix; however, it is not limited to this methodology and proteins can also be denatured by adding heat, strong acids or bases. The method of denaturation is selected based on the physiochemical properties of the analyte of interest [41]. After denaturation, samples are centrifuged to draw denatured proteins to the bottom of the vial, leaving other components in the liquid layer [62]. The supernatant is subsequently removed and used for quantitation [41]. PP is widely considered as the least favourable extraction protocol for LC-MS/MS, since it only removes proteins and leaves various other matrix constituents in the processed sample [41].

LLE is centred around affinity-based separation, where two immiscible solvents are added, allowing the analyte of interest to migrate to the solvent for which it has the most affinity [41,62]. This is based on the hydrophobicity of the analyte of interest and solvents are strategically selected according to their immiscibility with the sample solution and compatibility with the analyte of interest [62]. Even though it is a widely used technique, LLE has various limitations, including low or fluctuating recovery, it requires large sample volumes, and it may lead to poor selectivity and matrix effects when used in conjunction with mass spectrometry methods [41]. Alternative extraction methods are required for polar analytes, due to the poor recovery of water-soluble polar analytes. This will lead to poor reproducibility and ultimately a less robust methodology [63].

SPE was originally developed to substitute LLE; however, SPE is also centred around affinity based separation, although this approach chemically separates the various components of the sample by the use of solid particles [64]. These particles usually consist of chromatographic packing material and analytes are separated based on the analyte's affinity for the stationary phase [41]. SPE offers various advantages over other techniques, although it also has limitations, especially when working with hard to retain polar compounds. However, problems associated with LLE, namely incomplete phase separation, the use of expensive specialty glassware, and the use of large volumes of expensive organic solvents, can be circumvented with the use of SPE [64,65]. SPE also does not require large sample volumes, making it ideal for DBS analysis.

2.5. Mass Spectrometry

Mass spectrometry (MS) is an analytical technique that isolates and detects species according to their mass-to-charge (m/z) ratio. MS consists of three main components, namely an ionisation source, a mass analyser and a detector, and this is shown in Figure 2.1 [66]. The mass spectrometer converts the analyte of interest into gas phase ions, which is an essential step required for the detection of compounds [66]. There are various MS sources that allow this reaction to take place, namely ESI, APCI, and atmospheric pressure photoionisation (APPI); however, only ESI will be discussed since it will be used in this study [66].

During ESI, ions are generated at atmospheric pressure by passing a solution of compounds through a small capillary that has a potential difference of more than 500 volts, relative to a counter electrode (Skimmer cone) [66]. The precise voltage depends on the capillary diameter and the solvents used. An aerosol-like spray of charged droplets is created through a nebulisation process which is usually facilitated by an inert gas, like N_2 [67]. Droplets will migrate from the capillary needle towards the skimmer cone as a result of the generated potential difference. As the droplets migrates, solvent evaporation occurs and the droplet diameter decreases as a result of the high temperature environment in the source [67]. The decrease in droplet size leads to an increase in charge density, until it becomes unstable after reaching its Raygleigh limit, after which a cascade of Coulomb fissions occurs until gaseous ions are formed [67].

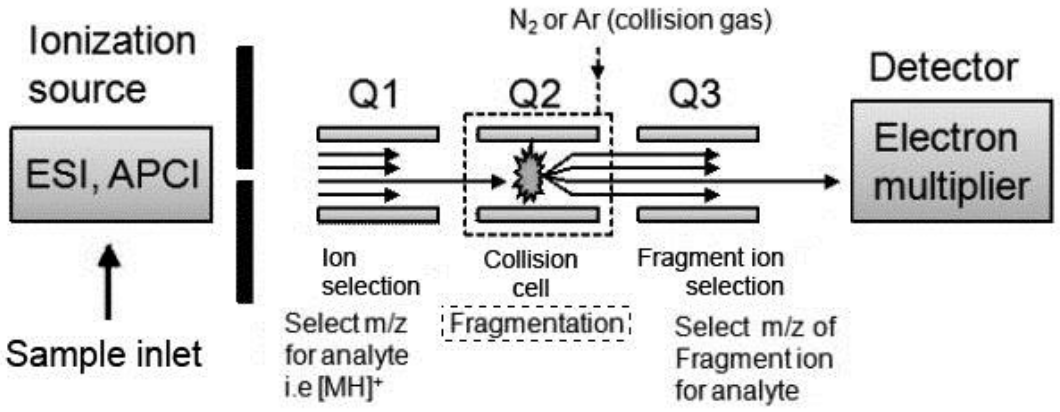


Figure 2.1: Basic schematic of the three main components of a MS system [68].

2.6. Liquid Chromatography

Liquid chromatography is a separation technique in which a mobile phase is passed through an analytical column, packed with a stationary phase (i.e., the sorbent), resulting in separation of solutes in mobile phase. The latter being the consequence of differences in the physiochemical properties of the solutes [69]. Each solute will have a certain affinity for the sorbent and during the transit time of solutes, they will interact with the sorbent to a varying extent based on the compounds affinity for each solute [69]. Therefore, separation of compounds can be achieved.

Conventional liquid chromatography is often used for preparative work and the purification and isolation of sample constituents. Recent advances in liquid chromatography utilizes small packing particles and high pressure for analytical separation of samples, referred to as high performance liquid chromatography (HPLC). The use of smaller particle sizes results in improved separation, resolution and column efficiency. This can be described by the Van Deemter equation, shown below:

$$H = A + {}^{B}/_{\mu} + C\mu$$

Where:

H= Theoretical plate height

A= Eddy (axial) diffusion

B= Longitudinal diffusion

C= Mass transfer

μ=Linear velocity

With the improvement in HPLC systems, lower linear velocities are attainable. This, accompanied with improved column chemistry, allows increased resolution and column efficiency [70].

Reversed-phase high-performance liquid chromatography (RP-HPLC) separates molecules based on hydrophobicity. The separation is determined by the hydrophobic binding of the solutes dissolved in mobile phase to the immobilized hydrophobic ligands attached to the sorbent [69]. Elution can proceed either by isocratic conditions, where the concentration of organic solvent is constant, or by gradient elution whereby the amount of organic solvent is increased over a period of time. The solutes are, therefore, eluted in order of increasing hydrophobicity [69]. RP-HPLC has excellent resolution properties and can be maintained over a wide range of chromatographic conditions. RP-HPLC also provides experimental ease with which chromatographic selectivity can be manipulated through changes in the mobile phase [69].

As a result of the highly polar nature of NRTIs and their phosphorylated metabolites, they are difficult to retain on conventional reversed-phase analytical columns. This encumbers the development of a multiplex method containing TFV and its metabolites. TFV has two pKa values at 3.8 and 6.7 and is highly hydrophilic (Log P= −1.6) [71]. TFV is very polar due to its phosphate group, making it difficult to retain on both reversed phase analytical columns and SPE cartridges [13]. The quantification of NRTI, like TFV and TFV-DP, presents various obstacles that need to be overcome to achieve reliable results. Nucleotides are negatively charged at pH values above 2, making them even more polar, and thus further decreases their retention on traditional reverse phase columns [72,73]. These negatively charged nucleotides can however, be separated with ionic interactions at the correct pH value [72]. Anion exchange liquid chromatography separate negatively charged analytes through ionic interactions with a charged stationary phase, although this is often incompatible with ESI mass spectrometry, due to the non-volatile nature of some of the inorganic salts that are required to act as competing ions [72]. These non-volatile inorganic salts often contaminate the ion-source and suppress ESI [72]. Alternatively, formic acid can be added as a mobile phase modifier as this helps facilitate ionization in the ESI positive mode and retention on the analytical column [13]. Improved retention is observed due to a decrease in the mobile phase pH, leading to NRTI reverting back to its unionised, and thus, less polar form.

All methods reviewed that allow the detection of TFV require an acidic solvent as a modifier. This facilitates ionisation when working in the positive mode of an ESI source and it also improves retention of TFV on a C_{18} analytical column [13,74]. LC-MS/MS-compatible acidic solvents that have been used to improve TFV retention and intensity, include formic acid, acetic acid, and trifluoroacetic acid, of which acetic acid has demonstrated the highest TFV sensitivity [13,74]. When working with an ionisable compound like TFV, the pKa can be used as an indication of the level of ionization at a certain pH. Working with a mobile phase pH that is close to the pKa value will result in instability in retention times, ultimately leading to a less robust method. Alternative approaches will be discussed in section 2.8. and these include derivatisation reactions, ion-pairing, and ion-exchange chromatography.

2.7. Existing Methods and Approaches for the Quantification of TFV and its Metabolites

Various methods have been developed to measure nucleosides and their phosphate metabolites; these can be grouped into two distinct categories, namely direct and indirect approaches. Indirect approaches focus on the separation of analytes and their phosphate metabolites into separate fractions with the use of strong anion-exchange SPE columns [13]. The separated metabolite fractions are subsequently dephosphorylated to allow reverse phase analysis [13]. The indirect approach is laborious, due to extensive sample pre-treatment, and often requires multiple analyses per sample. This leads to extra costs and is instrument-time demanding. The use of a direct approach can avoid these issues; however, greater chromatographic separation is required, due to the absence of the fractionation and subsequent dephosphorisation step in this method. The greater separation is especially necessary due to the possible degradation of analyte phosphate moieties in the ion-source [13]. Direct methods often have to employ the use of ion-pairing reagents that increase the capacity factor of the analytes, leading to improved retention, resolution and baseline separation [75]. A schematic depicting the two approaches can be seen in Figure 2.2.

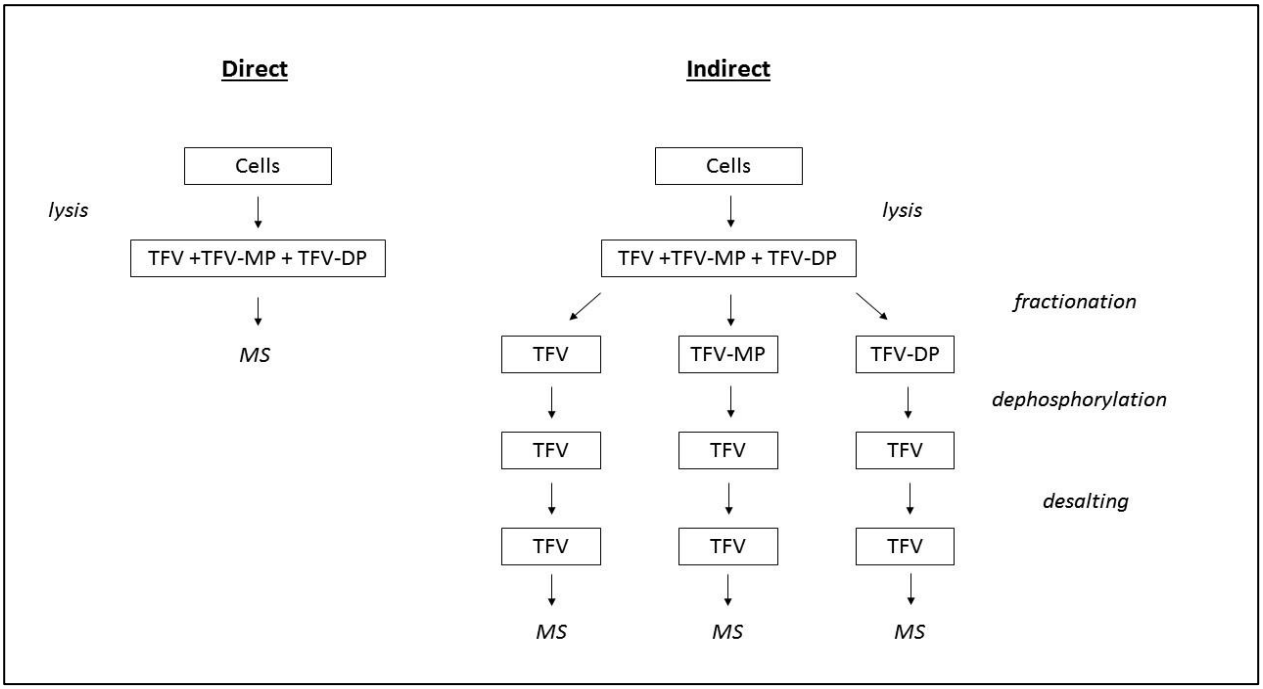


Figure 2.2: A schematic representation of the direct and indirect approaches of analysing TFV and its metabolites.

2.7.1. Indirect Approach

To achieve reliable quantification, the indirect method requires the separation and subsequent dephosphorisation of nucleotide analogues and their metabolites [13]. This is necessary due to the unstable nature of TFV-DP, TFV-DP degrades to TFV in the ion-source, thus adequate baseline separation is required for a direct approach. The indirect method avoids this drawback through separation and subsequent dephosphorisation of the metabolites during SPE.

A widely used method for the quantification of TFV and TFV-DP, was developed by Bushman *et al.* and includes the separation and quantification of mono-, di- and tri-phosphate metabolites of FTC, lamivudine and zidovudine [13]. In the developed method, a lysed cellular matrix is run through Waters QMA SPE cartridges, followed by the elution and isolation of the nucleotide analogue phosphate fractions. Various solutions with differing potassium chloride concentrations are used to elute nucleoside analogues [13]. The isolated nucleotide analogue fractions are subsequently dephosphorylated by adding acid phosphatase and incubating at 37^0C for 60 mins. Phosphatase hydrolyses the phosphate of TFV-DP and reverts the metabolite back to TFV. The fractions are then sent through a Phenomenex Strata-X SPE cartridge, to desalt and concentrate the fractions [13]. This method achieved measurable signals of reference standards at levels as low as 1 fmol/sample, which is equivalent to 0.30 fmol of TFV on an analytical column; however, similar levels were not measurable in extracted samples, due to the variable process efficiency. With the aim of increasing recovery, trifluoroacetic acid-containing SPE (Waters Oasis HLB cartridge) were tested. Even though the improved recovery was non-significant, trifluoroacetic acid is necessary for the retention of TFV on HLB cartridges. This may not be the case for other nucleotide analogues

and the acetic conditions may even lead to instability. However, acetic conditions are required for TFV due to the difficulty encountered when attempting to retain TFV, which is mainly as a result of its polar nature and phosphate group [13,74,76]. Considering that TFV-DP has added phosphate groups, more severe retention problems may be encountered when analysing this compound.

One of the notable advantages of the indirect method is that it allows the quantitation of mono-, di-, and tri-phosphates, without requiring the reference standards of the metabolites, which are often expensive. This is as a result of the dephosphorylation process reverting the metabolites back to the parent drug which is more stable, hence there are also less stability issues [13].

2.7.2. Direct Approach

The direct approach does not involve the separation and dephosphorisation of nucleotide metabolites, but rather the extraction and injection of the analytes in a single run. This minimises cost, labour and time; however, this approach requires adequate baseline separation, which may be problematic when working with polar molecules like TFV and TFV-DP.

A TFV-DP direct method has been developed, although it was designed for PBMCs and not DBS [26]. In the method, PBMC samples were processed by PP and analysed on a LC-MS/MS system. PBMC samples were centrifuged and a PP step (1:1 methanol: 1 mM ammonium phosphate solution) was subsequently performed on the samples. Followed by an evaporation step under an inert gas, after which the analytes were reconstituted with 1 mM ammonium phosphate and subsequently analysed on an AB Sciex API-5000 triple quadrupole mass spectrometer [26]. The method had a dynamic range of 1–2500 ng/ml. The recoveries for TFV-DP in PBMC was approximately 100% using the methodology described in this study. This however, was not the case for the upper layer plasma cells studied, where the recovery of TFV-DP was a dismal 20% [26].

Recently, a direct on-line LC-MS/MS method was developed that allows the quantitation of TFV, FTC and 3TC and their phosphate metabolites [73]. The method uses an automated on-line weak anion exchange (WAX) SPE method, coupled with ion-pair chromatography to clean and separate the analytes of interest. The WAX column allows the concentration and separation of the analytes of interest [73]. 1,5-dimethylhesylamine (1,5-DHMA) was used as an ion-pairing reagent to increase the retention time of polar analytes [73]. However, this method was completed in macaque PBMCs and requires a dedicated mass spectrometer with 6 and 10 port column switching valves coupled to six single solvent line pump systems. The analytical method also had a total run time of 18 mins [73]. Taking into consideration instrument requirements and the lengthy run time, this method is not suitable for routine laboratory work.

Stability is an issue when working with nucleotide analogue triphosphates, thus all extractions need to be carried out on ice to maintain analyte stability. This is especially applicable for the direct method, since metabolites are not dephosphorylated back to the more stable parent analyte.

The mobile phase pH values of previous developed methods for nucleoside triphosphates including TFV-DP and its parent drug TFV, ranging from 5–10.5, depending on the mobile phase modifiers, presence of an ion-pairing reagent, and the organic solvent used. When using an ion-pairing reagent, most studies used a gradient to elute the analytes [26,77]. Both isocratic and gradient analytical runs have been reported for the analysis of TFV and TFV-DP, although the direct method and methods that employ ion-pairing reagents use gradient elutions [26,77].

2.8. Measurement of Nucleosides and Nucleotides

Apart from retention issues, sensitivity and selectivity are also paramount in the detection of intracellular concentrations of nucleotide triphosphates, such as TFV-DP [38]. The use of a chemical derivatisation reaction [i.e., using dansyl chloride (Dns-Cl)] will increase the hydrophobicity and chromatographic retention of analytes, on a traditional reverse phase column and will also increase the selectivity and sensitivity. As an alternative to Dns-Cl an ion-pairing reagent and ion-exchange chromatography will also be discussed.

2.8.1. Dansyl Chloride Derivatisation

Amine derivatisation reagents like Dns-Cl, fluorescamine, fluorenyl-methyl chloroformate, dabsyl chloride and a myriad of thiol containing *o*-phthalaldehydes (OPA) have extensively been used with the aim of improving selectivity and sensitivity of free amino acids assays and various other amine-containing analytes. Even though Dns-Cl and OPA are the most widely used amine derivatisation reagents, studies have shown that both Dns-Cl and OPA are suitable derivatisation reagents in the nano-mole concentration range; however, Dns-Cl is a superior derivatisation reagent in the pico-mole concentration range [38,78–81]. Dns-Cl derivatisation products are also more stable than those formed using OPA. This adds to its suitability as a derivatisation reagent; however, it does react with water, leading to the production of various hydrolysis products [78]. Hydrolysis can be prevented by using minimum amounts of Dns-Cl or working at the optimal pH values [80].

Dns-Cl reacts with primary aliphatic and aromatic amines (as seen in Figure 2.3) and should react with the amine groups on TVF and TVF-DP, leading to increased specificity and selectivity [38,76]. This could allow the design of a direct multiplex method that could lead to a cost-effective and less laborious assay.

Dns-Cl derivatisation yields are significantly influenced by pH, reaction time, reaction temperature, and Dns-Cl concentration [80]. Derivatisation yields increase drastically with rising temperatures, as demonstrated in a study comparing the yield of reactions carried out at different incubation temperatures [80]. A reaction temperature of 95^0C, yielded up to 10 fold higher derivatised products when compared to solutions incubated at 40^0C [80]. However, the evaporation of the mobile phase at higher temperatures limit reaction temperature and most studies use temperatures ranging between 60–80^0C [79–82]. The optimal reaction time varied between most methods and appeared to be analyte and concentration specific, although most methods used an incubation period ranging between 30–60 mins [76,80,82]. A pH of 9.5 is optimum for the derivatisation of unprotonated amine groups. When the reaction is carried out at pH values higher than 10.0, the reaction of primary amine groups with Dns-Cl is in

direct competition with the hydrolysis of Dns-Cl by hydroxyl groups or water [78]. Adducts also form during the derivatisation reaction and should be minimised to achieve optimal sensitivity and selectivity. A study showed that Dns-Cl adduct yield decreases with increased Dns-Cl volumes [80]. This was attributed to the decomposition of adducts, due to excess Dns-Cl [80]. Most studies report using acetone to dissolve Dns-Cl, before addition to the biological samples [76,79,81].

To ensure the reproducibility of the Dns-Cl derivatisation reaction, the reaction should be stopped after a designated reaction period. This is often accomplished by adding an acid to the reaction mixture after a specified incubation period [81,83]. To decrease the interference of excess Dns-Cl in HPLC systems, alternatives like L-alanine can be added to stop the dansylation reaction, this works by reacting with excess Dns-Cl and stopping the formation of further derivatised products [78]. Glycine has also been used to react with excess Dns-Cl, after which the dansyl glycine was extracted with the use of C_{18} Sep-Pak cartridges [83]. However, the addition of extra extraction steps should be avoided when working with small sample volumes or struggling with sensitivity.

Special consideration should be given when adding a Dns-Cl derivatisation step to a method, since Dns-Cl is highly reactive and may react with internal standards (ISTD), especially if they contain an amine group. This is a problem when working with non-deuterated ISTD. When working with non-deuterated ISTD, the ISTD are chosen due to their ability to separate completely from analytes and an inertness to Dns-Cl [80]. However, when working with a deuterated ISTD the ISTD should be derivatised in order to compensate for reaction variability. Other reported complications include a decrease in analytical column life and most methods require a post run wash step, to remove impurities from the column. These wash steps consist of either MeOH or acetonitrile (ACN) [78,80]. Incorporation of a reversed column flush before the injection of a new batch, has been shown to significantly prolong the life of an analytical column [78]. Derivatised polyamines have also been reported to stick to polypropylene tubes, with larger polyamines binding more strongly to the tubes due to their higher charge [78]. Recovery issues have been encountered when reconstituting derivatised polyamines, due to their solubility in certain organic solutions [78].

A sensitive and selective LC-MS/MS method was developed for the simultaneous determination of gemcitabine (dFdC), an anti-cancer chemotherapy drug, and 2,2-difluoro-2-deoxyuridine (dFdU), its metabolite, using a Dns-Cl derivatisation step [76]. The derivatisation step was essential for retention and sensitivity of the compounds. The method used a pre-column derivatisation step that was performed in plasma without prior sample clean-up. Samples were subsequently extracted, using methyl tertiary-butyl ester (MTBE) [76]. dFdC is similar to TFV in its chemical properties and mode of action. Much like TFV, dFdC is phosphorylated to its pharmacologically active metabolite intracellularly. The di- and tri-phosphorylated metabolites are responsible for the drug's cytotoxic effect and dFdC and dFdU are very polar, like TFV, resulting in difficulty in attaining good resolution and retention on a reverse phase column [76]. These issues were circumvented by adding a Dns-Cl derivatisation step. The derivatisation step was carried out by adding a 100 µl of 10 mg/ml Dns-Cl in acetone and 100 µl 100 mM sodium bicarbonate (pH 11) to plasma samples, followed by a vortex step and incubation at 60^0C for 5 mins [76]. The dansyl-derivatives of dFdC and dFdU were then extracted using LLE by adding 1 ml of MTBE [76]. Even though the method achieved its aim of determining dFdC and dFdU, no mention was made of determining the concentration of the phosphorylated metabolites of either dFdC or dFdU. The authors also do not mention whether these metabolites

were degraded back to the parent drug during the Dns-Cl derivatisation reaction or whether the different fractions were used to determine the total dFdC and dFdU concentrations separately.

The bulk of methods reported for the determination of amino acids contain an offline pre-column derivatisation step [80]. SPE and LLE methods have been reported for the extraction of dansyl derivatives [80,83,84]. A study utilising off-line pre-column derivatisation added samples to 2 mol/l $KHCO_3$-KOH with a pH of 9.8 and 20 mg/ml Dns-Cl. This was incubated at 80^0C for 30 mins and the reaction was subsequently stopped by adding 20 µl of acetic acid [80].

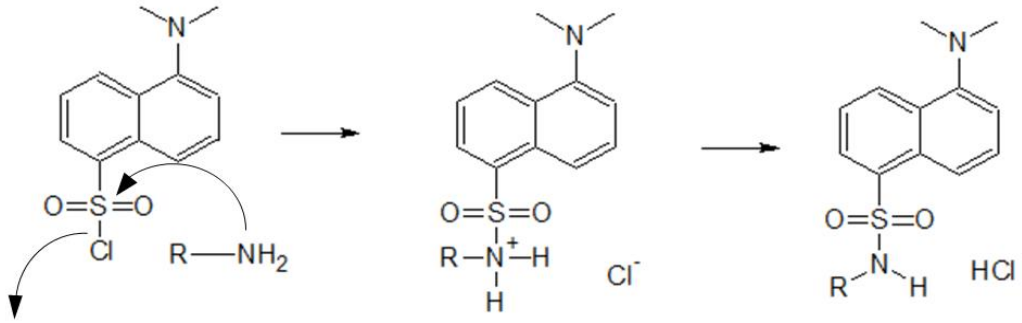


Figure 2.3: A depiction of the reaction of Dns-Cl with an amine group, leading to the formation of hydrochloric acid. Adapted from Hernández-Borges *et al*. [85].

2.8.2. Ion-Pairing Chromatography

Ion-pair chromatography is a promising alternative to anion exchange chromatography, since it is compatible with mass spectrometers and leads to improved retention and chromatography [75]. Using ion-pairing reagents as mobile phase additives allows the separation of ionic and highly hydrophilic substances on conventional reversed-phase analytical columns [75]. Various ion-pairing reagents have previously been used in methods that quantify nucleotide analogues and were selected based on the physiochemical properties of analyte of interest [72,73]. The addition of an ion-pairing reagent is necessary, since conventional reversed-phase separation is based on the hydrophobic interactions between the stationary phase and the analyte of interest. Hence, polar compounds, like TFV and its phosphorylated metabolites, will not interact with the hydrophilic stationary phase, leading to little to no retention and poor separation. The addition of an ion-pairing reagent leads to increased retention and separation of polar analytes [81,86,87].

Ion-pairing reagents consist of an ionic end and a non-polar tail (hydrocarbon chain) [88]. After addition to the mobile phase, the non-polar tail has strong interactions with the non-polar stationary phase. This leaves the ionic group

exposed and ionic species of the opposite charge will interact with the ion-pairing reagent, leading to significant increases in retention [88]. This mechanism of action is shown in Figure 2.4.

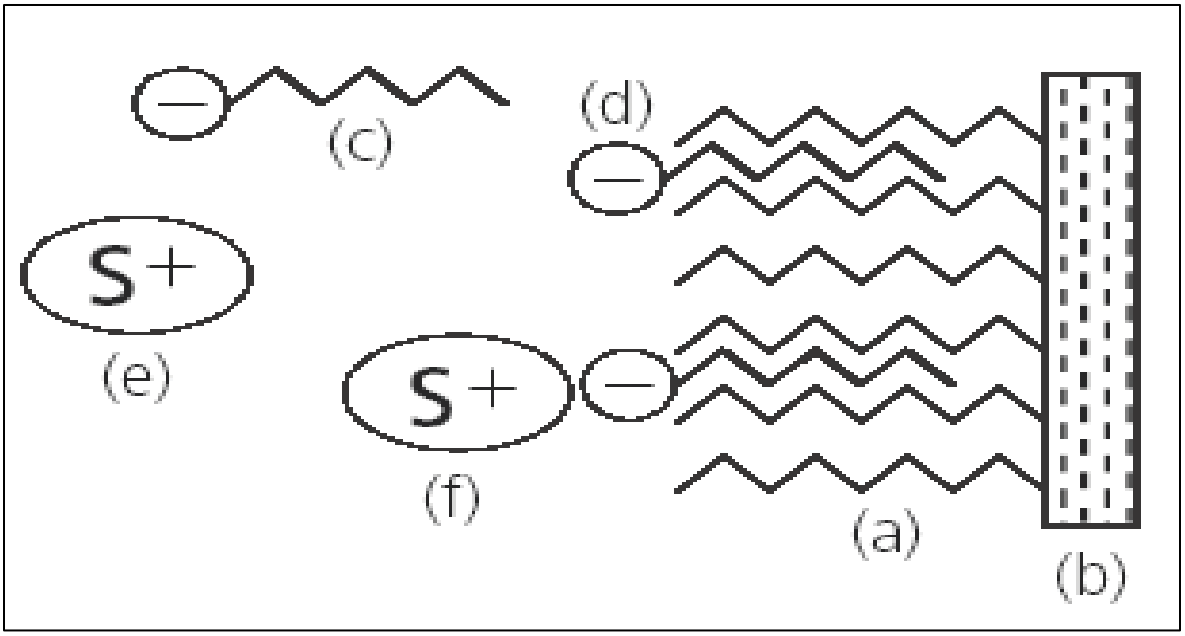


Figure 2.4: A visual representation of the mechanism of action of ion-pairing is shown: As can be seen a) is the bonded phase that is attached to b) the silica particles. The c) unattached ion-pairing reagent d) interacts with the stationary phase. The e) analyte of interest is injected into the mobile phase and f) interacts with the ionic group attached to the stationary phase [89].

TFV and TFV-DP studies have reported the use of ion-pairing reagents and the most commonly used is 1,5-DHMA [73,77,90]. However, its use may lead to a loss of sensitivity when compared to other more volatile ion-pairing reagents [72]. The use of hexafluoroisopropanol and trimethylamine have been suggested as an alternative to 1,5-DMHA. The combination of these ion-pair reagents have shown increases in selectivity and sensitivity compared to 1,5-DMHA [72]. This is proposed to be a result of dynamic pH changes of the electrospray droplet that are caused by the selective evaporation and elimination of the more volatile HFIP anionic counter-ion. This approach has been used in a method designed to measure nucleotide triphosphates [72]. In this method the mobile phase pH is approximately 8.3; but after evaporation of the electrospray droplets, the pH rises to 10 and, at this higher pH, the trimethylamine dissociates from the nucleotides and are released into the gas phase, resulting in increased ionisation; however, these ion-pairing reagents have not been used in combination with TFV or its metabolites [72].

The mobile phase pH is of the utmost importance for ion paring reagents, since profoundly different elution characteristics have been reported due to pH changes. At higher pH values certain functional groups, like carboxyl groups, will ionise and lose affinity for the charged species of the ion-pairing reagent [88].

2.8.3. Ion-Exchange Chromatography

Ion-exchange chromatography is a chromatographic technique that separates polar and ionic compounds based on ionic or electrostatic interactions [91]. Ion-exchange analytical columns have charged ionic functional groups derivatised to the column's stationary phase and can be subdivided into either cation-exchange or anion-exchange columns, depending on the charge of the attached functional groups [91]. The type of ion-exchange column is selected based on the charge of the analyte of interest. Cation-exchange columns contain negatively charged functional groups and are used to separate cations; whereas, anion-exchange columns contain positively charged functional groups and are used to separate anions [91].

The ionic strength of the mobile phase plays an essential role in the retention of compounds and elution is facilitated through the use of a mobile phase with a high ionic strength. Analyte elution can be achieved by either neutralisation of the analyte, neutralisation of surface functional groups, or the use of strong counter ions [91]. This can be achieved using one of two approaches, namely a salt or pH gradient.

When using a salt gradient, the ion of the analyte of interest must face competition from similarly charged counter ions present in the eluent. These ions will compete for the same oppositely charged ionic functional groups and will be displaced based on their charge density, thus, allowing separation of various analytes [91]. However, this is often incompatible with ESI mass spectrometry, due to the non-volatile nature of some of the inorganic salts that act as counter ions, leading to ion suppression and the contamination of the ion-source [91].

The pKa of the analyte of interest is essential when working with a pH gradient, since the pKa determines at which pH conditions a functional group will hold a charge. An acidic compound will be charged at two pH units above the pKa of the analyte, leading to the formation of an anion. The opposite is true when working with basic compounds [91]. This is not only applicable for the analyte of interest, but also for the functional groups derivatised to the stationary phase. Thus, changing the pH of the mobile phase will lead to the formation of either a charged or neutral functional group, leading to the retention or elution of the analyte of interest [91]. Various studies have reported the direct determination of TFV-DP using an anion exchange column with an ammonium acetate and pH gradient [26,92–94]. Most studies used a pH of 6 for mobile phase A and a pH ranging between 9.5 and 10.5 for mobile phase B [26,92–94]. The ammonium acetate concentrations varied significantly across the reviewed procedures; however, all the studies used a significantly lower ammonium acetate concentration in mobile phase A, ranging between 1–5 mM, and a high ammonium acetate concentration in mobile phase B, ranging between 10–750 mM [26,92–94]. This allowed the separation of TFV, TFV-MP and TFV-DP [92].

2.9. Method Validation

The validation of a bioanalytical method is a mandatory step that is required to demonstrate the suitability of an analytical method for its intended purpose, before the method may be implemented in routine application. Validation determines the reliability of the results obtained using the analytical method and is vital for pharmacokinetic, pharmacodynamic, bioequivalence, bioavailability, or toxicological studies [46]. It is vital to

establish that the quantification of an analyte in a biological matrix is reproducible, reliable, and suitable for the application [95]. General and specific standard operating procedures and good record keeping are key components to a successfully validated method.

The method can undergo either a full, partial or cross-validation and the validation strategy is determined by the degree of method development. A full validation is required when a new drug or metabolite is added to the bioanalytical method or a newly developed method is implemented for the first time [46]. According to EMA and FDA guidelines, a full validation must study all fundamental parameters including: selectivity, accuracy, precision, recovery, calibration curve, sensitivity, reproducibility, matrix effect, carry-over, and stability [46,95,96]. In the present study a full validation of the newly developed method was conducted. The various validation parameter and special considerations are listed below:

2.9.1. Internal Standards and Stock Solutions

An ISTD is added to improve the precision of an assay and aids in the quantification of an analyte [44]. The addition of an ISTD corrects for variability caused by dilutions, degradation, recovery, derivatization, evaporation, and various other instrumental parameters [44]. The addition of an ISTD is essential when working with a multi-step sample preparation procedure [44]. As has been mentioned before, LC-MS/MS is plagued by matrix effects and this is especially evident when working with an ESI source. When the matrix is introduced into the ion-source, the analyte of interest will compete for ionisation with various matrix constituents, leading to ion suppression [44]. The degree of ion suppression varies between matrices and may vary between patient samples [44]. Thus, this emphasises the need for an ISTD to correct for any variability in ionisation. However, the degree of ion suppression depends on the chemical structure of the analyte of interest; hence, an ISTD and the analyte of interest must have a sufficiently similar chemical structure to allow comparison. If the ISTD and the analyte of interest are not sufficiently similar, the degree of ion suppression may vary, leading to differences in the detector response and compromising the quantitation [44]. Therefore, ISTD are usually either structural analogues or stable isotopically labelled analogues of the analyte of interest [44]. Isotopically labelled analogues are preferred when used in combination with a mass spectrometer, since improved assay performance has been reported [44]. Stable isotopes, such as deuterium ^{2}H, ^{13}C, ^{15}N, and ^{17}O, are usually substituted for atoms in isotopically labelled analogues. Three to eight atoms are usually substituted and the most commonly used isotopes are ^{2}H and ^{13}C [44]. It is important that the stable isotopically labelled analogues contain three or more isotopes, since it should theoretically co-elute with the analyte of interest and a mass shift of three will allow the separation of the two compounds on the mass analyser. This should also help prevent cross-talk, which may impact the quantification of the analyte of interest. Cross-talk is due to the slow removal of ions from the collision cell and is often observed when analytes with the similar mass properties, structures, or monitored fragment ions are present [97]. Deuterated ISTDs are often used since they are less costly and more abundant than ^{13}C-, ^{15}N-, and ^{17}O-containing alternatives [44].

Stock solutions must be made of certified analytical reference standards of a known purity. If the stock solution exists in a different state or buffer composition than the reference standards, stability must be determined [98].

Corrections must be made if the reference standard is not in the same chemical form as the analyte of interest (i.e. a free base or acid) and the solubility of the compound must be taken into account when choosing a reconstitution solution [98].

2.9.2. Sensitivity

Sensitivity refers to the minimum concentration level at which a certain analytical method and instrument can deliver reliable quantitation results [96]. At this concentration any sample analysed by the analytical method and instrument will deliver reliable results. This concentration is established as the LLOQ and any result below this concentration cannot be trusted and is labelled as below the limit of quantitation (BLOQ) [98].

2.9.3. Selectivity and Specificity

These two terms are often confused and according to the FDA and EMA, selectivity is defined as the ability to distinguish the analyte of interest and the ISTD from matrix components that may be present in the sample [46,95,96]. However, EMA defines specificity as the ability to unequivocally measure the analyte of interest in the presence of exogenous or endogenous matrix constituents of the sample [46,95]. These two terms are almost indistinguishable; however, specificity is absolute and selectivity is graded, thus a method is only considered to be specific when it is perfectly selective [46]. The selectivity of an analyte is determined at the LLOQ by analysing six independent blank matrices as well as the analyte of interest spiked in matrix. The selectivity of the analytical method is assured when the response of interfering components is less than 20% of the analyte response at LLOQ [46].

2.9.4. Accuracy and Precision

Accuracy is described as the closeness of agreement of measured results to the true value of the analyte [46,95,96]. Within-run accuracy and between-run accuracy should be evaluated, and this is expressed as Relative Error (%RE). Regulatory organisations recommend the use of a minimum of five replicates per concentration level, at a minimum of three concentrations levels [46,95,96]. These three concentration levels consist of QCs and are made up of a low QC (which is within three times the LLOQ), a high QC (which is at least 75% of the upper calibration curve range) and a medium QC (which is between QC high and QC medium) [46,95]. The deviation of the mean value should be within 15% of the true value, except at LLOQ, where a deviation of 20% is allowed [46,95,96]. Thus, the deviation of the mean from the true value serves as the measure of accuracy. The formula for calculating accuracy is shown below:

$$\%\ Nominal = \frac{Mean\ concentration}{Nominal\ concetration} \times 100$$

The precision of an analytical method is defined as the closeness of multiple individual measures of a single homogeneous sample [46]. It can either be expressed as relative standard deviation or as the absolute coefficient of variation (%CV). The criteria are similar to that of accuracy, since the %CV should not exceed 15% for the QC samples and 20% for the LLOQ [46]. The formula for the %CV is shown below:

$$\%CV = \frac{Standard\ deviation}{Mean} \times 100$$

Precision can be evaluated with the same experiments used for the determination of accuracy. Within-run and between-run precision must also be determined. Within-run describes measurement of accuracy and precision by in a single run, while between-run describes the measurement of accuracy and precision from at least three runs analysed, on at least two different days [46].

2.9.5. Recovery

Recovery is defined as the ability of the method to extract the analyte of interest from a biological matrix [46,95,96]. Recovery is determined by comparing the response of blank matrix spiked with a known concentration that has been extracted and one that hasn't, thus representing 100% of the known concentration [46]. There are no criteria for the recovery value; however, the recovery of an analyte and of the ISTD must be consistent, precise, and reproducible. Recovery experiments should be performed by comparing the analytical results for extracted samples at QC low, QC medium and QC high, with theoretical samples that represent 100% recovery. Recovery is calculated as:

$$\%\ Recovery = \frac{Mean\ response\ of\ test\ samples}{Mean\ response\ of\ theoretical\ samples} \times 100$$

2.9.6. Matrix Effects

The Matrix effect is specifically related to methods that use mass spectrometry and is defined as the effect of matrix constituents on the analysis of the analyte of interest. This may manifest as signal suppression or enhancement, impact on retention times, or elevated baseline signals [46]. The presence of an internal standard

may minimise the effect of some matrix components. Matrix effects may be calculated using a minimum of six different blank sources of the appropriate biological matrix. Each individual biological matrix must be spiked at low, medium and high concentration levels and all matrix sources must be spiked with a single concentration of the ISTD. The %CV for the six different matrix sources should not exceed 5% [99].

2.9.7. Carry-Over

Carry-over is caused when a residual amount of analyte remains on the analytical system after an injection has been completed. Carry-over can be a serious obstacle for LC-MS/MS analysis, since it can affect the accuracy and precision of an assay [46]. Carry-over has a greater effect on lower concentration samples and this is especially evident after the injection of a high concentration sample. Carry-over is assessed by including a blank sample after a high concentration sample or calibration standard at the upper limit of quantification (ULOQ) [46,95]. The response of carry-over should not be greater than 20% of the LLOQ, and 5% for the ISTD [46]. Carry-over is calculated as:

$$\%\text{Carry} - \text{over} = \frac{Peak\ area\ of\ a\ blank\ sample, after\ injection\ of\ UPLOQ}{Peak\ are\ of\ analyte\ at\ LLOQ} \times 100$$

2.9.8. Stability

To guarantee reliable and reproducible results, the stability of an analyte must be determined in stock solutions and in the relevant biological matrix [46]. The conditions used in stability experiments should mimic conditions that may occur during sample handling and analysis [46]. Stability samples should be compared to freshly made calibrators or QCs. At least three replicates at each of the low and high concentrations should be assessed. According to regulatory bodies, the deviation of calculated and nominal results should be within 15% for all stability analyses [46,95]. Stability is calculated by the formula shown below:

$$Stability = \frac{Mean\ response\ of\ stability\ samples}{Mean\ response\ of\ reference\ samples} \times 100$$

Regulatory bodies also require several other stability tests to ensure proper handling and storage. These tests include freeze/thaw stability, bench-top stability, long-term stability, stock solution stability, and auto-sampler stability [95].

2.10. Conclusion

As has been discussed in detail, various alternative approaches are available for the quantification of TFV and TFV-DP in DBS. The current methodology entails the separation of the parent drug from the metabolite, followed by the back-conversion of all metabolites to the parent drug before analysing the samples on LC-MS/MS, which is a time-consuming process. The new method aims to reduce the time taken for each assay and the associated cost for the consumables, making the technology more accessible to local researchers with limited funding available. To do so, the various retention and sensitivity limitations associated with TFV and its metabolites will have to be addressed. The use of either a Dns-Cl derivatisation reaction, an ion-pairing reagent or anion-exchange chromatography should compensate for the associated limitations and allow for the development of a less laborious and cost-effective assay.

Chapter 3: Bioanalytical Method Development

3.1. Introduction

Prior to the implementation of a bioanalytical method, the method must be developed and subsequently validated, according to FDA and EMA guidelines [95,96]. Possible approaches for the development of a direct LC-MS/MS method have been discussed in the previous chapter and this chapter will discuss the implementation of these approaches, with the aim of developing a direct LC-MS/MS analysis for TFV-DP. Three main approaches were investigated during the method development, namely derivatisation reactions, ion-pairing reagents and anion exchange chromatography.

3.2. Experimental Development of Bioanalytical Method

3.2.1. Analytes and Internal Standards

TDF is an oral prodrug, which requires diester hydrolysis to form TFV, which is subsequently absorbed into the cell and becomes phosphorylated, leading to the formation of TFV-MP and, ultimately, TFV-DP [24]. This study's main analyte of interest is TFV-DP, since it can provide an indication of long-term adherence and is the pharmacologically active component. However, if the method also allows for the analysis of TFV it would be an added bonus, since TFV can be used as an indication of short-term adherence.

For the development of the direct method TFV, TFV-MP and TFV-DP were monitored, to evaluate the chromatography of the analytes and allow the optimisation of baseline separation. Baseline separation is essential for the mitigation of cross-talk or back-conversion of the analytes in the ion-source.

Stock solutions of TFV, TFV-MP and TFV-DP were prepared by weighing out the mass of the analyte into a container and dissolving the weighed-out mass in the appropriate volume of water to obtain the target concentration of 1000 µg/ml. All stock solutions were stored at approximately -80°C until required.

An ISTD was added to the bioanalytical method to compensate for any variation, which may occur as a result of evaporation, derivatization, instrument parameters, degradation or recovery [44]. The addition of an ISTD should also help mitigate the matrix effects, which affect the reproducibility and accuracy of the assay [44,100–102]. As has been discussed in the previous chapter, matrix effects are caused by undetectable coeluting sample constituents that influence ion intensity by either causing ion suppression or enhancement. The degree of ion suppression or enhancement is also largely determined by the chemical structure of the analyte [44]. Therefore, the choice of the ISTD is important, since it should mimic the analyte of interest as closely as possible [44]. Structural analogues and stable isotopically labelled ISTDs are commonly used for this reason. In this study we selected stable isotopically

labelled ISTDs, since they will co-elute with the analytes of interest. Tenofovir diphosphate-$^{13}C_5$ (Adenine-$^{13}C_5$) and Tenofovir-D6 were selected as internal standards for TFV-DP and TFV, respectively.

The following sections will discuss the development and implementation of the various assay approaches. The various limitations and restrictions encountered with each method will also be presented.

3.3. Dansyl-Chloride Derivatisation Reaction

As has been discussed in section 2.8.1., Dns-Cl is an amine reagent and reacts with the primary amine group on TFV and TFV-DP as shown in Figures 3.1 and 3.2, respectively. However, the rate and extent of this reaction is dependent on the reaction conditions and these must be optimised to achieve an optimal and reproducible yield. According to Kang *et al.* the following conditions impact yield, namely reaction temperature, Dns-Cl concentration and reaction time [80]. The optimisation of these parameters will be discussed in section 3.1.1.-3.1.3. below. TFV-DP was not used for the optimisation of the reaction parameters, due to stability concerns, thus all the reaction parameters were optimised using TFV. The reaction yield was monitored using an AB Sciex API 2000 Qtrap mass spectrometer at unit resolution in the multiple reaction monitoring (MRM) mode and Analyst® software version 1.6.1. was used for all chromatographic data acquisition, peak integration and quantification. An ESI source was used for ion production and a Poroshell 120 (4.6 mm x 150 mm, 2.7 µm) analytical column was used to achieve chromatographic separation.

Infusion solutions were prepared to identify the transitions of TFV and the TFV derivative. A solution of 500 ng/ml TFV in water was prepared for TFV, while the TFV derivative was investigated by preparing a solution containing 1 µg/ml of TFV and of 1 µg/ml Dns-Cl in water. The solutions were infused into an AB Sciex Qtrap 2000 mass spectrometer using a Hamilton syringe set at a constant flow rate of 10 µl/min.

The transitions of the protonated precursor ions that were monitored were *m/z* 288 to the product ion *m/z* 176 for TFV. Stable Q1 precursor ions at m/z 521 were not observed for the dansyl derivative. The final Q1 scan of TFV is shown in Figures 3.3. The initial product ions for TFV is shown in Figures 3.4.

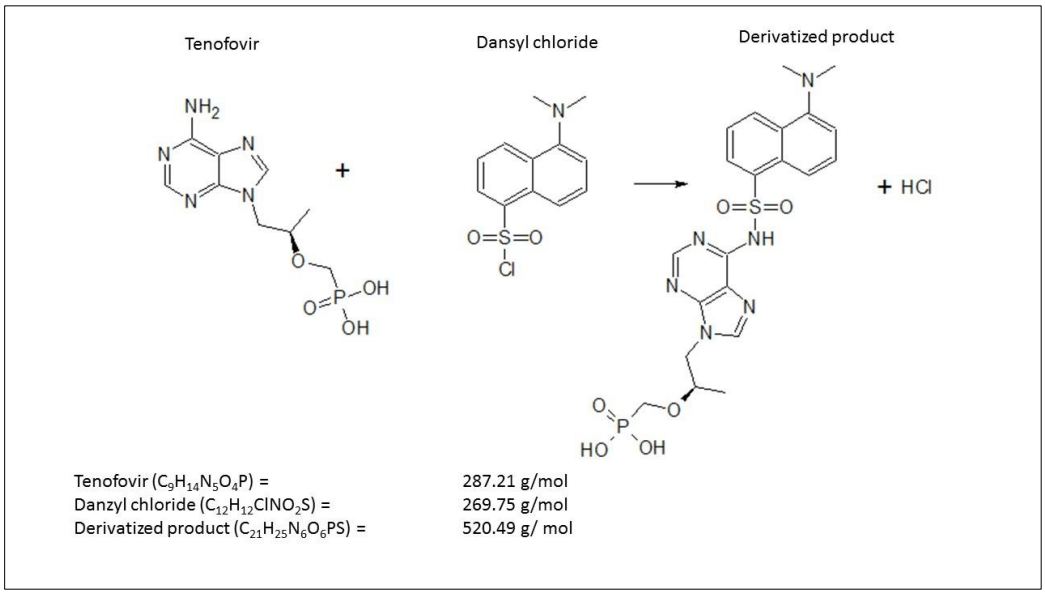


Figure 3.1: Depiction of Dns-Cl reaction with TFV with the empirical formula and molecular weight of the products and reagents.

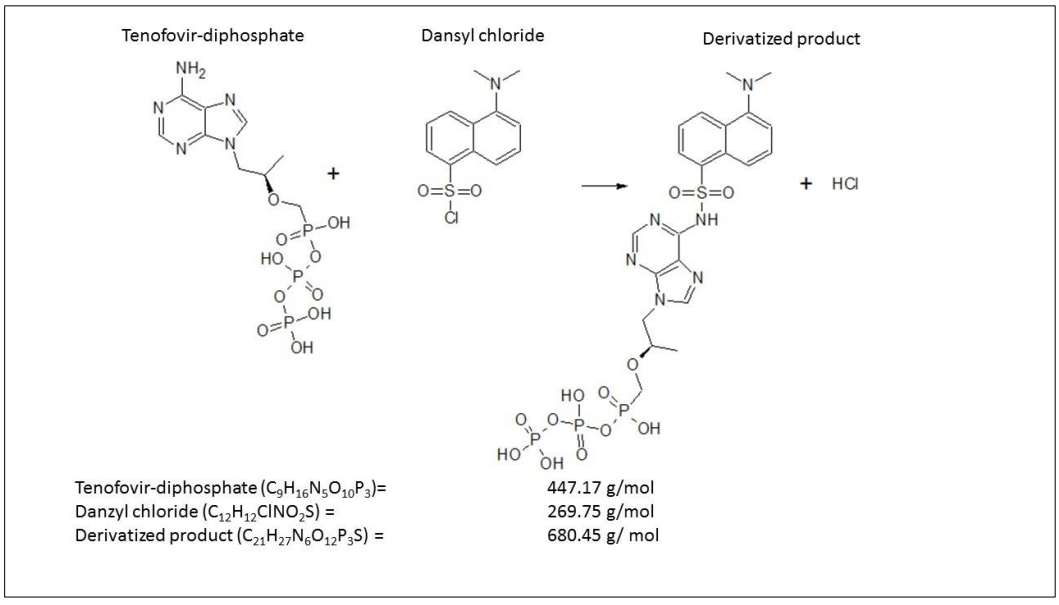


Figure 3.2: Depiction of Dns-Cl reaction with TFV with the empirical formula and molecular weight of the products and reagents.

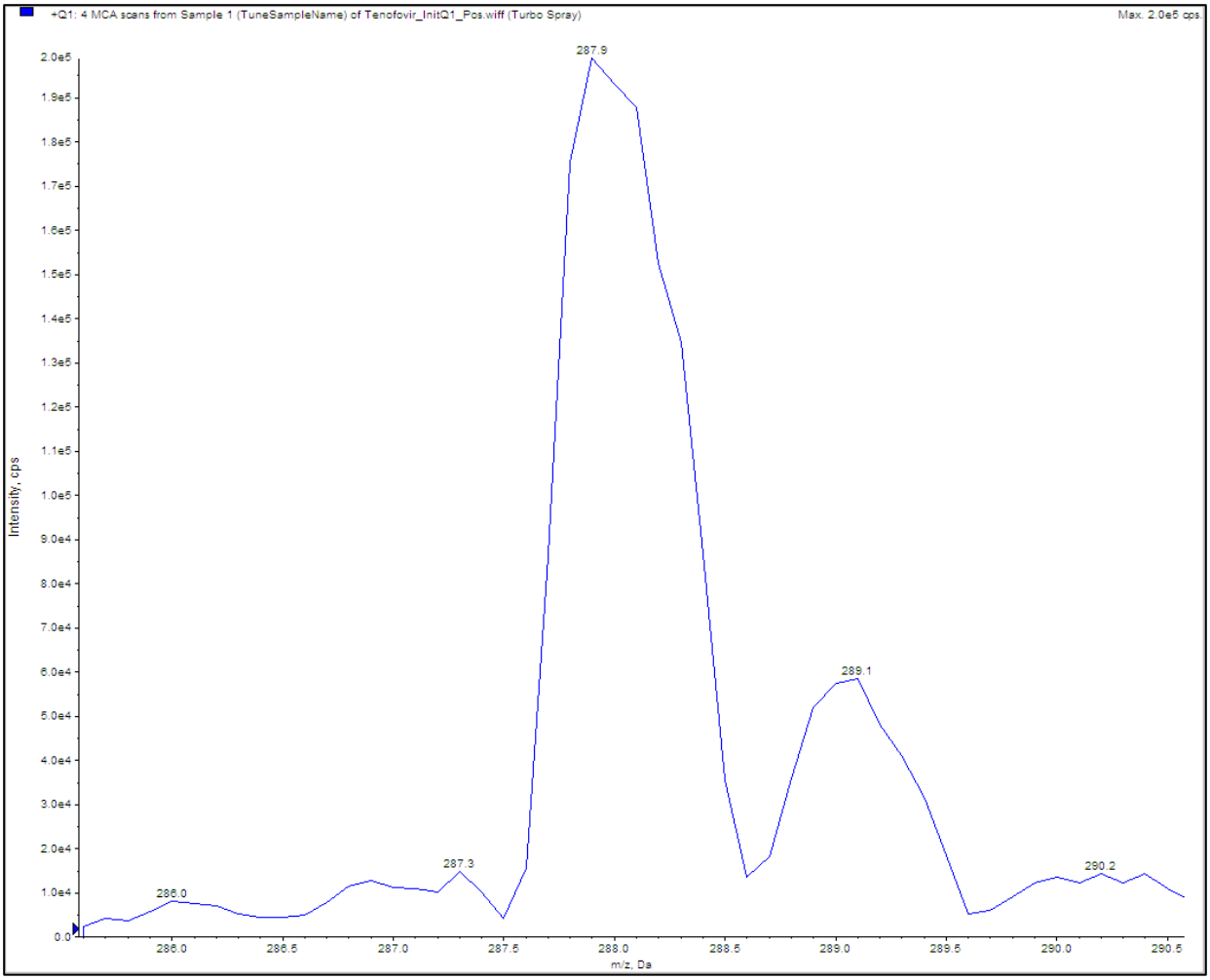


Figure 3.3: Final Q1 scan of TFV, following an infusion of 500 ng/ml TFV in a mixture of water and methanol (50:50, v/v).

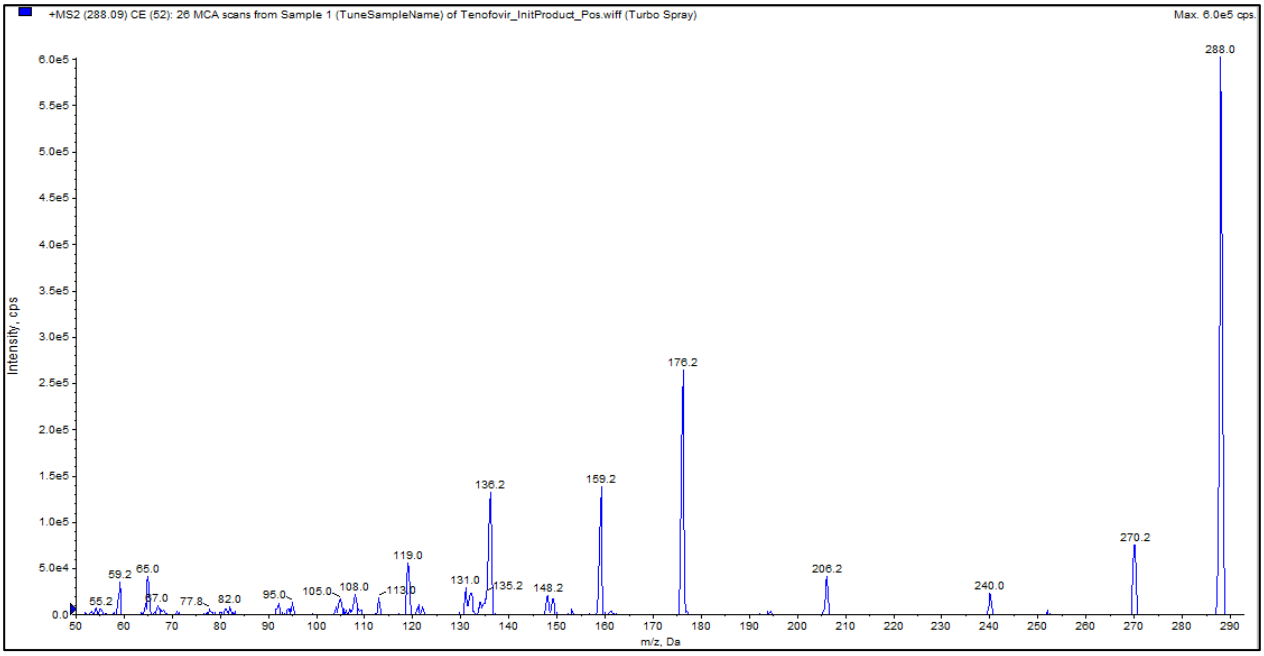


Figure 3.4: The initial product ions of TFV are presented. Exact molecular weight of TFV is 287.07 g/mol.

The monitored dansyl derivative could not be seen in any of the chromatographic runs; however, a decrease in TFV intensity was observed with the addition of Dns-Cl. A representative chromatogram is shown in Figure 3.5 comparing TFV before and after the addition of Dns-Cl. As can be seen in Figure 3.5, the TFV intensity decreased as Dns-Cl was added. In an attempt to improve the chance of observing a dansyl derivative, the reaction conditions were optimised. However, due to the inability to observe the dansyl derivative itself, the reaction yield had to be indirectly monitored. To do so the reaction yield was elucidated by monitoring the decrease in TFV in each sample. As the reaction took place, the initial TFV concentration would decrease and this decrease was used as an indication of reaction yield; a decrease in TFV taken to indicate an increase in reaction yield. However, to confirm that the decrease in concentration was not due to degradation, all the optimisation experiments contained a sample containing only TFV as a reference.

There were also concerns that the decrease in TFV concentration was only due to a possible influence of Dns-Cl on the ion intensity and that the addition of Dns-Cl may lead to ion suppression. However, this was shown to not have a substantial effect, since the TFV signal did not significantly differ at room temperature with and without Dns-Cl (before any optimisation was completed) as can be seen in Figure 3.6.

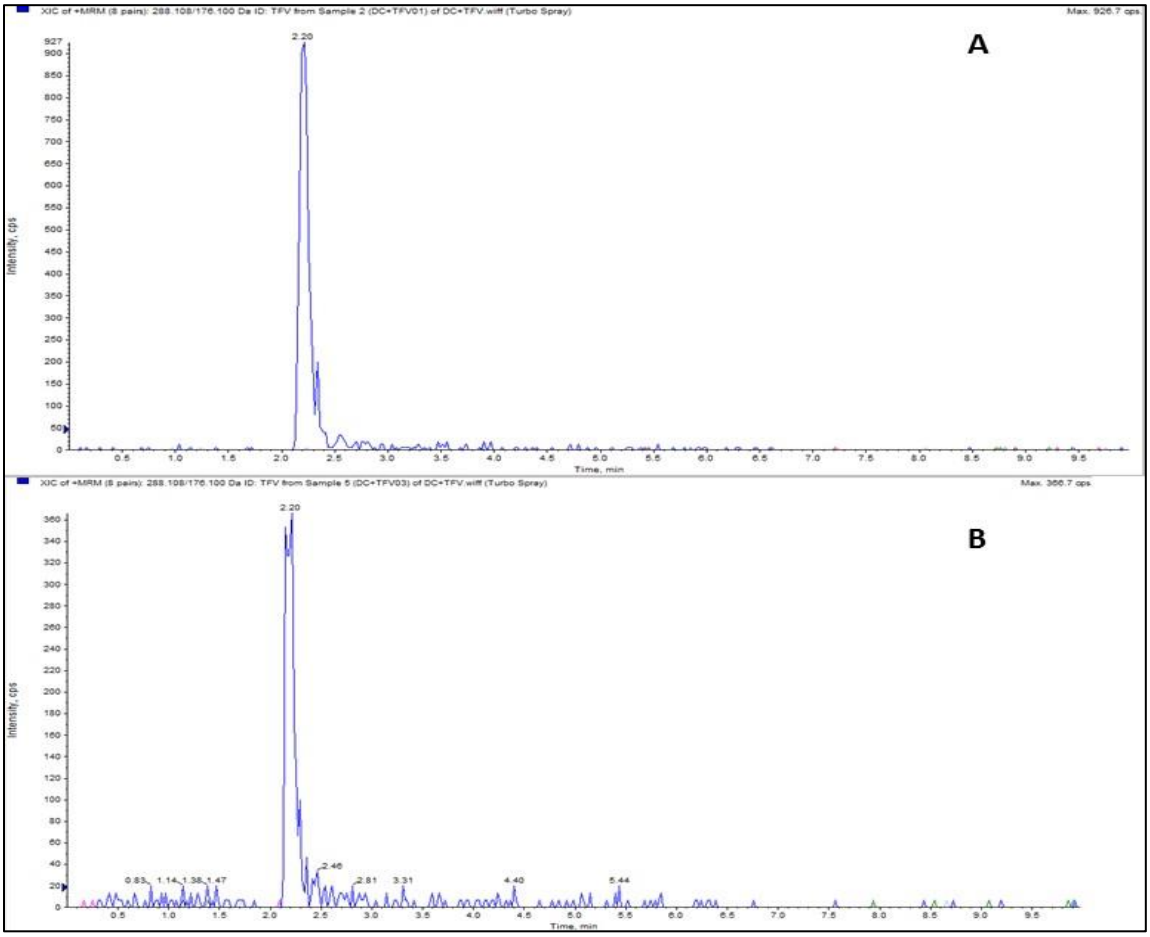


Figure 3.5: A representative chromatogram of TFV A) before the addition of Dns-Cl and B) after the addition of Dns-Cl. The solutions used in chromatogram A and B were both incubated at 40 ^{0}C for 10 mins.

3.3.1. Reaction Temperature

The reaction temperature plays an important role in yield; therefore, the optimisation of this parameter is essential [80]. Various incubation temperatures were tested to determine the optimal reaction temperature, and this was achieved by adding 1 µg/ml of TFV and 1 µg/ml Dns-Cl into a 1.5 ml Eppendorf and incubating the sample at the appropriate temperature for 10 mins. Samples were evaluated at 5 temperatures, namely room temperature, 40^{0}C, 60^{0}C, 70^{0}C and 100^{0}C. Samples stored at room temperature were used as a reference and a second sample set containing 1 µg/ml TFV without Dns-Cl was placed at each temperature to monitor any temperature-related TFV degradation. Each reaction was repeated six times and the results of the quantification are shown in Figure 3.6.

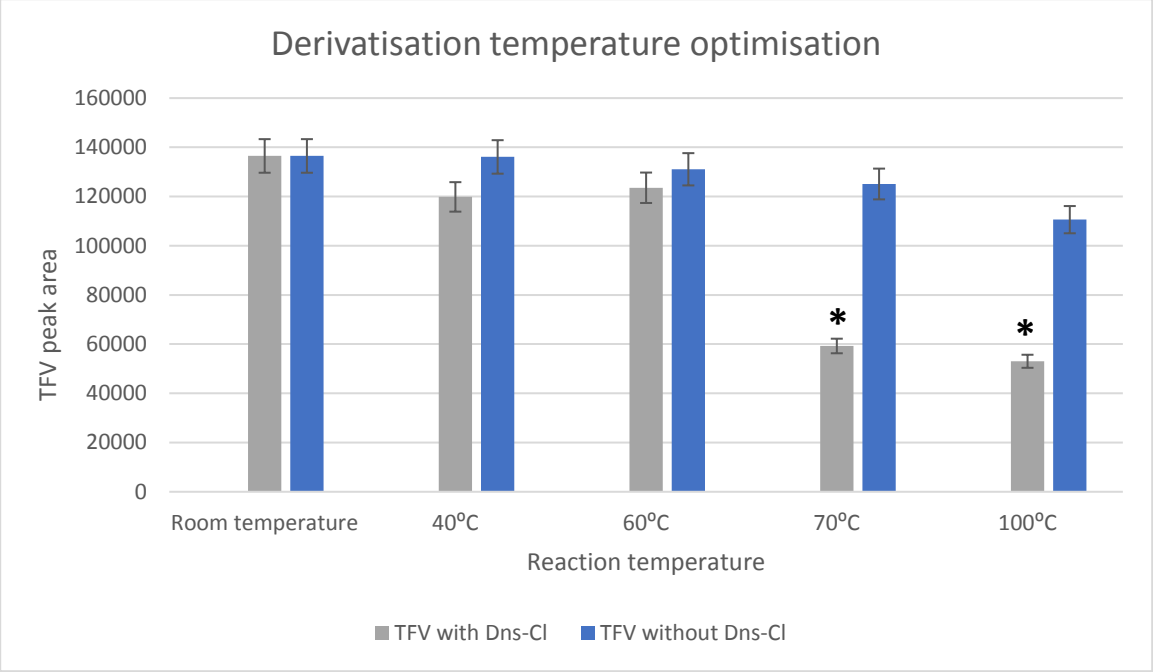


Figure 3.6: Change in TFV concentrations due to the derivatisation of TFV and Dns-Cl at various incubation temperatures. Statistically significant yield changes are indicated with an asterisk ($p \leq 0.05$).

A statistically significant increase in the derivatisation reaction was observed with an increase in temperature above 60^{0}C. This corresponds to observations made by Kang *et al.* [80]. A two-sample t-test assuming unequal variance was used to determine statistical significance (P≤0.05). This was done by comparing the each of the temperatures to the room temperature sample, which served as the reference. Samples stored at 70^{0}C and 100^{0}C showed statistically significant (n=6, t=2.10, p=0.0017 and n=6, t=1.73, p=0.0039, respectively) reaction yield differences when compared to the room temperature reference. The highest reaction rate was seen at 100^{0}C; however, TFV degradation was also observed at 100^{0}C. The use of 100^{0}C as an incubation temperature also posed practical problems, since the mobile phase evaporates at this temperature, leading to increased pressure. Therefore, 70^{0}C was chosen as the ideal incubation temperature, and this temperature was used in all further optimisation experiments.

3.3.2. Dns-Cl Concentration

The ratio of the analyte to Dns-Cl also plays an essential role in the product yield. According to Kang *et al.*, higher Dns-Cl ratios not only lead to a higher yield, but also reduce the amount of adducts formed [80]. However, the Dns-Cl concentration can only be increased to a certain level, due to possible loss of sensitivity attributed to ion suppression or contamination of the mass spectrometer. The optimal ratio of Dns-Cl to TFV was determined by

adding 0.1, 0.5, 1, 10 and 100 µg/ml Dns-Cl, respectively to 1 µg/ml TFV. This was subsequently incubated at 70^{0}C for 10 mins and injected onto the LC-MS/MS system.

The 1 µg/ml Dns-Cl sample set was used as a reference and each concentration was compared to the reference separately. Statistical significance was determined by completing a two-sample t-test assuming unequal variance (P≤0.05). As can be seen in Figure 3.7, statistically significant differences) could only be observed when comparing the 0.1 µg/ml and 0.5 µg/ml sample set intensities to those of the reference (n=6, t=1.83, p=0.0021 and n=6, t=1.92, p=0.0024, respectively). Thus, increasing the concentration to a level higher than 1 µg/ml Dns-Cl would not lead to significant increases in yield.

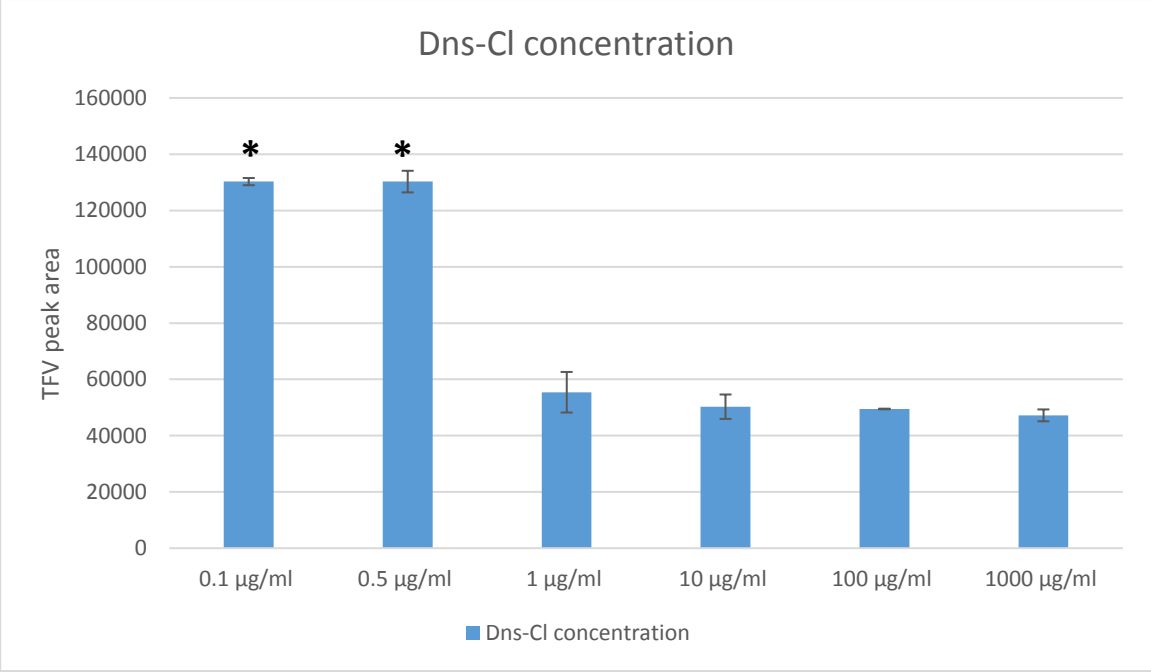


Figure 3.7: Changes in production yield as a result of varying Dns-Cl concentrations.

3.3.3. Reaction Period

According to Kang *et al.*, the reaction period also has a significant effect on the reaction yield [80]. To determine the optimal reaction period; 1 µg/ml Dns-Cl was added to 1 µg/ml TFV and subsequently incubated at 70^{0}C for up to 1 hour. The solutions were injected onto the LC-MS/MS system at time point zero (T0) and in 10-minute intervals thereafter. This was repeated in triplicate and the mean peak area values are shown in Figure 3.8.

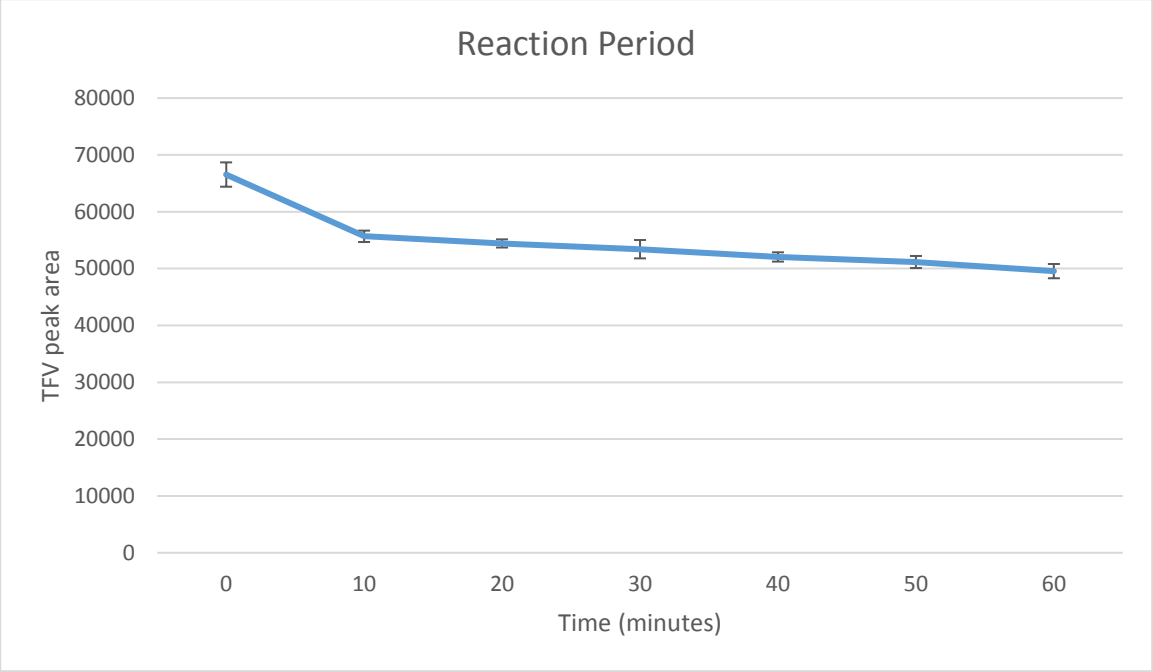


Figure 3.8: The mean response of TFV samples incubated at 70^{0}C were determined in 10-minute intervals and were repeated in triplicate.

Figure 3.8 demonstrates that a decline in TFV is observed as reaction time increases and this reflects an increase in the reaction yield. However, only a marginal increase in reaction yield was observed after an incubation period of 10 mins. Therefore, extending the incubation period past a period of 10 minutes would not lead to a substantial increase in reaction yield.

3.3.4. Dansyl Derivative Identification

After the optimisation procedure, a mixture of 1 µg/ml Dns-Cl and 1 µg/ml TFV in water was reinfused after incubation at the optimised conditions. The solution was infused into an AB Sciex Qtrap 2000 mass spectrometer using a Hamilton syringe set at a constant flow rate of 10 µl/min. The expected transition of the dansyl derivative protonated precursor ion was not observed, as can be seen in the initial Q1 scan shown in Figure 3.9.

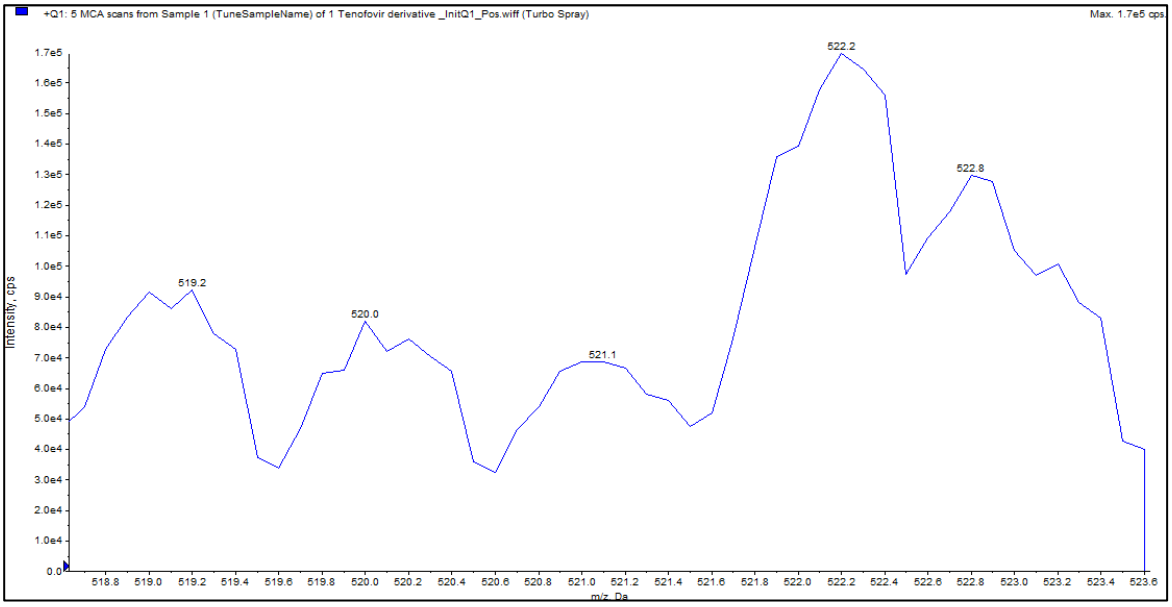


Figure 3.9: Initial Q1 scan of the expected protonated dansyl derivative precursor ion, which was generated by infusing a mixture of 1 µg/ml Dns-Cl and 1 µg/ml TFV in water after a 10-minute incubation step at 70^0C.

Protonated dansyl derivative precursor ions at m/z 521 were not observed with the optimised reaction and an incomplete reaction caused the decision to abandon this approach.

3.4. Ion-Pairing Chromatography

Ion-pairing chromatography was investigated as an alternative chromatography system for the direct determination of TFV-DP. This approach has been used in various studies to quantify NRTIs [36,77,103–111]. Ion-pairing reagents can be utilized to increase the retention of polar compounds, which are difficult to retain on traditional reverse phase analytical columns. TFV-DP and TFV-MP may revert to their parent compound once inside the heated mass spectrometer ion source. Therefore, it is essential to obtain baseline separation between TFV, TFV-MP and TFV-DP. To normalise and simplify the data and allow a comparison between various solvents, pH values, ion-pair reagent concentration and various analytical columns, capacity factors of each analyte were calculated. The formula used to calculate capacity factors is shown below:

$$K' = \frac{(T_R - T_0)}{T_0}$$

Where:

- T_R is the retention time of the analyte
- T_0 is the dead volume.

3.4.1. Determining the Optimal Ion-Pairing Reagent

The correct pH of a solution is essential when working with ion-pairing reagents and dictates whether a paired ion will form or not [112]. The correct pH is dependent on the pKa of either the analyte or ion-pairing reagent, since both the analyte and ion-pairing reagent must be charged to interact [112]. Therefore, to identify the optimal ion-pairing reagent the capacity factors must be determined for each analyte over a broad pH range.

The capacity factor of each analyte was determined using a 1 µg/ml working solution prepared in water. These solutions were injected onto a Kinetex EVO C18 (50 mm x 2.1 mm, 1.7 µm) column coupled to a HPLC-UV spectrometer. A UV detector was used for method development, due to possible source contamination that may occur due to the ion-pairing reagents and the column was selected due to its high pH range that could withstand the unadjusted pH of the solvents. All capacity factors were determined with isocratic methods. Each analyte was injected individually to determine its retention time and capacity factor over a pH range of 6–10, and an unadjusted mobile phase of pH 10.6. Two ion-paring reagents were investigated over the pH range, namely 1,5-DMHA and N,N-DMHA. The structure of the two ion-pairing reagents are shown in Figure 3.10. A concentration of 10 mM of either 1,5-DMHA or N,N-DMHA was added to a mixture of 5 mM ammonium acetate in water and methanol (70:30, v/v). The solutions were adjusted to the desired pH using acetic acid.

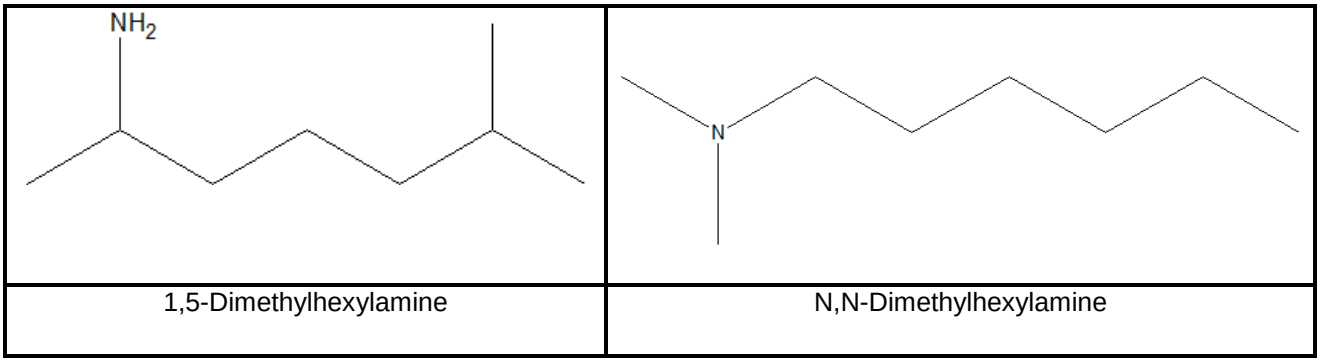


Figure 3.10: The structures of 1,5-DMHA and N,N-DMHA.

Most studies use 1,5-DMHA to retain TFV; however, N,N-DMHA is less expensive and more readily available and therefore it was also investigated as a prospective affordable alternative [106,108].

Representative chromatograms of each ion-pairing reagent are shown in Figure 3.11. The chromatograms were observed by adding 10 mM of the respective ion-pairing reagent to a mixture of 5 mM of ammonium acetate in water and methanol (70:30, v/v) with an unadjusted pH. A 10 µg/ml of each analyte was injected into the HPLC system. Figures 3.12 and 3.13 depict separate injection of each of these analytes along with a blank sample. Figure 3.12 and Figure 3.13 presents the chromatograms for 1,5-DMHA and N,N-DMHA, respectively.

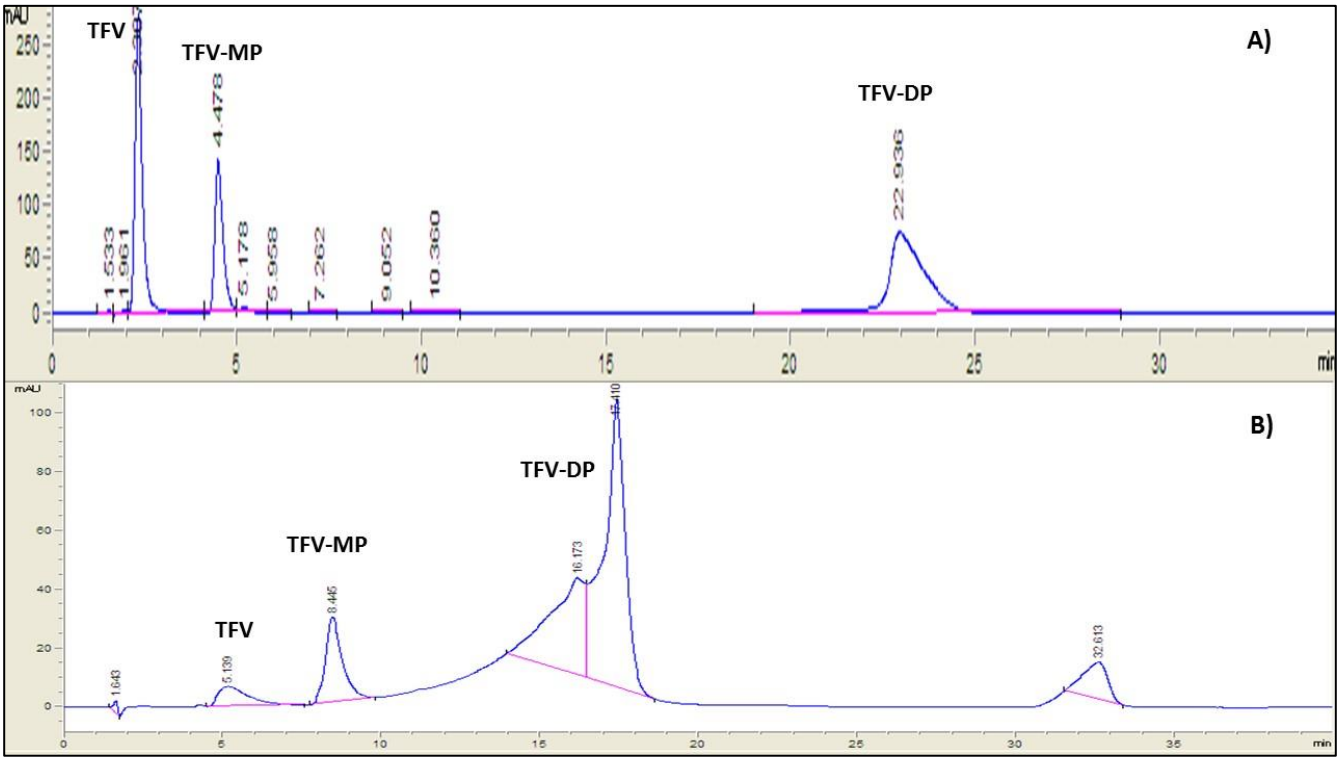


Figure 3.11: The chromatograms above show TFV, TFV-MP and TFV-DP separated with an isocratic run using 10 mM of A) 1,5-DMHA and B) N,N-DMHA as ion-pairing reagents.

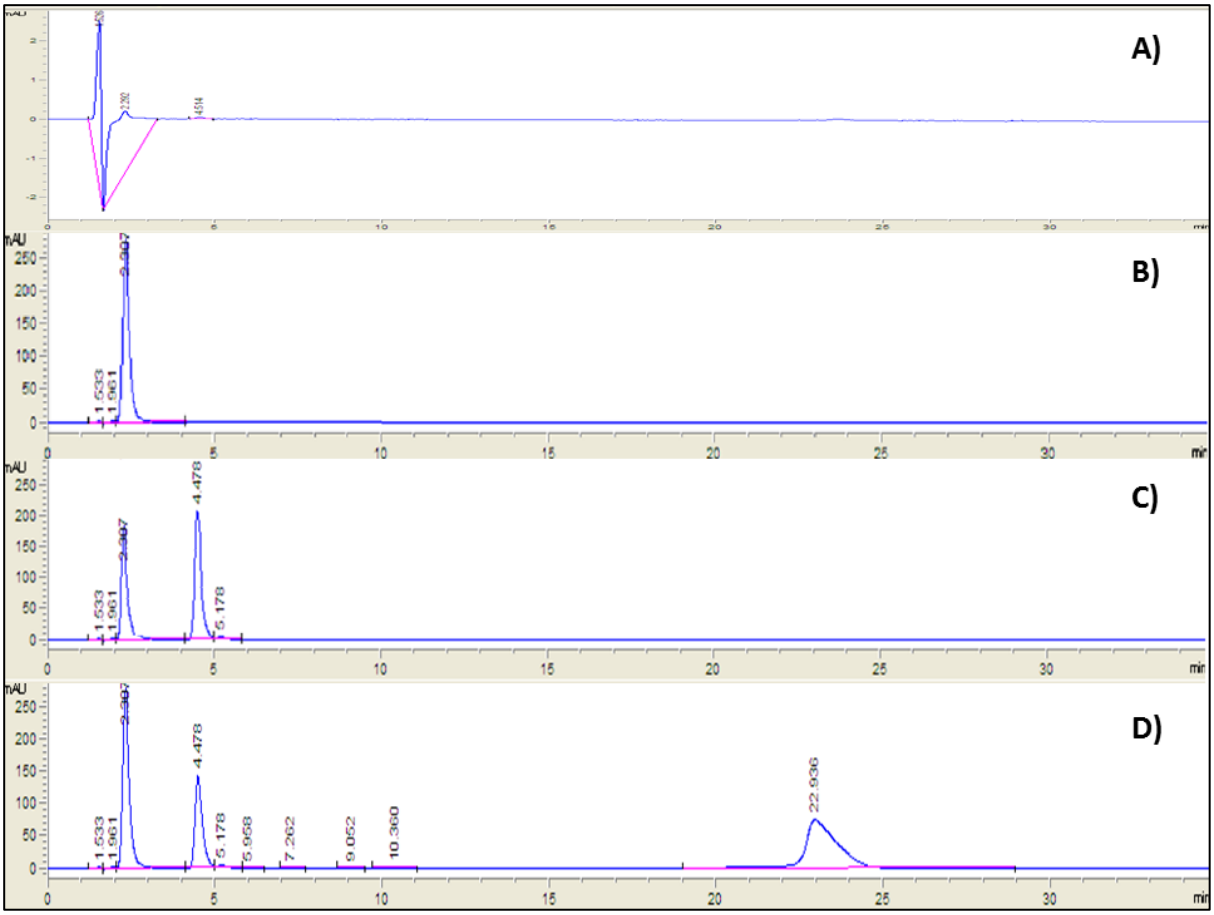


Figure 3.12: The representative chromatograms above were generated using an isocratic run with a mobile phase consisting of a mixture of 10 mM 1,5-DMHA and 5 mM of ammonium acetate in water and methanol (70:30, v/v) with an unadjusted pH. The following solutions were injected A) Blank, B) 10 µg/ml TFV, C) 10 µg/ml TFV-MP and D) 10 µg/ml TFV-DP.

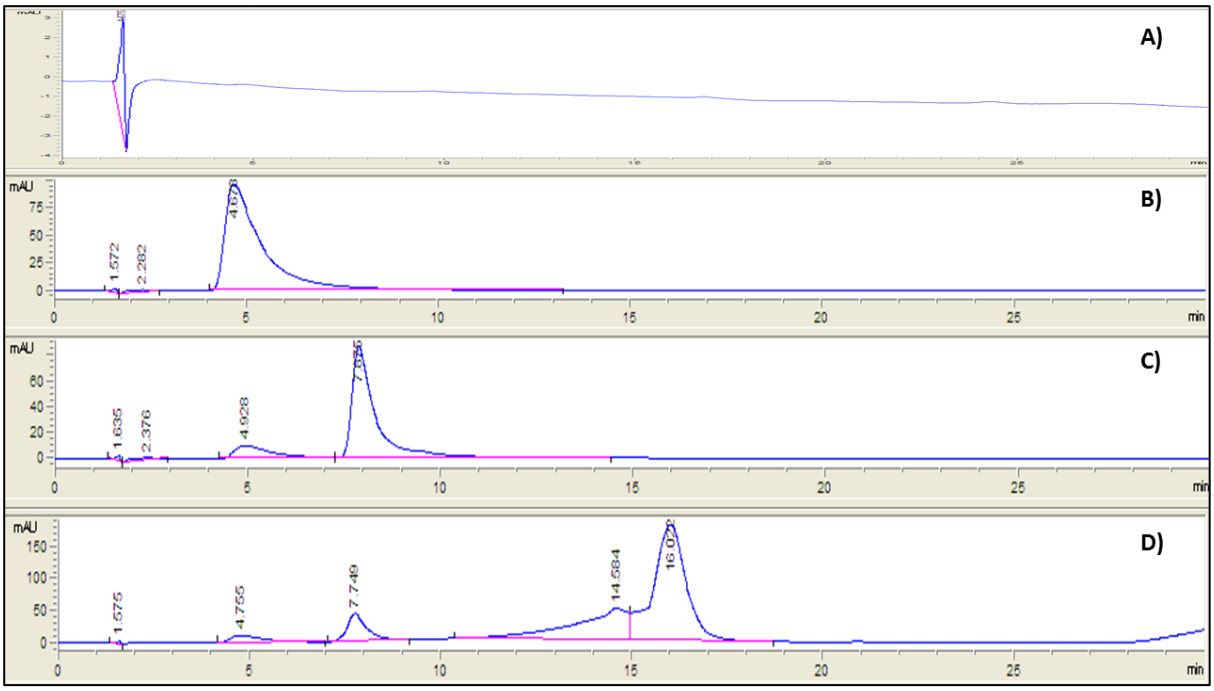


Figure 3.13: The representative chromatograms above were generated using an isocratic run with a mobile phase consisting of a mixture of 10 mM N,N-DMHA and 5 mM of ammonium acetate in water and methanol (70:30, v/v) with an unadjusted pH. The following solutions were injected A) Blank, B) 10 µg/ml TFV, C) 10 µg/ml TFV-MP and D) 10 µg/ml TFV-DP.

From the representative chromatograms it is clear that the 1,5-DMHA (Figure 3.13A) has a far superior separation capacity. Adequate baseline separation was observed in N,N-DMHA chromatograms; however, the resolution was poor and thus further optimisation was required. With the addition of TFV-DP an additional peak was also observed with a retention time of 32 minutes.

There are various reports of decreased signal intensities associated with the use of ion-pairing reagents on ESI mass spectrometers [102,113,114]. Therefore, the difference in signal suppression between 1,5-DMHA and N,N-DMHA was investigated. A marked decrease in signal intensity was observed when using N,N-DMHA as mobile phase additive. A 30% decrease in TFV-DP signal intensity was observed when comparing TFV-DP infused with N,N-DMHA to TFV-DP infused with 1,5-DMHA, as seen in Table 3.2. Therefore, N,N-DMHA was not used for further experiments.

Table 3.1: Summary of infusion results of TFV-DP with either 1,5-DMHA or N,N-DMHA

	500 ng/ml TFV-DP	
	1,5-DMHA TFV-DP signal intensity	**N,N-DMHA TFV-DP signal intensity**
Sample 1	380000	250000
Sample 2	390000	270000
Sample 3	380000	280000
Average	383333.33	266666.67
STDEV	5773.5027	15275.2523
%CV	1.5	5.7
%Difference		-30.4

3.4.2. Determining the Optimal pH for 1,5-DMHA

Capacity factors of each analyte at different pH values were determined using 1,5-DMHA as the ion-pairing reagent and a Kinetex EVO C18 (50 mm x 2.1 mm, 1.7 µm) analytical column. Clear baseline separation of the three analytes were observed at mobile phase pH values higher than 9 (a representative chromatogram is shown in Figure 3.11 A).

The front of the Kinetex C18 EVO column was determined to be ~1.570 min and retention time and capacity factors for TFV, TFV-MP and TFV-DP are shown in the Table 3.3. From the results it is evident that the retention time for TFV-DP is the greatest at the unadjusted pH and that the opposite is true for both TFV and TFV-MP. Therefore, the greatest baseline separation for TFV-DP would be achieved with a mobile phase with an unadjusted pH.

Table 3.2: Capacity factors of TFV and its metabolites at various mobile phase pH values.

Mobile Phase conditions	**TVF**		**TFV-MP**		**TFV-DP**	
	T_R	K'	T_R	K'	T_R	K'
10 mM 1,5-DMHA Unadjusted pH, 30% MeOH	4.76	2.04	7.75	3.94	16.02	9.21
10 mM 1,5-DMHA pH 10, 30% MeOH	4.93	2.14	8.15	4.19	13.81	7.80
10 mM 1,5-DMHA pH 9, 30% MeOH	4.94	2.14	8.15	4.19	13.64	7.69
10 mM 1,5-DMHA pH 8, 30% MeOH	5.11	2.26	8.44	4.37	13.84	7.82
10 mM 1,5-DMHA pH 7, 30% MeOH	5.07	2.23	8.39	4.35	13.62	7.67
10 mM 1,5-DMHA pH 6, 30% MeOH	4.90	2.12	8.23	4.24	13.52	7.61

3.4.3. Determining the Optimal Concentration of 1,5-DMHA

The concentration of 1,5-DMHA was increased to observe the effect on the retention time of the analytes. A higher concentration of 20 mM was tested and a significant increase in retention time was observed accompanied by an increase in the baseline separation between TFV and TFV-MP. This can be seen in the representative chromatograms in Figure 3.14.

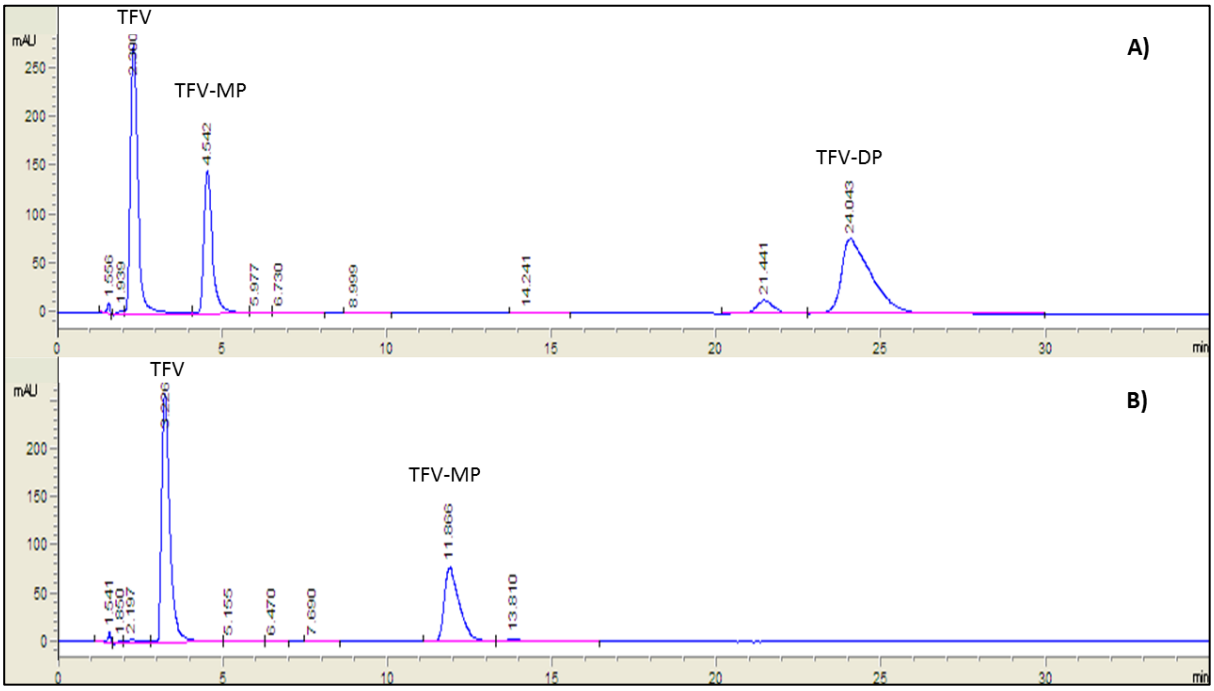


Figure 3.14: The chromatograms above shows TFV, TFV-MP and TFV-DP separated in an isocratic run with A) 10 mM of 1,5-DMHA and B) 20 mM Ammonium Acetate in 30:70 MeOH/H2O (v/v).

3.4.4. Determining the Optimal Mobile Phase

In order to develop a gradient method, which would reduce runtime and improve peak shape, the effect of the ratio of the organic to the aqueous phase was investigated. The capacity factors for each analyte was determined over an organic mobile phase range of 20% to 80% (MeOH/H_2O, v/v). The results of this experiment are shown in Figure 3.15. All the mobile phases consisted of a mixture of 10 mM 1,5-DMHA with 5 mM ammonium acetate and an unadjusted pH at the respective organic/aqueous ratios.

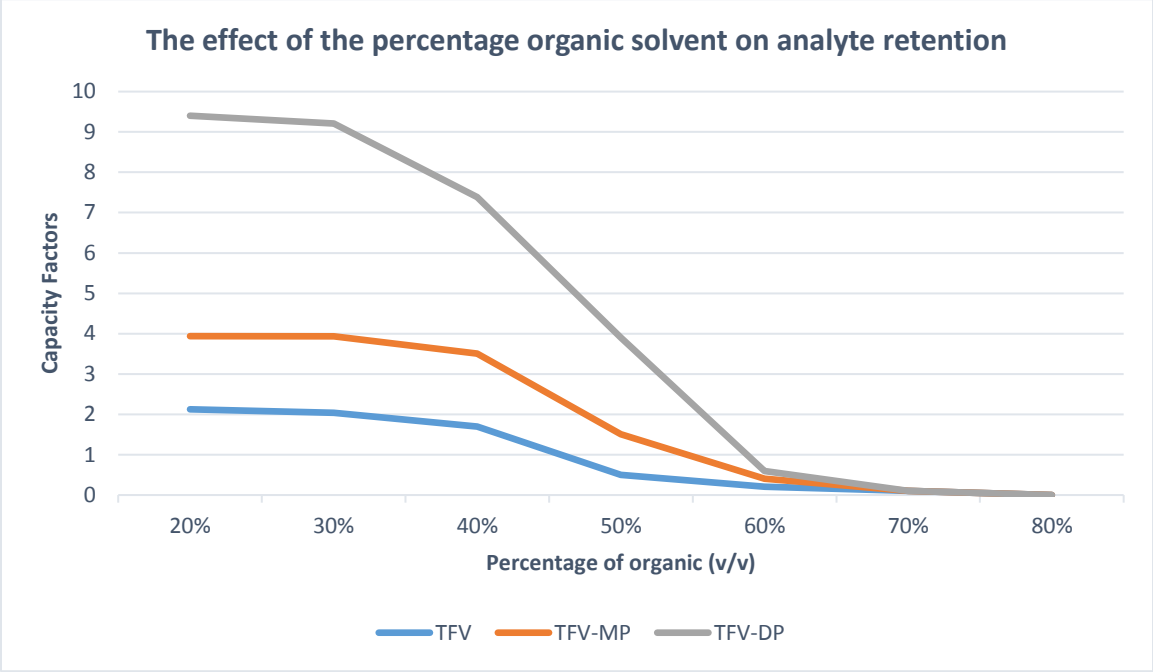


Figure 3.15: The figure shows the capacity factors of TFV, TFV-MP and TFV-DP in a mobile phase consisting of a mixture of 10 mM 1,5-DMHA and 5 mM ammonium acetate in water and methanol with an unadjusted pH.

Figure 3.15 illustrates the relationship between the percentage of organic solvent (v/v) and capacity factors of TFV, TFV-MP and TFV-DP. An increase in the percentage of organic solvent (methanol) resulted in a decrease in the capacity factors of all three analytes. There is a marked decrease in the capacity factor of TFV-DP when the percentage of organic exceeds 30% (v/v).

All previous experiments were carried out using isocratic runs; however, a gradient method was developed to improve resolution and decrease the runtime. Initially the mobile phases would have consisted of a mixture of 10 mM 1,5-DMHA and 5 mM ammonium acetate in 100% (v/v) water for mobile phase A and 100% (v/v) methanol for mobile phase B. However, 1,5-DMHA was not soluble in 100% water and thus required a portion of methanol. The 1,5-DMHA ion-pairing reagent was only soluble in mobile phases consisting of 30% MeOH (v/v) and higher. Therefore, the following two mobile phases were selected:

A: A mixture of 10 mM 1,5-DMHA with 5 mM ammonium acetate in water and methanol (70:30, v/v) with an unadjusted pH.

B: A mixture of 10 mM 1,5-DMHA with 5 mM ammonium acetate in water and methanol (10:90, v/v) with an unadjusted pH.

The gradient method used can be seen in Table 3.4 and a representative chromatogram achieved using this method is shown in Figure 3.16. Baseline separation was achieved between TFV and its two metabolites.

Table 3.3: The gradient flow schedule of ion-pairing method.

Time (min)	Flow rate (µl/min)	Mobile phase	
		A (%)	B (%)
0.0	100	90	10
2.0	100	90	10
8.0	100	10	90
9.0	100	10	90
9.3	100	90	10
12.0	100	90	10

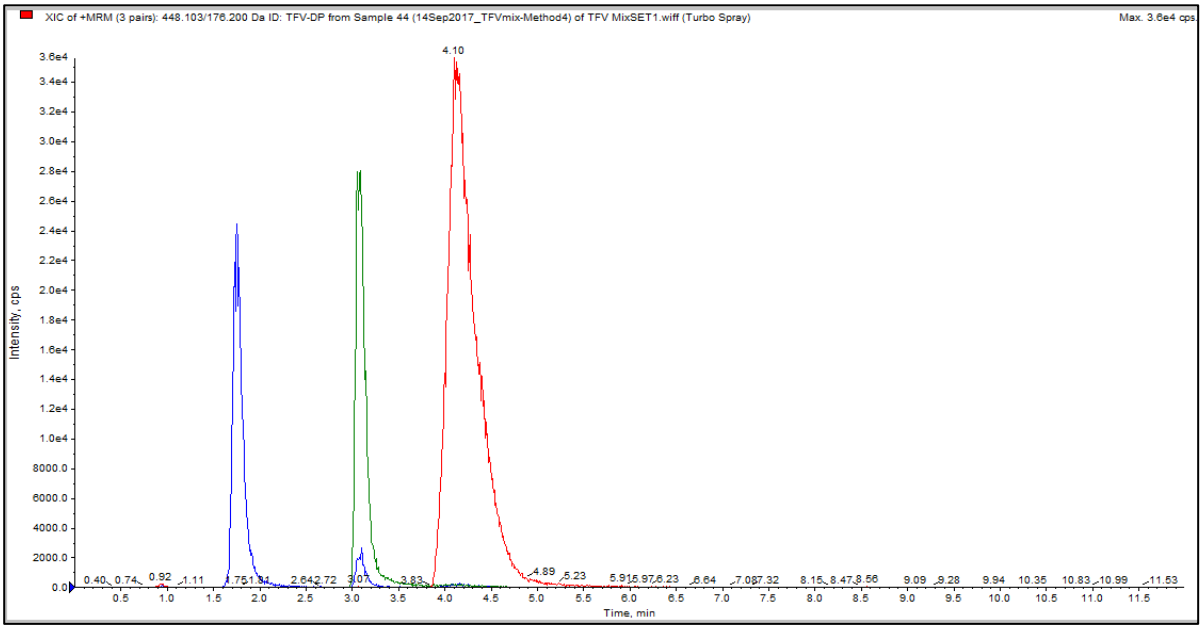


Figure 3.16: A representative chromatogram achieved using a gradient method, showing TFV-DP (Red), TFV-MP (Green) and TFV (Blue).

The method was subsequently transferred from an AB Sciex 2000 QTRAP to an AB Sciex 5500 with the aim of improving sensitivity. After optimisation, a calibration curve consisting of eight TFV-DP working solutions ranging from 40 ng/ml to 4800 ng/ml was designed to assess the linearity of the method over the calibration range. Representative chromatograms that formed part of the curve are shown in Figure 3.17. The curve was calculated with a quadratic regression using a weighting of 1/x. The calibration curve is shown in Figure 3.18 and had a r-value of 0.9945.

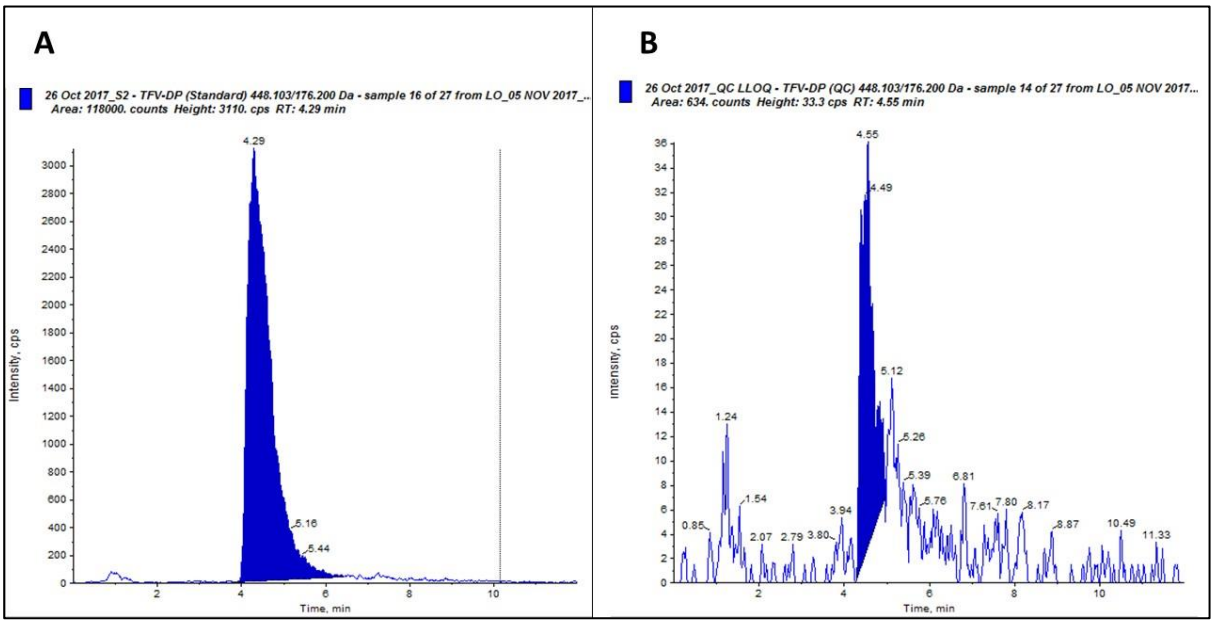


Figure 3.17: The representative chromatograms of TFV-DP working solutions with concentrations of A) 2400 ng/ml and B) the LLOQ at 40 ng/ml.

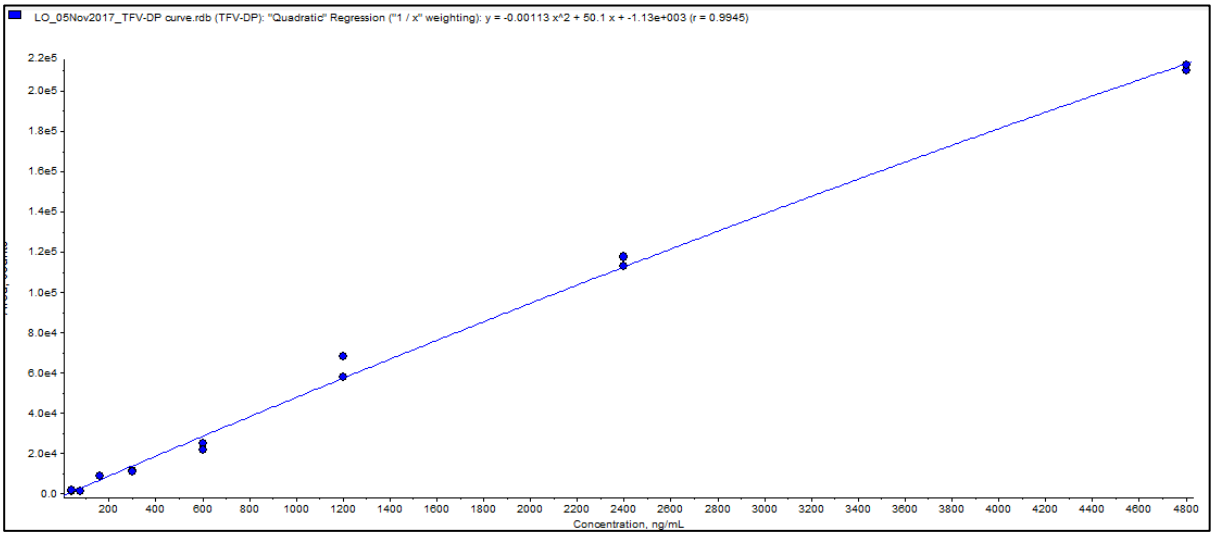


Figure 3.18: A TFV-DP working solutions ranging from 40 ng/ml to 4800 ng/ml (r-value of 0.9945).

It is important to note that the current indirect DBS method has been validated over a range of 139–5546 fmol/punch (2–124 ng/ml) and, therefore, the developed method would require a similar or lower LLOQ, otherwise it could not be used to replace the current indirect method since a similar level of sensitivity is required. The LLOQ reached using this ion-pairing method was 40 ng/ml, which is equivalent to 2785 fmol/punch. Therefore, this method was clearly not sensitive enough to be used as an alternative to the current indirect method.

3.5. Anion Exchange Chromatography

The polar nature of nucleotides makes it difficult to retain these analytes using traditional reverse phase chromatography. Nucleotides are negatively charged at pH values above 2, making them even more polar [72,73]. These negatively charged nucleotides can, however, be separated with ionic interactions at the correct pH value.

The retention and separation of nucleotides can thus be achieved using anion exchange liquid chromatography, which separates negatively charged analytes through ionic interactions with a charged stationary phase. This is especially useful because of the formation of ion pairs between the negatively charged nucleotide phosphate groups and the stationary phase [72].

Therefore, anion exchange chromatography was investigated as an alternative to ion-pairing chromatography. A Thermo BioBasic AX, (50 mm × 2.1 mm, 5 µm) column was selected as the anion exchange column of choice. The chromatography was developed using an AB Sciex QTRAP 5500 mass spectrometer coupled to an Agilent 1260 HPLC system. This system was selected due to its higher sensitivity, which would allow the detection of lower analyte concentrations.

3.5.1. Mass Spectrometry

For MS analysis infusion solutions of TFV-DP, TFV-MP, TFV, and the TFV-DP deuterated internal standard were made from the previously prepared stock solutions. The stock solutions were used to prepare working solutions with a concentration of 0.1 µg/ml in a mixture of water and acetonitrile. The solutions were infused into an AB Sciex Qtrap 5500 mass spectrometer using a Hamilton syringe and set at a constant flow rate of 10 µl/min. This was done to obtain the mass spectra of the protonated molecular ions and their fragments. The quantitation method was set at unit resolution and was in the MRM mode. The transitions of the protonated precursor ions monitored were *m/z* 448.0 and 452.9 to the product ion *m/z* 350.0 and 354.9 for TFV-DP and the TFV-DP deuterated internal standard, respectively (Figure 3.19 and Figure 3.20). TFV-MP and TFV transitions were also added to the quantitation method to observe the retention times of these moieties during the chromatography development and to ensure adequate baseline separation of these compounds. The transitions of the protonated precursor ions monitored for TFV and TFV-MP were *m/z* 288.0 and 368.0 to the product ion *m/z* 176.0 and 269.9, respectively (Figure 3.21 and Figure 3.22). A DELL® Windows® XP computer was used as interface for the LC-MS/MS system and Analyst® software version 1.6.2. was used for all chromatographic data acquisition, peak integration and quantification.

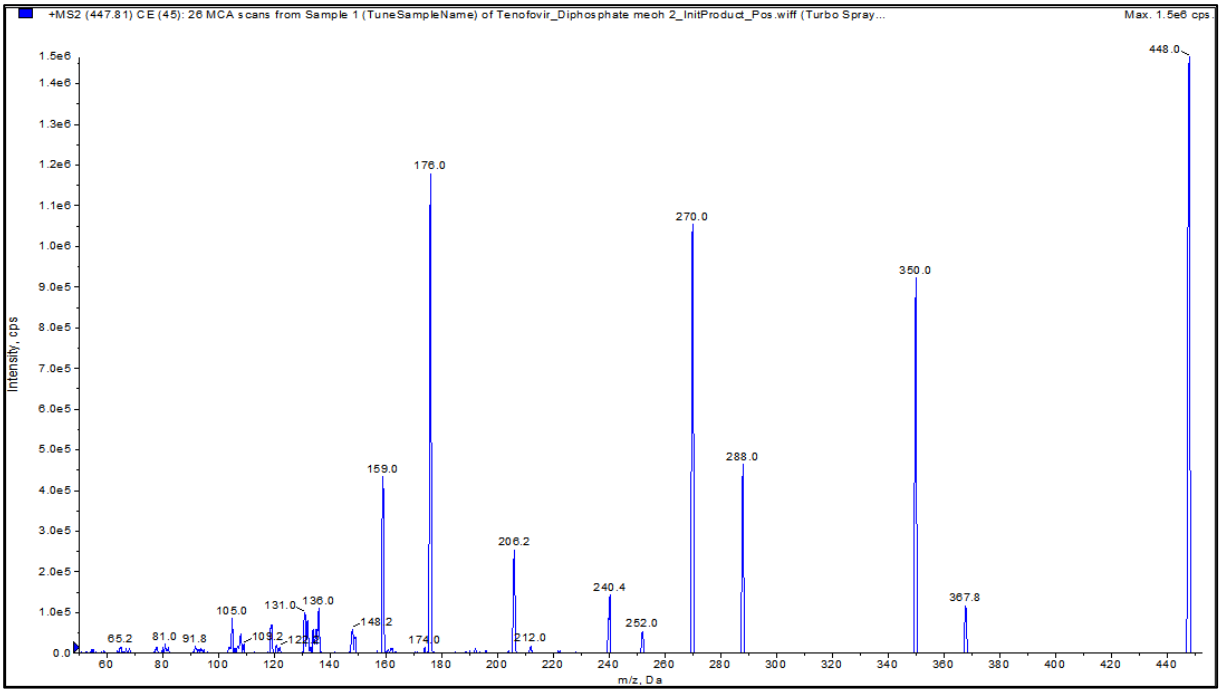


Figure 3.19: Initial product ion mass spectrum of TFV-DP. The molecular weight of TFV-DP is 447.17 g/mol and therefore the expected protonated precursor ion is observed at *m/z* 448.

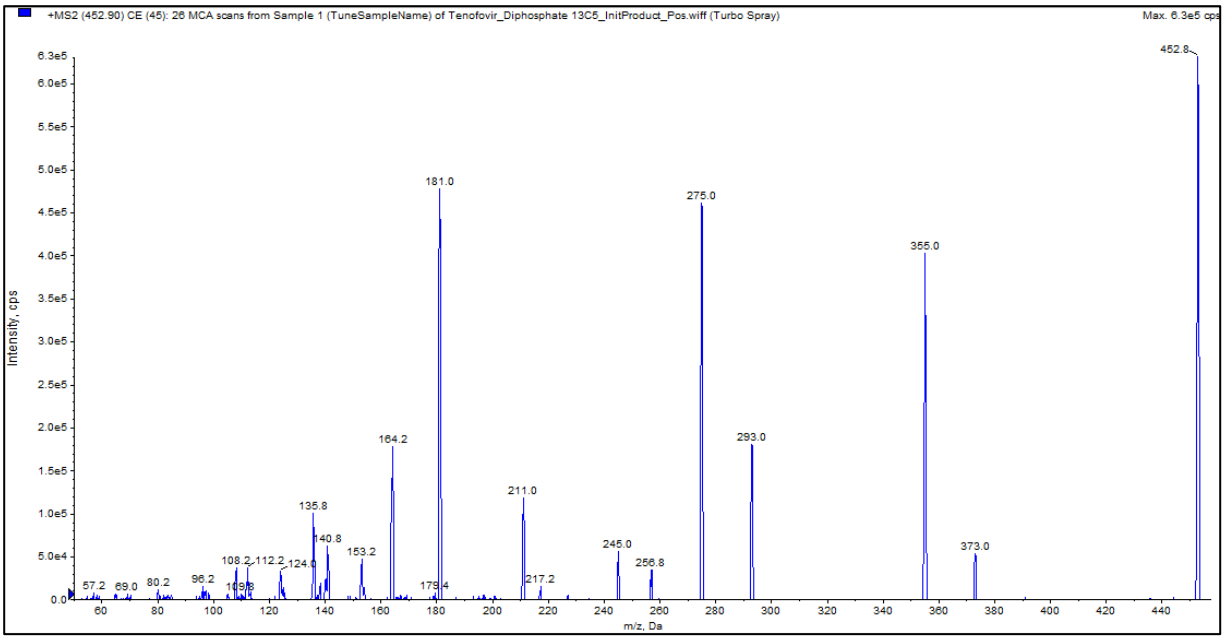


Figure 3.20: Initial product ion mass spectrum of the deuterated internal standard of TFV-DP. The molecular weight of the deuterated internal standard is 452 g/mol and therefore the expected protonated precursor ion is observed at *m/z* 453.

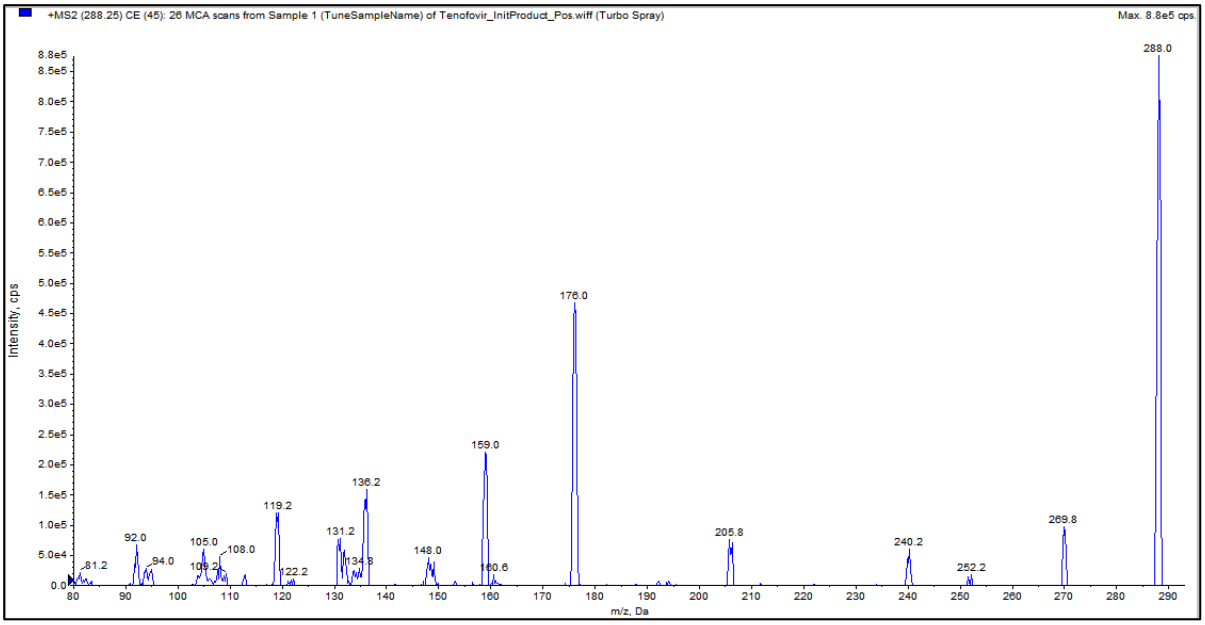


Figure 3.21: Initial product ion mass spectrum of TFV. The molecular weight of TFV is 287.21 g/mol and therefore the expected protonated precursor ion is observed at *m/z* 288.

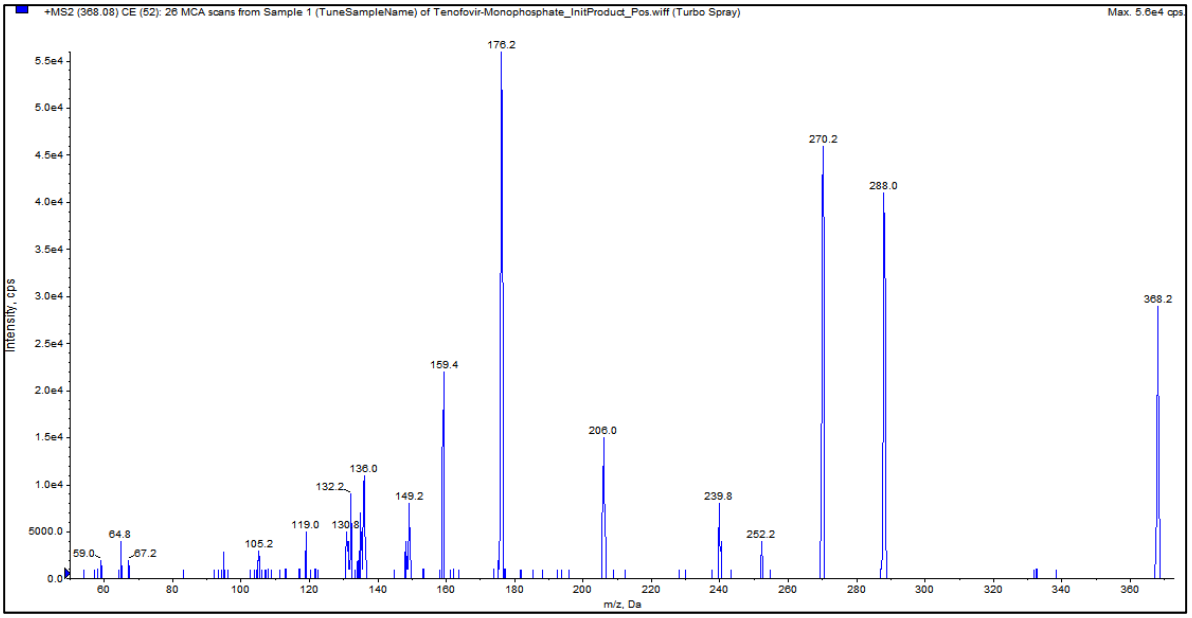


Figure 3.22: Initial product ion mass spectrum of TFV-MP. The molecular weight of TFV-MP is 367.19 g/mol and therefore the expected protonated precursor ion is observed at *m/z* 368.

3.5.2. Detection

Mass spectrometer parameters are presented in Table 3.5 to 3.7.

Table 3.4: Scan Description

Scan Type:	MRM
Polarity:	Positive
Pause Time (ms)	5

Table 3.5: Source settings

Split	1:5
CUR (curtain gas)	50
IS (Ion-spray voltage) (V)	4500
TEM (source temperature) (˚C)	500
GS1 (Nebuliser gas)	40
GS2 (Turbo gas)	70
CAD (Collision gas)	2
CEM (Channel electron multipliers) (eV)	2700

Table 3.6: Tandem mass spectrometer settings

	TFV-DP	**TFV-DP ISTD**	**TFV**	**TFV-MP**
Dwell time (msec)	150	150	150	150
Entrance potential (V)	10	10	10	10
Delustering potential (V)	136	151	121	111
Collision energy (eV)	23	23	33	27
Collision cell exit potential (V)	28	26	14	22

3.5.3. Chromatography

A Thermo BioBasic AX (50 mm × 2.1 mm, 5 µm) column was selected as the analytical column of choice. One of the main aims during chromatography development was to separate TFV and TFV-MP from TFV-DP (analyte of interest) on the analytical column. The reason for the concern relates to possible cross-talk, thermal back-conversion in the ion source, and ion enhancement or suppression that may be caused by TFV and TFV-MP during TFV-DP quantification. Various organic solvents, mobile phase pH's and additives were investigated with the aim of obtaining optimal chromatography, adequate retention, and baseline separation between TFV-DP and its precursors analytes. The organic solvents evaluated consisted of either methanol and water or acetonitrile and water, with different solvent strengths ranging from 10–50% organic (v/v). The analytes were eluted using a pH gradient. A solvent with a low pH was used at the start of the run, allowing analyte retention. The gradient was switched over time to a high pH to allow the elution of the analytes. The two mobile phases that yielded the optimal chromatography and baseline separation are shown below:

Mobile phase A: A mixture of 10 mM ammonium acetate in water and acetonitrile (70:30, v/v), adjusted to pH 6 with acetic acid.

Mobile phase B: A mixture of 2 mM ammonium acetate in water and acetonitrile (70:30, v/v), adjusted to pH 10 with ammonium hydroxide.

A gradient elution method was designed using the mobile phases shown above and the gradient flow schedule is shown in Table 3.8. The gradient method was designed to limit the amount of time in which mobile phase B (pH 10) was pumped at a maximum percentage of the flow. This was done to increase the longevity of the analytical column, since the mobile phase pH is on the upper limit of the analytical column's pH range. The flow rate was initially set at 400 µl/min; however, the effect of increased flow rate on the column pressure and chromatography was investigated in increments of 50 µl/min. The peak shape and separation of the analytes improved with an increase in flow rate; however, this could only be increased to 700 µl/min due to pressure concerns.

Table 3.7: Gradient flow schedule

Total Time (min)	Flow Rate (µl/min)	Line A (%)	Line B (%)
0.0	700	100	0
0.4	700	50	50
1.1	700	50	50
1.2	700	5	95
2.4	700	5	95
2.6	700	100	0
3.5	700	100	0

Representative chromatograms demonstrating the separation achieved with the developed gradient method are shown in Figure 3.23. It is important to note that TFV-DP is the analyte of interest. Therefore, chromatography was only optimised for TFV-DP, which is shown in the top panel (A). Chromatography of the other two analytes, namely TFV and TFV-MP, was not optimised as the only aim was to separate the analytes from TFV-DP.

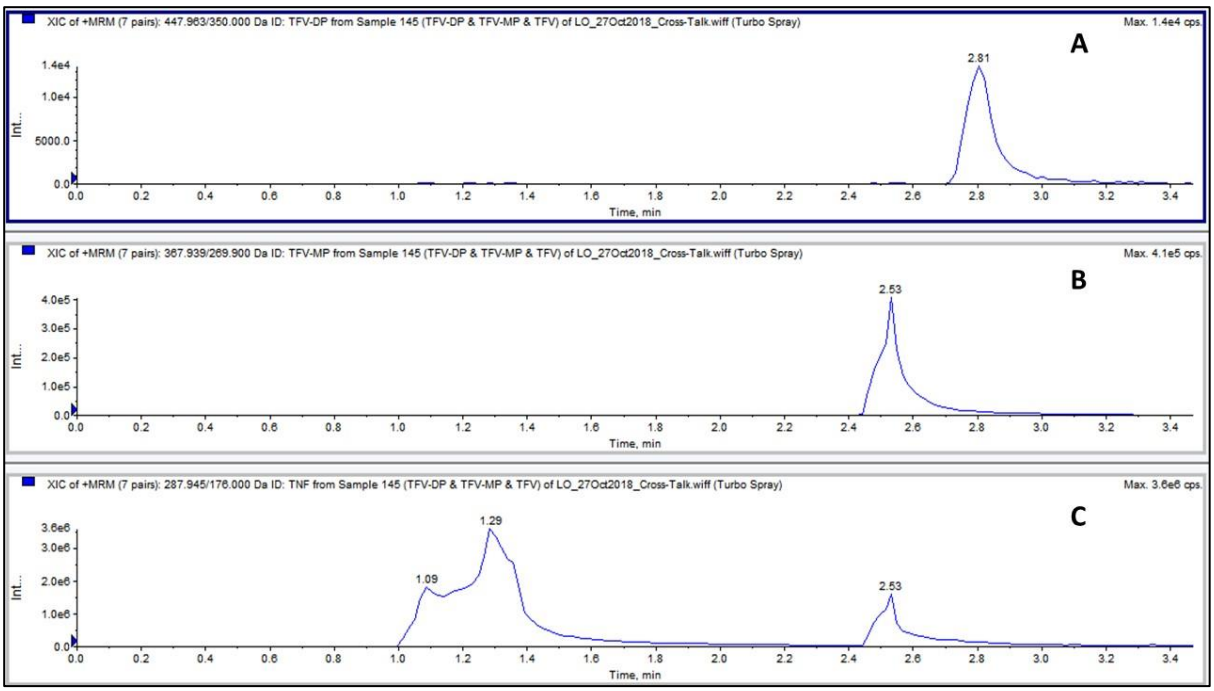


Figure 3.23: Representative chromatographs of A) TFV-DP (Top panel) B) TFV-MP and C) TFV.

Various needle wash solutions were investigated for their ability to reduce HPLC-injection carry-over. These solutions consisted of several organic solvents, including methanol, acetonitrile and isopropanol. The solutions were investigated with different solvent strengths ranging from 10–60% organic (v/v). The pH of the needle wash solution was also adjusted with pH's ranging between 5–10.5. The needle wash solution that resulted in the least amount of carry-over consisted of a mixture of 5 mM ammonium acetate in water and acetonitrile (90:10, v/v), adjusted to pH 9 with ammonium hydroxide. The percentage carry-over was calculated by injecting a TFV-DP neat solution equivalent to a concentration of 6400 fmol/punch and subsequently injecting a blank sample, consisting of water (reconstitution solution). The peaks in the blank sample were then quantified and compared. A decrease in carry-over was observed in all needle wash solutions; however, carry-over was still present. We hypothesised that this may have been due to some analyte being retained on the injector switching valve and therefore a custom injection program was created to decrease any residual analyte that may have remained on the autosampler switching valve. This custom injection program is presented in Table 3.9, which also depicts the autosampler settings. The use of the custom injection program led to a 10% decrease in the peak area of the peak seen in the blank. However, carry-over was still observed.

Table 3.8: Autosampler settings

Pump type	Agilent 1260 Binary Pump
Flow Rate	0.700 ml/min
Autosampler Type	Agilent 1260 High Performance Autosampler
Sample arrangement	96-well plate
Injection volume	20 µl
Autosampler Temperature	8°C
Custom injection program	1. DRAW def. amount from sample, def. speed, def. offset 2. WASH NEEDLE with default wash parameters 3. INJECT 4. REMOTE start pulse, duration 60*12.5 msec 5. WAIT 3.3 min 6. VALVE bypass 7. WAIT 0.50 min 8. VALVE mainpass 9. VALVE bypass 10. VALVE mainpass 11. REMOTE start pulse duration 120*12.5 msec

In an attempt to mitigate any possible carry-over, stainless-steel tubing was exchanged for peek tubing and two wash injection steps were added to rinse both the column and the tubing. The two wash steps both consisted of gradient methods and these are depicted in Tables 3.10 and 3.11. The aim of the two wash steps were to remove the residual analyte from the HPLC system. The wash step injection solution consisted of a mixture of 2 mM ammonium acetate in water and acetonitrile (70:30, v/v), adjusted to pH 10 with ammonium hydroxide. No carry-over was observed after the addition of the two wash steps. As can be seen in Figure 3.24.

Table 3.9: The gradient method of wash step 1.

Total Time (min)	Flow Rate (µl/min)	Line A (%)	Line B (%)
0.00	700	5	95
1.00	700	5	95
1.10	700	100	0

Table 3.10: The gradient method of wash step 2.

Total Time (min)	Flow Rate (µl/min)	Line A (%)	Line B (%)
0.00	700	5	95
0.50	700	5	95
0.65	700	100	0
3.50	700	100	0

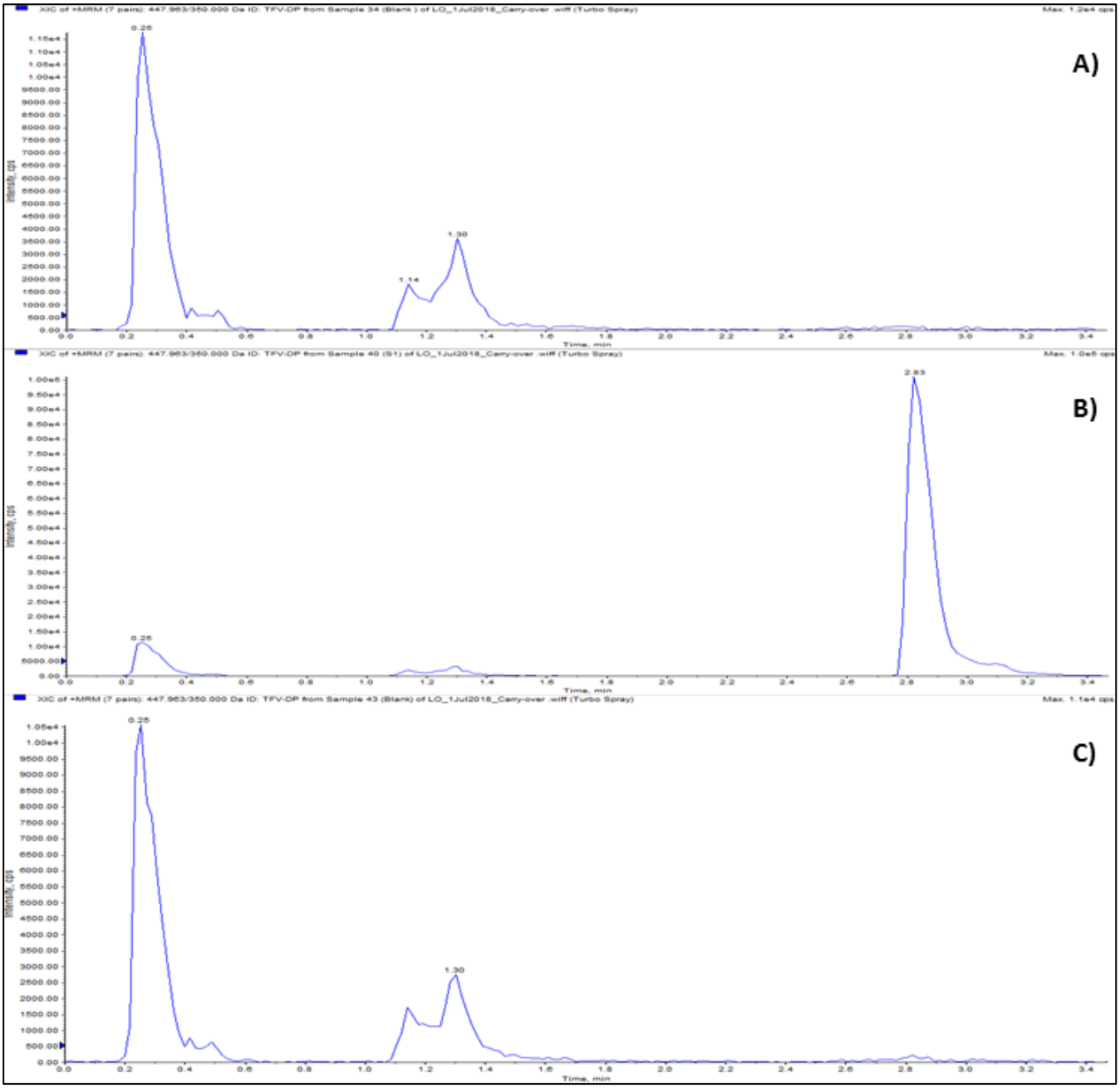


Figure 3.24: Representative chromatograms are shown illustrating the carry-over after adding the wash steps. The following injections were made: A) Blank; B) 6400 fmol/punch neat solution; and C) Blank.

3.5.4. Extraction Method

Sample processing and extraction are essential for the removal of endogenous matrix components that may hinder or interfere with the analysis of the analyte of interest [101]. There are three main approaches to sample extraction, namely, PP, LLE, and SPE [41]. The use of an ion-pairing solid phase extraction (IP-SPE) was initially investigated to allow for the optimal removal of sample constituents. IP-SPE follows the same steps and procedure required

for traditional SPE; however, it utilises ion-pairing reagents to increase the retention of the analyte of interest on the extraction cartridge [106]. This is especially useful when working with highly polar compounds, like TFV-DP. During traditional SPE, a solution is added to the SPE cartridge prior to the biological sample to equilibrate the cartridge. In IP-SPE an ion-pairing reagent is added to the solutions used for the equilibration, loading and wash steps to help retain the analyte of interest [106]. 1,5-DMHA was selected as the ion-pairing reagent and the equilibration solution consisted of a mixture of 10 mM 1,5-DMHA and 5 mM ammonium acetate in water and methanol (80:20, v/v) with an unadjusted pH. One millilitre of the equilibration solution was added to the cartridge, which would prepare the cartridge for sample loading. The lysate of the biological sample was subsequently added to the cartridge in the loading step. This was followed by a wash step, which consisted of the addition of one millilitre of a mixture of 10 mM 1,5-DMHA with 5 mM ammonium acetate in water and methanol (70:30, v/v) with an unadjusted pH. In the final step, namely the elution step, one millilitre of a mixture of 5 mM ammonium acetate in water and methanol (10:90, v/v) with an unadjusted pH was added to the cartridge to elute the analyte of interest. The elution solution was subsequently dried down and reconstituted with 150 µl of water and injected onto the LC-MS/MS system. However, the recovery of this extraction method was only 40%. Various studies have shown that the use of an ion pairing reagent may lead to signal suppression and decreased sensitivity [102,114]. Thus, the low recovery accompanied with concerns of possible exogenous ion suppression due to residual 1,5-DMHA in the elution solution, led to the dismissal of this approach.

A simpler extraction approach was followed. PP with phospholipids removed using Phree columns was investigated.

The sample extraction procedure started with the removal of the DBS sample from storage, which were subsequently left on the bench at room temperature for 20 minutes. After the 20 minutes three 3 mm DBS aliquots were punhed from the respective DBS cards and placed into a 1.5 ml Eppendorf tube.

The TFV-DP internal standard was added to the lysate solution, which consisted of a mixture of water and methanol (30:70, v/v). The internal standard concentration in the lysate was prepared to a concentration equivalent to 200 fmol/punch. Each Eppendorf tube received 0.5 ml of the lysate solution; however, when the STDs and QCs were prepared, 60 µl of each STDs and QC level were added to their respective tubes. To compensate for the addition of the 60 µl in the STDs and QCs, 60 µl of water was added to the Eppendorf tubes of the blank and unknown samples. The tubes were subsequently sonicated for approximately 10 minutes at room temperature.

A 1 ml Phree phospholipid removal SPE cartridge was used to help reduce the exogenous matrix effect. SPE was performed using a Speedisk® 48 SPE positive pressure system. The cartridges were conditioned with 1 ml methanol, before samples were added. After the sonication step 500 µl of the lysate was removed from the Eppendorf tube and placed into the conditioned 1 ml Phree phospholipid removal cartridge. The analytes were subsequently eluted into disposable borosilicate glass tubes. The glass tubes were placed in a heating block (Stuart® block heater, 40°C) and evaporated to complete dryness under a gentle stream of nitrogen for approximately 30 minutes. The extract was reconstituted in 150 µl of water and subsequently vortexed for 30 seconds, before being transferred to a 96-well plate.

The newly developed direct LC/MS/MS assay covered a range of 50 to 6400 fmol/punch as presented in Figure 3.25.

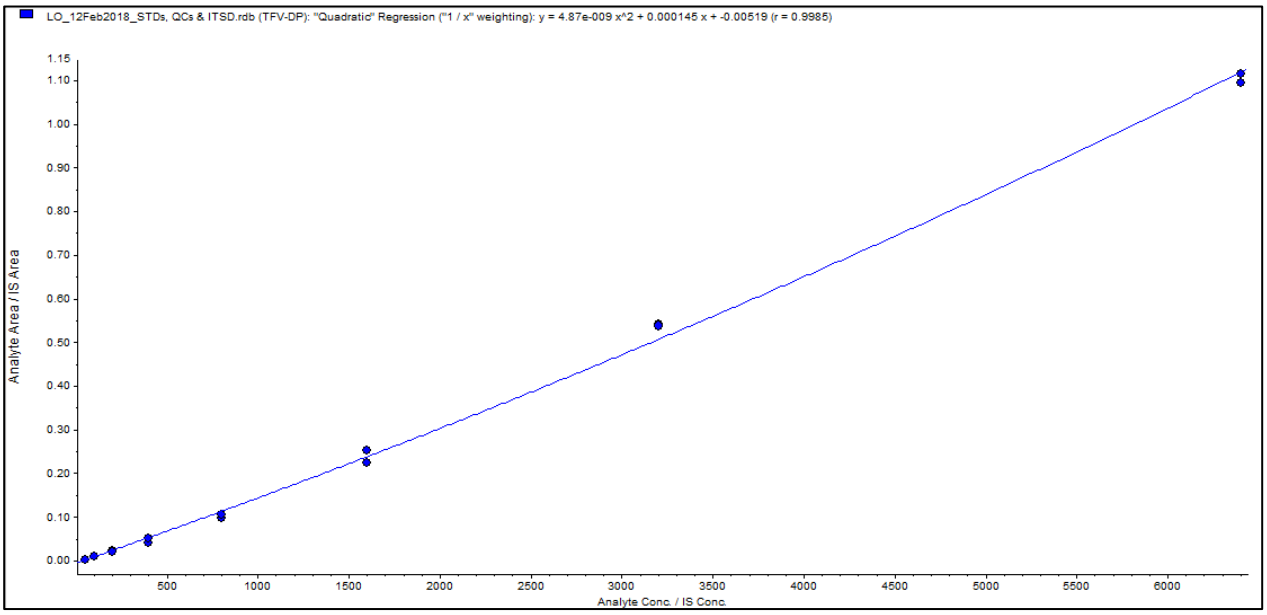


Figure 3.25: Calibration curve of standards that range from 50 to 6400 fmol/punch (r=0.9985).

Chapter 4: Final Method

Due to the sensitivity issues and the highly polar nature of TFV-DP, various approaches were investigated in an attempt to develop a more cost effective and less laborious direct method. The investigation of the various approaches were discussed in Chapter 3; however, this chapter will summarise the final bioanalytical method procedure, which ultimately underwent the validation procedure.

4.1. Sample Conditions

4.1.1. Specimen Collection Procedures

Sample collection procedures are summarised in Table 4.1.

Table 4.1: Summary of sample collection procedures

Volume required per assay:	Five, 50 µl whole blood aliquots spotted onto filter paper (Whatman - Protein Saver 903 Card)
Matrix:	Human Whole Blood
Anticoagulant used:	K3EDTA
Stability concerns:	TFV-DP is prone to dephosphorisation. Therefore, whole blood samples must be kept on ice until spotting on filter paper.
Sample preparation considerations:	A calibrated pipette must be used to accurately aliquot 50 µl of whole blood from the collection tube and spot it onto the pre-marked circles on the filter paper (Whatman-Protein Saver 903 Card). The filter paper sample must be left at room temperature for two hours to dry and subsequently placed into a resealable bag with desiccant. The bag must be sealed and stored at approximately -80^{0}C.
Conditions pertaining to the rejection of samples:	Insufficient sample volume blood spot or smeared blood spot
	Temperature inconsistencies
	Undue exposure to light
	Anticoagulant confusion (K2EDTA has been shown to have minimal anticoagulant matrix effects)
	Unsure sample identity
	Other conditions at the direction of the laboratory principal investigator, which would make sample analysis impossible.

4.1.2. Summary of stability data for TFV-DP

Information regarding the stability of the analyte of interest is essential for the correct storage and handling of samples during the analytical process. A summary of the stability data of TFV-DP is discussed in Table 4.2 below.

Table 4.2: TFV-DP stability data

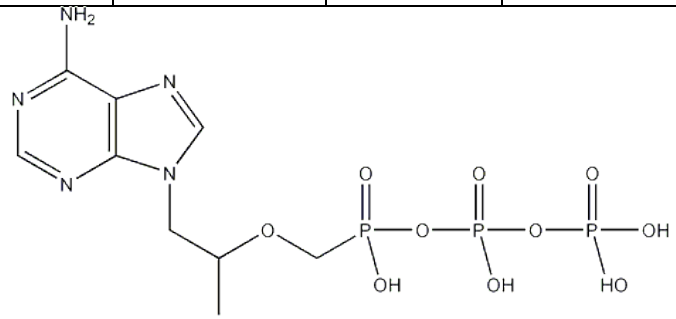
Stability	
Stability in Matrix (at ~-20°C)	TFV-DP has been shown to be stable in DBS for at least 198 days when stored at approximately -20°C.
Stability in Matrix (at ~4°C)	TFV-DP is stable in DBS for up to 14 days when stored at ~4°C.
Stability in Matrix (at RT)	TFV-DP is stable in DBS for at up to 7 days when stored at room temperature.
Stability in Matrix (at ~37°C)	TFV-DP is NOT stable in DBS when stored at ~37°C for up to six days.
Stock Solution Stability (methanol)	Stock solutions of TFV-DP in methanol were found to be stable when stored at ~-80°C for at least 150 days, and for ~24 hours when stored at room temperature, ~4°C, ~-20°C. Stock solutions of TFV in methanol were found to be stable when stored at ~-80°C for at least 330 days, and for ~24 hours when stored at room temperature, ~4°C, ~-20°C.
Working Solution Stability (water)	Working solutions of TFV-DP in water were found to be stable when stored at room temperature, ~4°C, ~-20°C and ~-80°C for ~4 hours and stable at ~-20°C and ~-80°C only when stored for 24 hours. Working solutions of TFV in water were found to be stable when stored at room temperature, ~4°C and ~-80°C for ~24 hours, and at ~-20°C for 20 days.
Freeze and Thaw Stability	TFV-DP was found to be stable in DBS when subjected to three freeze-thaw cycles.
Stability in whole blood	TFV-DP is not stable in whole blood when stored at room temperature.
Benchtop stability	TFV-DP was found to be stable in a DBS for up to 12 hours in ambient light at room temperature.
Reinjection reproducibility	TFV-DP is stable and can be reinjected up to five days after extraction, if samples are stored at ~4°C.

4.2. Method description

4.2.1.TFV-DP

Information regarding the TFV-DP analytical reference standard, used during the validation of the method, is shown in Table 4.3.

Table 4.3: TFV-DP analytical reference standard properties

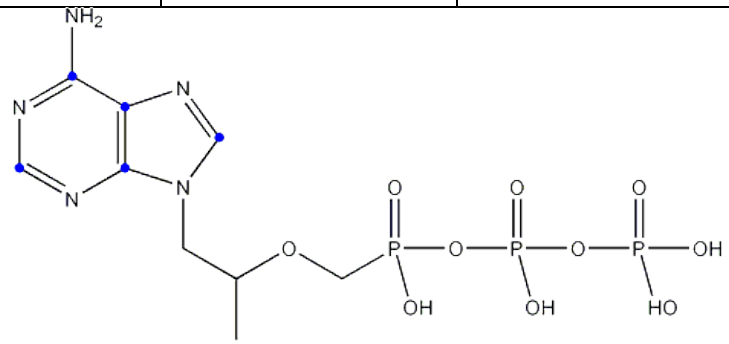
Name:	**TFV-DP**				
Analytical Reference standard:	Tenofovir-Diphosphate Tetraammonium Salt				
Synonyms:	PMPApp Triethylamine Salt, Diphosphoric Acid Triethylamine Salt				
Molecular Formula:	$C_9H_{16}N_5O_{10}P_3 \bullet x(C_6H_{15}N)$				
Molecular formula of free acid/base:	$C_9H_{16}N_5O_{10}P_3$				
Molecular Weight:	515.29	Free acid/base:	447.17	Exact Mass:	447.01
Chemical structure:					
In-house catalogue number	543/544				
Manufacturer:	Moravek				
Lot No.	M-1731				
Purity	95.7%	Adjustment made?	**YES**	NO	
Expiry date:	Sept 2018 (retest)				

4.2.2. ISTD: TFV-DP Adenine-$^{13}C_5$

Information regarding the deuterated internal standard is summarised in Table 4.4.

Table 4.4: Internal standard analytical reference standard

Name:	**TFV-DP -$^{13}C_5$**
Analytical Reference standard:	**TFV-DP, tetraammonium salt, adenine -$^{13}C_5$**
Synonyms:	None

Molecular Formula:	$C_{15}H_{31}N_6O_{10}P_3 \bullet x(C_{16}H_{36}N)$			
Molecular formula of free acid/base:	$C_{15}H_{31}N_6O_{10}P_3$ – The reference standard comes as a 2 µmol powder and therefore no correction was made.			
Molecular Weight:	520.26	Free acid/base:	452.14 g/mol	
Chemical structure:				
In-house catalogue number	551			
Manufacturer:	Moravek			
Lot No.	MG-135			
Purity	96.0%	Adjustment made?	YES	**NO**
Expiry date:	No expiry date on COA			

4.3. Biological matrix

The method was designed for the analysis of DBS consisting of 50 µl of whole blood spotted onto filter paper. Information regarding the biological matrix used in the validation of the method is summarised in Table 4.5.

Table 4.5: Summary of the biological matrix information used in validation

Identity:	Whole Blood
Anticoagulant:	K3EDTA
Source (including internal reference):	Inhouse donation

4.4. Solution preparations

4.4.1. Stock Solution Preparation

Stock solutions (SS) were prepared by weighing a mass of the analyte into a container and dissolving the weighed-out mass in the desired volume of water to obtain target concentrations of 1000 µg/ml. The weighed mass of the analyte was also adjusted where applicable (purity, salt, etc.). All stock solutions were stored at approximately -80°C until required. Stock solutions were used to prepare working solutions or to spike blank biological matrix as required. The ISTD stock solution (ISS1) was made by dissolving 2.00 µmol of isotopically labelled TFV-DP (adenine $^{13}C_5$) in 1.00 ml of water, with the use of a calibrated pipette to prepare a 2.00 µmol/ml stock solution.

This was subsequently aliquoted and stored at approximately -80°C until required. A summary of the TFV-DP stock solution preparations (SS1 & SS2) are shown in Tables 4.6 and 4.7. The preparation of the TFV-DP ISTD (ISS1) is shown in Table 4.8.

Table 4.6: Preparation of TFV-DP stock solution (SS1)

Solvent used	**Volume solvent (ml)**	**Weighed mass of analyte (mg)**	**Adjusted* mass of analyte (mg)**	**SS1 concentration (µg/ml)**
Water	0.648	0.780	0.648	1000

* *Reason for Adjustment (e.g. purity, salt, hydrate):* Purity 95.7%; Tetraammonium salt

* *Calculation:* = 0.780 x 0.957 x 447.173/515.297

Table 4.7: Preparation of TFV-DP stock solution (SS2)

Solvent used	**Volume solvent (ml)**	**Weighed mass of analyte (mg)**	**Adjusted* mass of analyte (mg)**	**SS2 concentration (µg/ml)**
Methanol	0.681	0.820	0.681	1000

* *Reason for Adjustment (eg. purity, salt, hydrate):* Purity 95.7%; Tetraammonium salt

* *Calculation:* = 0.820 x 0.957 x 447.173/515.297

Table 4.8: Preparation of $^{13}C_5$ TFV-DP ISTD stock solution (ISS1)

Solvent used	**Volume Solvent (ml)**	**Moles (µmol)**	**Adjusted* Mass of analyte (µmol)**	**ISS1 Concentration (µmol/ml)**
Water	1.00	2.00	2.00	2.00

* *No adjustment was made*

4.4.2. Preparation of ISTD Working Solutions

ISTD working solution (IWS1) was prepared daily by spiking 10.0 µl of ISS1 stock solution into 0.990 ml water. An aliquot of this solution was added to the lysate solution to achieve a final ISTD concentration equivalent to 500 fmol/ punch in the lysate.

4.4.3. Preparation of TFV-DP Calibration Standards Working Solutions

Calibration standard working solutions (WS) were prepared volumetrically in water by spiking 10.0 µl of TFV-DP stock solution (SS1) into 9.99 ml of water to make Stock A. A volume of 0.668 ml of Stock A was subsequently added to 4.00 ml of water, using a calibrated pipette. This was then serially diluted with water to attain the desired working solutions as presented in Table 4.9. The working solutions were kept on ice until storage or spiking. Multiple 80 µl aliquots of each working solution were stored in individual 1.5 ml Eppendorf tubes at approximately -80°C. Literature has shown the stability of TFV-DP in water for at least two months, when stored at approximately -80°C [77].

Table 4.9: Preparation of TFV-DP Calibration Standards Working Solutions

Standard	Blank Water Volume (ml)	Volume SS1 spiked (µl)	Dilution Source	Dilution Source Volume (ml)	Total Volume of Dilution (ml)	TFV-DP spiking solution conc.		
						(pg/ml)	(pmol/ml)	(fmol/punch)
Stock A	9.99	10.0			10.0	1000000	2236	44726
WS1	4.00		*Stock A*	*0.668*	4.67	143102	320	6400
WS2	2.00		*WS1*	*2.00*	4.00	71551	160	3200
WS3	2.00		*WS2*	*2.00*	4.00	35775	80.0	1600
WS4	2.00		*WS3*	*2.00*	4.00	17888	40.0	800
WS5	2.00		*WS4*	*2.00*	4.00	8944	20.0	400
WS6	2.00		*WS5*	*2.00*	4.00	4472	10.0	200
WS7	2.00		*WS6*	*2.00*	4.00	2236	5.00	100
WS8	2.00		*WS7*	*2.00*	4.00	1118	2.50	50.0

4.4.4. Preparation of TFV-DP Quality Controls Working Solutions

QC working solutions (WSs) were prepared in water by adding a volume of 10.0 µl of TFV-DP stock solution (SS2) to 9.99 ml of water to make Stock B. A volume of 1.11 ml of Stock B was subsequently added to 3.39 ml of water, using a calibrated pipette. This was subsequently diluted with water to attain the desired working solutions as

presented in Table 4.10. Multiple 80 µl aliquots of each working solution were stored in individual 1.5 ml Eppendorf tubes at approximately -80°C.

Table 4.10: Preparation of TFV-DP QC Working Solutions

Quality control	Blank Water Volume (ml)	Volume SS2 spiked (µl)	Dilution Source	Dilution Source Volume (ml)	Total Volume of Dilution (ml)	TNF-DP spiking solution conc.		
						(pg/ml)	(pmol/ml)	(fmol/punch)
Stock B	9.99	10.0			10.0	1000000	2236	44726
QCDil	3.39		*Stock B*	*1.11*	4.50	245945	550	11000
QC H	2.30		*QC Dil*	*2.00*	4.30	114473	256	5120
QC M	2.00		*QC H*	*2.00*	4.00	57237	128	2560
SYS	6.53		*QC M*	*2.00*	8.53	13420	30.0	600
QC L	6.00		*SYS*	*2.00*	8.00	3355	7.50	150
LLOQ	4.00		*QC L*	*2.00*	6.00	1118	2.50	50.0

**The QC Dilute sample is used to qualify the dilution process in the first validation batch and is not included in subsequent validation or sample batches*

4.4.5. Verification of Standards and Quality Controls

The calibration standards (STDs) and QC samples were analysed in a batch prior to sample analysis to confirm their accuracy. A second stock solution was prepared independently by another analyst and used to verify the stock solution used for the preparation of STDs and QCs. Prior to sample analysis, QC working solutions was analysed against freshly prepared calibration standard working solutions to verify their accuracy and to determine stability at ~-80°C. Should this verification meet the acceptance criteria, the curve was deemed valid for sample analysis and the validation purposes. Verified standards and QCs were stored as batches.

4.4.6. Buffers and Solutions

The constituents and preparation of the necessary solutions are described below. As with the stock solutions and STDs and QCs, the specific stability of the solutions were determined and the solutions were replaced after the expiry date.

- **Mobile Phase A:** Add 0.771 g ammonium acetate to 300 ml acetonitrile and 700 ml water (10.0 mM), adjust pH by adding 200 µl of acetic acid (pH 5.9). Keep at room temperature and replace after five days.
- **Mobile Phase B**: Add 0.154 g ammonium acetate to 300 ml acetonitrile and 700 ml water (2.00 mM), adjust pH by adding 2.00 ml ammonium hydroxide (pH 10.1). Keep at room temperature and replace after five days.
- **Autosampler needle wash:** Add 0.385 g ammonium acetate to 100 ml acetonitrile and 900 ml water, adjust pH by adding 0.500 ml ammonium hydroxide (pH 9.0). Keep at room temperature and replace after one month.
- **Lysate:** Add 300 ml of water to 700 ml of methanol. Store at ~4°C and replace after two weeks.

4.5. Extraction procedure

This section lists the extraction protocol, which is used for the analysis of analytical batches. However, prior to the discussing the extraction protocol, it is important to define what an analytical batch is and what it consists of.

4.5.1. Criteria for an Analytical Batch

An analytical batch is a single extraction procedure applied to a series of unknown samples, which must include a system suitability sample (SYS) of sufficient volume to inject at least ten times, a minimum of eight STDs analysed in duplicate, three levels of QCs analysed in duplicate, a blank and a double blank sample. These are all required to assess various parameters, including accuracy, precision, carryover and system suitability, in order to ensure the validity of the results. STDs are spread throughout the run, in order to capture instrument drift. QC samples are also spread over the run, in order to control the analysis appropriately. Blanks and double blanks are run after the highest calibration standard, consecutively in order to ascertain the level of carryover for the batch.

4.5.2. Extraction Methodology

Preceding the extraction procedure of analytical batches, the appropriate DBS cards were removed from their storage at -80°C, and subsequently placed at room temperature for approximately 20 minutes. Three, 3 mm punches were made within the designated circle on the Whatman Protein Saver 903 Cards containing the DBS. The 3 mm punches were then placed in 1.5 ml Eppendorf tubes. The same procedure was followed for the sample preparation of STD, QC, blank, and double blank samples; however, the DBS used for these samples were prepared with blank whole blood.

The ISTD stock solution (ISS1) was used to make a fresh ISTD working solution (IWS1), which was subsequently added to the lysate to achieve a concentration of 500 fmol/punch as outlined in Section 4.4.2. A volume of 500 µl lysate, containing ISTD, was added to each of the 1.5 ml Eppendorf tubes containing the three 3 mm DBS punches. All samples received 500 µl of the lysate with ISTD except for the double blank, which received ISTD-free lysate.

To reach the desired concentration in the STD and QC samples a volume of 60 µl of each STD and QC working solution level was added to their respective samples. To compensate for the added volume in the STD and QC tubes, 60 µl of water was added to all unknown, blank and double blank samples.

After the sample preparation was completed, the Eppendorf tubes were placed into an ultrasonic bath (Instrulab®), set at room temperature, and sonicated for 10 minutes at room temperature. The samples were subsequently removed from the ultrasonic bath, before being aliquoted into Phree™ phospholipid removal cartridges. However, the Phree™ cartridges had to be conditioned before samples could be added and this was done by adding 1 ml of methanol to each cartridge and subsequently eluting the methanol into a waste container. The Phree™ columns were placed into a SPEEDISK® 48 SPE positive pressure system to aid in the elution of the samples and other solutions. A volume of 500 µl of lysate was removed from each sample with a calibrated pipette and added to the Phree™ cartridge, while taking care not to remove the 3 mm DBS punches. The lysate was eluted into disposable borosilicate glass tubes, which were subsequently placed in a Stuart® Evaporator with a heating block. The extract was left under a gentle stream of nitrogen at 40^0C for 30 minutes to evaporate. After the samples had evaporated, they were reconstituted with 150 µl of water. To ensure the complete reconstitution of the samples, each borosilicate glass tube was vortexed for 30 seconds before being transferred to a 96-well plate.

4.6. Instrument Setup and Special Precautions

The following alterations were made to the LC-MS/MS system to ensure the accuracy and reliability of the analytical method. A custom injection program, as outlined in Section 4.8, was used to help prevent carry-over. Each sample injection was followed by two subsequent wash steps, which consisted of a 20 µl injection of mobile phase B. The gradient flow schedules of each of the methods are summarised in Section 4.8. All post autosampler stainless steel tubing was replaced with Peek tubing and the column switching valve was bypassed. A post-column mobile phase split (1:5) was also included at the source.

4.7. Reagents, Chemicals, Consumables, and Equipment Used

The specific chemicals and reagents, consumables and equipment that were used for this method are summarised in Tables 4.11, 4.12 and 4.13, respectively.

Table 4.11: Chemicals and reagents

Reagent	Catalogue No.	Grade	Supplier
Acetic Acid	49199-50ml F	LC/MS	Sigma-Aldrich
Acetonitrile	LC015-2.5	LC/MS	Anatech
Ammonium Acetate	431311#50g	ACS	Sigma-Aldrich
Ammonium Hydroxide	338818-100ML	ACS	Sigma-Aldrich
Methanol	LC230-2.5	LC/MS	Anatech
Water	In house Millipore	18.2 mΩ.cm @ 25°C	N/A
TFV-DP	M-1731	95.7%	Moravek
TFV-DP, tetraammonium salt, adenine -$^{13}C_5$	MG-135	96.0%	Moravek

Table 4.12: List of required consumables

Description	Catalogue No.	Supplier
96 well plate	5042-1385	Agilent Technologies
Sealing mats	PAXG AN-2ml-RD-PK	Lasec SA
Pipette tips (White)	771290	Lasec SA
Pipette tips (Yellow)	P2TIP018Y-010200R	Lasec SA
Pipette tips (Blue)	P2TIP018B-001000R	Lasec SA
Borosilicate Culture Tubes (Kimble)	Item 1505(12 x 75)	Lasec SA
Filter Paper: Protein Saver 903 Card	10531018	Whatman
Phree Phospholipids Removal columns (1 ml)	8B-S133-TAK	Phenomonex
Analytical column: BioBasic AX, 5µm, 50 x 2.1 mm	73105-052130	ThermoFisher Scientific

Table 4.13: Equipment

Name	Model	Manufacturer
Pipette	2-20 µl	Various
Pipette	20-200 µl	Various
Pipette	200-1000 µl	Various
Vortex	Genie 2	Scientific Industries
Analytical Microbalance	CPA2P	Sartorius
SPEEDISK 48 SPE positive pressure system	48 SPE	Speedisk
Evaporator with heating block (incubator)	SBH 130D/3	Stuart
Ultrasonic Bath	UMC5	Instrulab

4.8. Mass Spectrometer and Chromatographic Conditions

Important details concerning the LC-MS/MS system and the parameters that were used during the analysis of samples during the validation process are summarised in Table 4.14. This includes the various mobile phases and their constituents and the custom injection program. These parameters are strictly adhered to during the validation process and any analysis of samples that follow.

Table 4.14: A summary of the instrument and chromatographic conditions

<table>
<tr><td>Instrument used</td><td>AB Sciex 5500 Qtrap</td></tr>
<tr><td>Project number</td><td>TFV-DP 2016/174</td></tr>
<tr><td>Acquisition method</td><td>Acquisition method: LO_Anion Exchange_20ul.dam
Wash run A: LO_Anion Exchange_wash AI.dam
Wash run B: LO_ Anion Exchange_wash B.dam</td></tr>
<tr><td>Analytical Column</td><td>Analytical column: BioBasic AX, 5µm, 50 x 2.1 mm</td></tr>
<tr><td>Column Temperature</td><td>~30^{0}C</td></tr>
<tr><td>Mobile Phase</td><td>The run contains a gradient consisting of the two mobile phases:
-A mixture of 10.0 mM ammonium acetate in water and acetonitrile (70:30, v/v), adjusted to pH 5.9 by adding acetic acid.
-A mixture of 2.00 mM ammonium acetate in water and acetonitrile (70:30, v/v), adjusted to pH 10.1 by adding ammonium hydroxide.

Analytical Run: LO_Anion Exchange_20ul.dam
<table>
<tr><th>Total Time (min)</th><th>Flow Rate (µl/min)</th><th>Line A (%)</th><th>Line B (%)</th></tr>
<tr><td>0.00</td><td>700</td><td>100</td><td>0</td></tr>
<tr><td>0.40</td><td>700</td><td>50</td><td>50</td></tr>
<tr><td>1.10</td><td>700</td><td>50</td><td>50</td></tr>
<tr><td>1.20</td><td>700</td><td>5</td><td>95</td></tr>
<tr><td>2.40</td><td>700</td><td>5</td><td>95</td></tr>
<tr><td>2.60</td><td>700</td><td>100</td><td>0</td></tr>
<tr><td>3.50</td><td>700</td><td>100</td><td>0</td></tr>
</table>
Wash run 1: LO_Anion Exchange_wash A.dam
<table>
<tr><th>Total Time (min)</th><th>Flow Rate (µl/min)</th><th>Line A (%)</th><th>Line B (%)</th></tr>
</table></td></tr>
</table>

<table>
<tr><td></td><td>
<table>
<tr><td>0.00</td><td>700</td><td>5</td><td>95</td></tr>
<tr><td>1.00</td><td>700</td><td>5</td><td>95</td></tr>
<tr><td>1.10</td><td>700</td><td>100</td><td>0</td></tr>
</table>
Wash run 2: LO_ Anion Exchange_wash B.dam
<table>
<tr><td>Total Time (min)</td><td>Flow Rate (µl/min)</td><td>Line A (%)</td><td>Line B (%)</td></tr>
<tr><td>0.00</td><td>700</td><td>5</td><td>95</td></tr>
<tr><td>0.50</td><td>700</td><td>5</td><td>95</td></tr>
<tr><td>0.65</td><td>700</td><td>100</td><td>0</td></tr>
<tr><td>3.50</td><td>700</td><td>100</td><td>0</td></tr>
</table>
</td></tr>
<tr><td>Pump type</td><td>Agilent 1260 Binary Pump</td></tr>
<tr><td>Flow Rate</td><td>0.700 ml/min</td></tr>
<tr><td>Autosampler Type</td><td>Agilent 1260 High Performance Autosampler</td></tr>
<tr><td>Sample arrangement</td><td>96-well plate</td></tr>
<tr><td>Injection volume</td><td>20 µl</td></tr>
<tr><td>Autosampler Temperature</td><td>~8°C</td></tr>
<tr><td>Custom injection program</td><td>DRAW def. amount from sample, def. speed, def. offset
WASH NEEDLE with default wash parameters
INJECT
REMOTE start pulse, duration 60*12.5 msec
WAIT 3.3 min
VALVE bypass
WAIT 0.50 min
VALVE mainpass
VALVE bypass
VALVE mainpass
REMOTE start pulse duration 120*12.5 msec</td></tr>
</table>

4.9. System Suitability Requirements

As has been discussed in Section 4.5, the system suitability test must be done before each analytical batch. A SYS, consisting of a mid-level extracted sample, is injected a minimum of 10 times. The acceptance criteria for system suitability tests requires the percentage covariance (CV(%)) of the last six injected sample peak area ratios to be ≤ 5%. All SYS samples are injected with the LO_ Anion Exchange_SYS_10ul.dam method, which is identical

to LO_Anion Exchange_20ul.dam (shown in Table 4.14); however, the injection volume is only 10 µl. The lower injection volume is used to allow more injections per extracted SYS sample.

4.10. Detection Details

The various instrument settings pertaining to the detection of the analyte of interest are summarised in Tables 4.15 to 4.18.

Table 4.15: Detection settings

Mass spectrometer Identity	API 5500 QTrap
APCI/ESI	ESI

Table 4.16: Electro Spray Ionisation Settings

Nebuliser gas (Gas 1) (arbitrary unit)	40
Turbo gas (Gas 2) (arbitrary unit)	70
CUR (curtain gas) (arbitrary unit)	50
CAD (collision gas) (arbitrary unit)	2
TEM (Source Temperature) (°C)	500
IS (Ion Spray Voltage) (V)	4500

Table 4.17: MS/MS Settings

	TFV-DP	**ISTD**
Protonated molecular ion mass (m/z) $[M+H]^+$	448.0	452.9
Product ion mass (m/z) Quantifier	350.0	354.9
Product ion mass (m/z) Qualifier	270.1	274.9
Dwell time (ms)	150	150
Declustering potential (V)	136	151
Entrance potential (V)	10	10
Collision energy (eV)	23	23
Collision cell exit potential (V)	28	26

Table 4.18: Scan description

Scan Type	MRM
Polarity	Positive
Pause time (msec)	5

4.11. Analyte Spectra

The mass spectra of the analyte of interest and the ISTD are show in this section. Both were observed after infusing a solution of 200 ng/ml of each analyte in the into an AB Sciex 55000 mass spectrometer using a Hamilton syringe set at a constant flow rate of 10 µl/min.

4.11.1 Analyte: TFV-DP

A mass spectrum of the analyte after collision-induced dissociation in the fragmentation cell, showing the TFV-DP precursor ion at m/z 448 as well as the product ions, Figure 4.1.

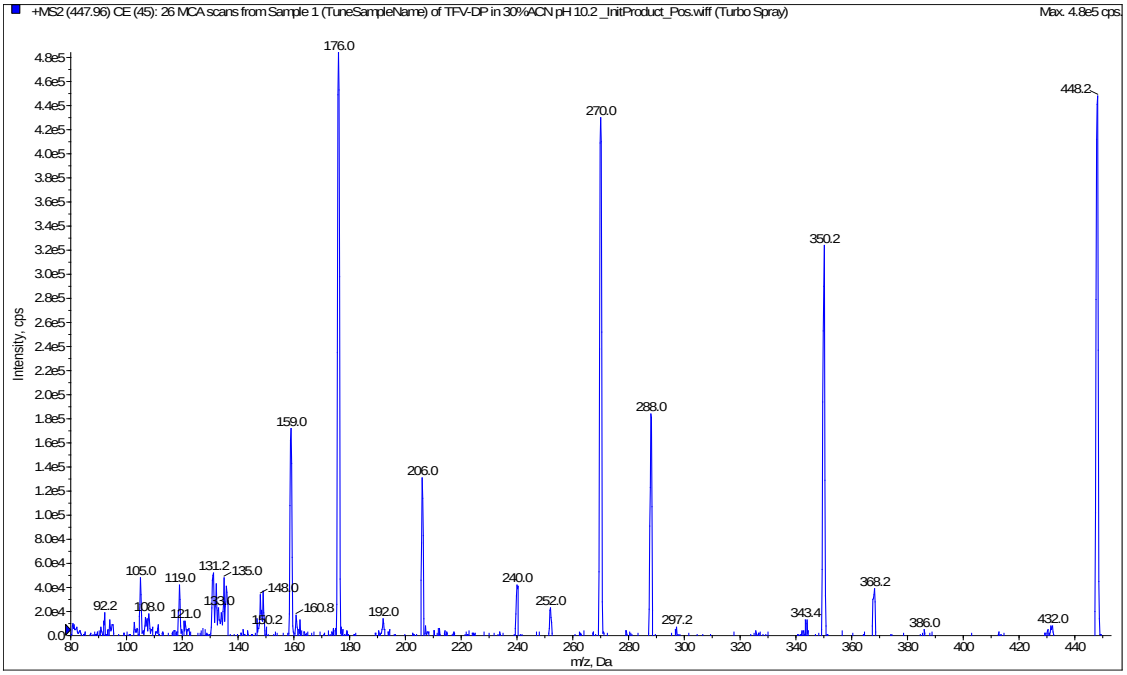


Figure 4.1: Mass spectrum of TFV-DP.

4.11.2. ISTD: TFV-DP, Adenine-$^{13}C_5$

A mass spectrum of the analyte after collision-induced dissociation in the fragmentation cell, showing the TFV-DP-ISTD precursor ion at m/z 453 as well as the product ions, Figure 4.2.

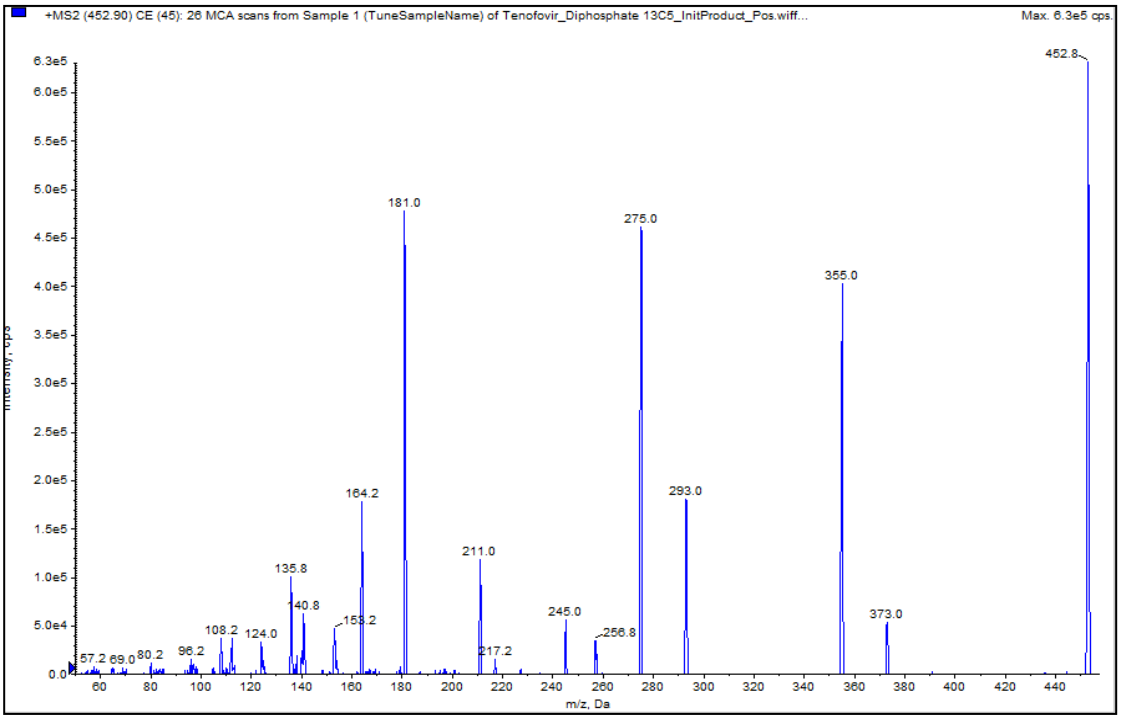


Figure 4.2: Mass spectrum of ISTD.

4.12. Recording and integration

4.12.1 Quantitation Parameters, Ions Monitored, and Retention Times:

Analyte concentrations were calculated using Analyst software with the Analyst Classic Algorithm. Duplicate calibration points were used to construct the calibration curve, using the validated regression model (fit type and weighting). Important information regarding the quantitation of both TFV and the ISTD is summarised in Tables 4.19 to 4.21 below.

Table 4.19: Summary of the quantitation parameters

	Analyte: **TFV-DP**	ISTD: **TFV-DP (adenine -$^{13}C_5$)**
Software Version	Analyst 1.6.2 using Analyst Classic Algorithm	
Algorithm	Analyst Classic	
Calibration fit type	Quadratic	Quadratic
Parameter	Area Ratio	Area Ratio
Curve Weighting	1/x	
Bunching Factor	1	1
Number of smooths	2	2

Table 4.20: Precursor and Product ions monitored

	Precursor Ion Mass (amu)	**Product Ion Mass (amu)**
Analyte: **TFV-DP**	448.0	350.0
ISTD: **TFV-DP (adenine -$^{13}C_5$)**	452.9	354.9

Table 4.21: Retention time of the analytes in minutes

Analyte: **TFV-DP**	2.72
ISTD: **TFV-DP (adenine -$^{13}C_5$)**	2.71

4.13. Acceptance criteria

To ensure the validity of the results obtained from the developed method, any analytical batch results must undergo a quality review procedure and meet the various acceptance criteria that are required for the STDs, QC's, blanks and double blanks. The review process and acceptance criteria are discussed below.

4.13.1. Quality Review Procedure

All data is subjected to a QC review by a designated analyst. This review considers the acceptance criteria listed below, as well as appropriate scientific criteria for data acceptance. Following the completion of a project, the project documentation is reviewed by Quality Assurance to confirm standard operating procedure compliance and regulatory adherence if applicable. This includes a complete data audit to ensure that the data is accurately reported in the dataset to be released. Following this, the data may be released.

4.13.2. Calibration Standards

Seventy five percent of the standards used must fall within 15% of the nominal concentration (i.e. 85–115%), except for the LLOQ, which should be within 20% (i.e. 80–120%) of the nominal value. Duplicate points are used to construct the calibration curve. Failed points must be excluded from the calibration curve's regression determination.

4.13.3. Quality Control

A minimum of 6 QC levels covering the range from ~80% of the ULOQ to within three times the LLOQ are injected in each analytical batch. Sixty seven percent of all controls assayed must meet the acceptance criteria of 85–115% accuracy. The allowed failures may not be of the same concentration, therefore 50% of controls run at every level must pass. Failure to meet these acceptance criteria will require the reanalysis of the unknown samples. The number of QC samples included in an analytical assay run must be ≥ 5% of the unknown samples in each run.

4.13.4. Blanks and Double Blanks

Blanks and double blanks are used to assess the carryover during an analytical batch. Blanks and double blanks are analysed immediately after the highest calibration standard. The double blank is injected first followed by a blank sample, in order to determine any possible interference caused by the ISTD. The observed peak areas of the analyte and ISTD in the blank and double blank samples should be less than 20% of the peak areas observed in the LLOQ samples.

4.13.5. Duplicate and Triplicate Analyses

Unknown samples are analysed singly. Without evidence to show that the analytical batch has failed, these results are accepted. When repeating a selected sample due to suspected experimental error, the sample is repeated in duplicate. The average of the repeat values is selected over the original value only if a >15% difference between the average of the repeat values and the original value is observed, otherwise the original value stands. When repeating a batch of samples due to a failed analytical batch or when assaying dilution requests, samples are repeated singly.

4.13.6. Peak Reintegration

As far as possible, method development ensures that reintegrations are not routinely necessary for an assay. Automatic reintegration is preferable, using a sample chromatogram from the run, should the analyst deem it

necessary to adjust the integration parameters for a batch. Chromatograms are reviewed as part of the batch acceptance procedure. The original data may not be overwritten, and the reintegrated chromatogram is saved separately and printed along with the entire chromatogram set. The reintegrations are reviewed by QC and quality assurance prior to data release.

Chapter 5: Method Validation

This chapter describes the validation process of the bioanalytical method, described in Chapter 4. The validation was carried out in accordance to FDA and EMA guidelines for the validation of a bioanalytical assay [95,96]. The validation process must be completed before the implementation of any bioanalytical method and is done to demonstrate the reliability and suitability of an assay for its intended use. This chapter will discuss and summarise the various validation experiments and their accompanying results.

According to the FDA and EMA, the validation of a bioanalytical method has to be completed to objectively demonstrate and document the accuracy, precision, specificity, sensitivity, and reproducibility of a bioanalytical method [95,96]. During the validation process, stability experiments are also completed to determine the stability of the analyte for the optimal storage, handling and processing of samples of unknown concentrations.

Demonstrating acceptable within- and between-day accuracy and precision over the desired calibration range is an essential step in demonstrating the robustness and reproducibility of the bioanalytical method. In order to do so, a set of calibration STDs and QCs were prepared and assayed in three consecutive runs of which at least two were on different days. A full set of STDs and QCs were prepared and stored at -80˚C, as outlined in Chapter 4. The required aliquots were removed from storage and subsequently thawed and assayed according to the method described in Chapter 4. Each run consisted of a duplicate of each STD level, in order to produce a calibration curve. This would be accompanied by six replicates of each of the QC levels.

5.1. Procedure

To demonstrate acceptable within- and between-day accuracy and precision over the desired concentration range, STDs and QCs were prepared and assayed in three consecutive runs. A full set of STDs and QCs were prepared and stored frozen (at ~-80˚C), and the required aliquots were thawed and assayed. Each run consisted of all the STDs in duplicate to produce one calibration curve and six replicates of the prepared QCs. A QC spiked to a concentration above the ULOQ was diluted (1:4) with blank plasma to validate the dilution of samples for which the concentrations potentially do not fall within the validated range.

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

5.2. Stock solutions, STD and QC Preparation

The preparation of stock solutions, STDs and QCs were described in Section 4.4 in Chapter 4 and this was strictly adhered to during the validation process. All stock solutions, STDs and QCs were stored at ~-80^{0}C until required.

5.3. Validation Results

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

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

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

5.4. Validation 1 (Day 1)

Within-batch accuracy and precision were assessed by assaying all the STDs in duplicate, to produce one calibration curve, and six replicates of each QC level in a single batch of assays. The within-batch accuracy and precision of the assay procedure were assessed by calculating the regression equation and constructing the calibration curve based on peak area ratios of analyte to ISTD.

5.4.1. Validation 1, Day 1

The calibration curve is presented in Figure 5.1. The regression equation used was Quadratic (weighted by 1/x concentration), $f(x) = a + bx + cx^2$, Table 5.1. A summary of the STD and QC results of validation batch one is presented in Tables 5.2 and 5.3.

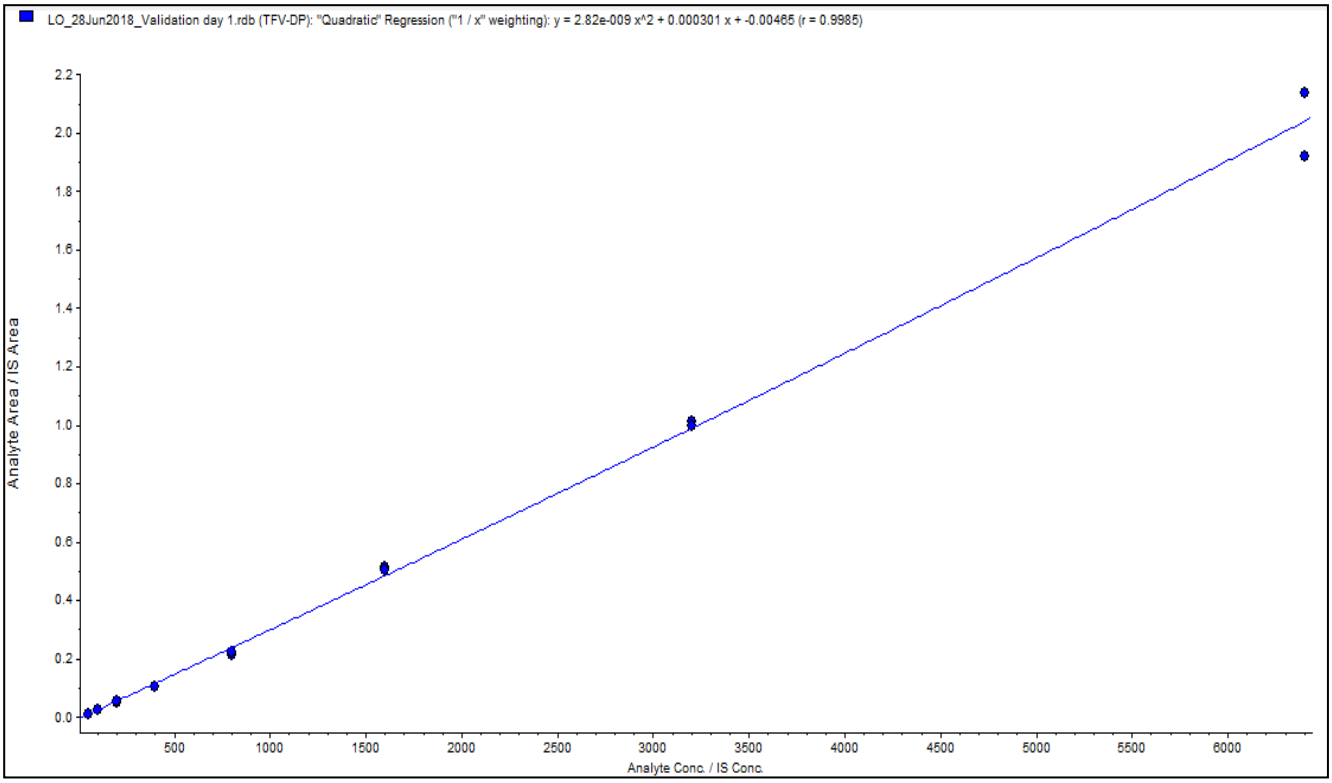


Figure 5.1: Representative calibration curve for TFV-DP: Validation batch one, day one. r =0.999.

Table 5.1: Regression equation for TFV-DP validation batch one, day one

Validation	Quadratic Calibration Curve Parameters			
Batch	**a**	**B**	**c**	**r**
1	-0.00000000282	0.000301	-0.00465	0.999

Table 5.2: Summary of accuracy and precision results for the STDs of validation batch one

Sample ID	Nominal concentration (fmol/punch)	Mean Observed concentration (fmol/punch)	Standard deviation	CV(%)	%Accuracy	n
S8 (LLOQ)	50.0	58.0	1.59	2.7	116.1	2 of 2
S7	100	97.8	0.554	0.6	97.8	2 of 2
S6	200	192	11.8	6.2	96.2	2 of 2
S5	400	369	1.00	0.3	92.1	2 of 2

S4	800	733	26.0	3.5	91.7	2 of 2
S3	1600	1680	21.1	1.3	105.0	2 of 2
S2	3200	3254	36.0	1.1	101.7	2 of 2
S1	6400	6365	449	7.1	99.4	2 of 2

Table 5.3: Summary of accuracy and precision results of the QCs of validation batch one

Sample ID	Nominal concentration (fmol/punch)	Mean Observed concentration (fmol/punch)	Standard deviation	CV(%)	%Accuracy	n
LLOQ	50.0	54.2	3.81	7.0	108.5	6 of 6
QC L	150	151	6.40	4.2	100.7	6 of 6
QC M	2560	2515	187	7.4	98.2	6 of 6
QC H	5120	5221	160	3.1	102.0	6 of 6

5.5. Validation 2 and 3

Between-batch accuracy and precision were determined by assaying two additional separate consecutive batches, each consisting of a double set of STDs designated for use in the assay of samples of unknown concentrations and six replicates of each of the QCs. The between-batch accuracy and precision of the assay procedure were assessed by constructing a calibration curve based on analyte/ISTD peak area ratios and calculating the regression equations.

5.5.1. Validation 2, Day 2

The calibration curve for validation batch two is presented in Figure 5.2. The regression equation used was Quadratic (weighted by 1/x concentration), $f(x) = a + bx + cx^2$, as presented in Table 5.4. A summary of the STD and QC results of validation batch two is presented in Tables 5.5 and 5.6.

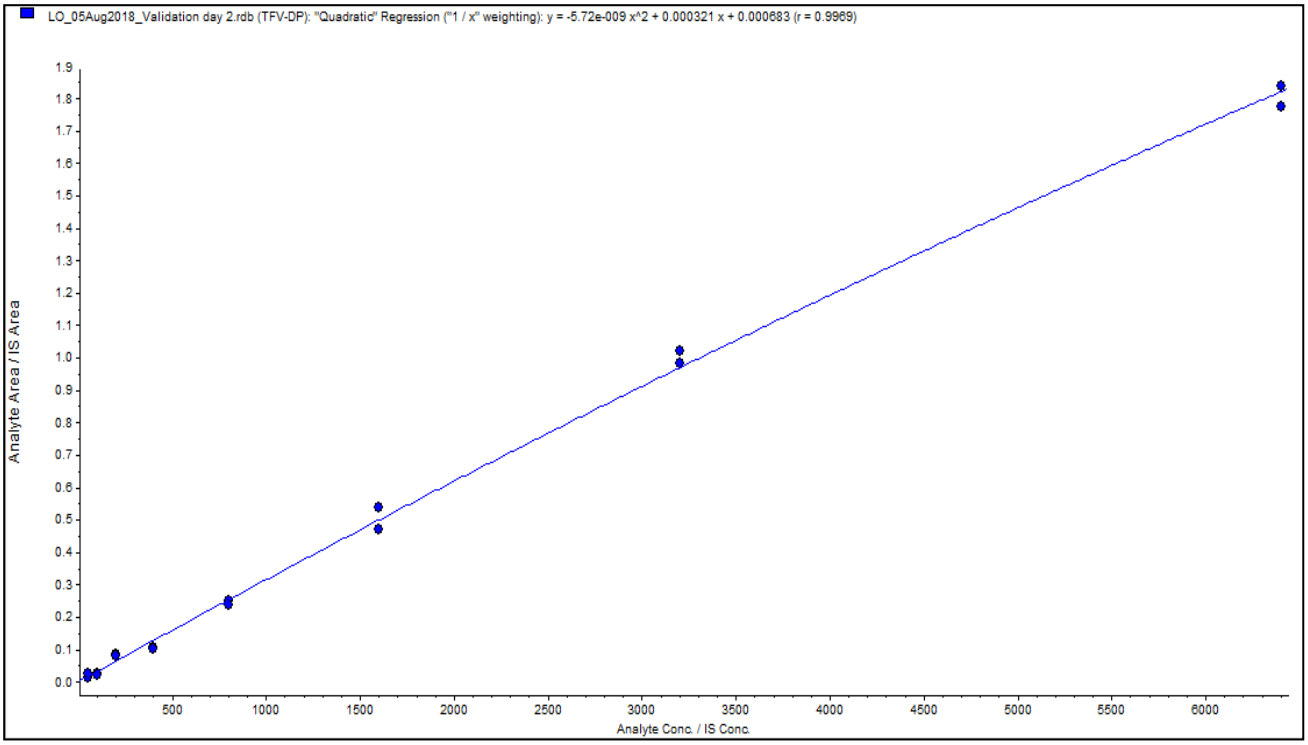


Figure 5.2: Representative calibration curve for TFV-DP: Validation batch two, day two. r =0.997.

Table 5.4: Regression equation for TFV-DP validation batch two, day two

Validation	Quadratic Calibration Curve Parameters			
Batch	**a**	**B**	**c**	**r**
2	-0.00000000572	0.000321	-0.00683	0.997

Table 5.5: Summary of accuracy and precision results of the STDs of validation batch two

Sample ID	Nominal concentration (fmol/punch)	Mean Observed concentration (fmol/punch)	Standard deviation	CV(%)	%Accuracy	n
S8	50.0	55.4	5.23	9.4	110.8	2 of 2
S7	100	105	4.82	4.6	105.0	2 of 2
S6	200	192	10.8	5.6	95.8	2 of 2
S5	400	356	7.04	2.0	89.0	2 of 2
S4	800	759	3.81	0.5	94.8	2 of 2
S3	1600	1642	56.7	3.5	102.6	2 of 2
S2	3200	3281	32.6	1.0	102.5	2 of 2
S1	6400	6359	173	2.7	99.4	2 of 2

Table 5.6: Summary of the accuracy and precision results of the QCs of validation batch two

Sample ID	Nominal concentration (fmol/punch)	Mean Observed concentration (fmol/punch)	Standard deviation	CV(%)	%Accuracy	n
QC LLOQ	50.0	60.0	4.07	6.8	120.0	6 of 6
QC L	150	158	12.1	7.6	105.5	6 of 6
QC M	2560	2662	44.8	1.7	104.0	6 of 6
QC H	5120	5237	216	4.1	102.3	6 of 6

5.5.2. Validation 3, Day 3

The calibration curve for validation batch three is presented in Figure 5.3. The regression equation used was Quadratic (weighted by 1/x concentration), $f(x) = a + bx + cx^2$, as presented in Table 5.7. A summary of the STD and QC results of validation batch three is presented in Tables 5.8 and 5.9.

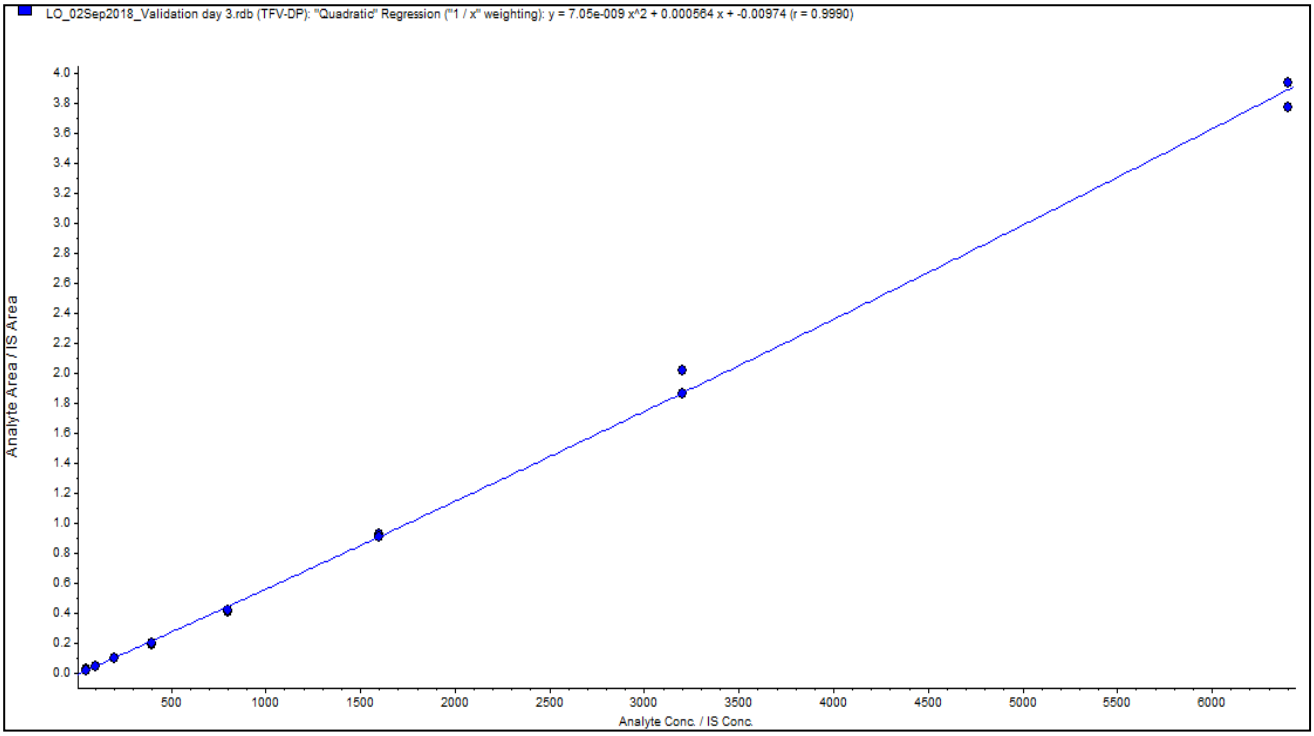


Figure 5.3: Representative calibration curve for TFV-DP: Validation batch three, day three. r =0.999.

Table 5.7: Regression equation for TFV-DP validation batch three, day three

Validation	Quadratic Calibration Curve Parameters			
Batch	**a**	**B**	**c**	**r**
3	-0.00000000705	0.000564	-0.00974	0.999

Table 5.8: Summary of the accuracy and precision results of the STDs of validation batch three

Sample ID	Nominal concentration (fmol/punch)	Mean Observed concentration (fmol/punch)	Standard deviation	CV(%)	%Accuracy	n
S8	50.0	58.1	2.14	3.7	116.1	2 of 2
S7	100	95.4	3.67	3.8	95.4	2 of 2
S6	200	200	2.97	1.5	100.1	2 of 2
S5	400	366	7.89	2.2	91.4	2 of 2
S4	800	745	16.1	2.2	93.1	2 of 2
S3	1600	1612	18.3	1.1	100.7	2 of 2
S2	3200	3327	179	5.4	104.0	2 of 2
S1	6400	6346	170	2.7	99.1	2 of 2

Table 5.9: Summary of the accuracy and precision results of the QCs of Validation batch three

Sample ID	Nominal concentration (fmol/punch)	Mean Observed concentration (fmol/punch)	Standard deviation	CV(%)	%Accuracy	n
QC LLOQ	50.0	57.0	3.48	6.1	114.0	6 of 6
QC L	150	153	4.90	3.2	101.8	6 of 6
QC M	2560	2464	35.4	1.4	96.3	6 of 6
QC H	5120	4951	87.1	1.8	96.7	6 of 6

5.6. Summary of the Combined STD and Quality Control Results

The overall accuracy and precision of the assay procedure is assessed by calculating the accuracy and precision statistics over the within- and between-batch validation batches (three in total). Accuracy is expressed as the concentration of the analyte found as a percentage of the nominal concentration (%Accuracy), while precision is expressed as the CV(%). The combined regression, STDs and QCs results (all three validation batches) of TFV-DP are summarised in Tables 5.10 to 5.12 below.

Table 5.10: Overall Summary of Calibration Curve Parameters

Validation Batch	Quadratic Calibration Curve Parameters			
	a	B	c	r
1	-0.00000000282	0.000301	-0.00465	0.999
2	-0.00000000572	0.000321	-0.00683	0.997
3	-0.00000000705	0.00564	-0.00974	0.999

Table 5.11: Summary of the three validation batches STD results

Validation Batch	Sample ID	STD 1 - ULOQ	STD 2	STD 3	STD 4	STD 5	STD 6	STD 7	STD 8 - LLOQ
	Nominal Conc.	6400 (fmol/ punch)	3200 (fmol/ punch)	1600 (fmol/ punch)	800 (fmol/ punch)	400 (fmol/ punch)	200 (fmol/ punch)	100 (fmol/ punch)	50.0 (fmol/ punch)
	Replicates	Observed Conc.	Observed Conc.	Observed Conc.	Observed Conc.	Observed Conc.	Observed Conc.	Observed Conc.	Observed Conc.
Validation 1	1	6050	3280	1690	715	369	184	97.4	59.2
	2	6680	3230	1660	752	368	201	98.2	56.9
Validation 2	1	6490	3310	1610	762	344	182	110	60.1
	2	6220	3250	1680	759	363	200	97.8	52.7
Validation 3	1	6470	3200	1620	733	360	198	98.0	59.6
	2	6230	3450	1600	756	371	202	92.8	56.5
	n	6	6	6	6	6	6	6	6
	Average	6357	3287	1643	746	363	195	99.0	57.5
	STDEV	229	88.7	38.3	18.4	9.93	9.03	5.75	2.77
	CV(%)	3.6	2.7	2.3	2.5	2.7	4.6	5.8	4.8
	%Accuracy	99.3	102.7	102.7	93.3	90.6	97.3	99.0	115.0

Table 5.12: Summary of the three validation batches QC results

Validation Batch	Sample ID Nominal Conc. Replicates	LLOQ 50.0 (fmol/punch) Observed Conc.	QC - L 150.0 (fmol/punch) Observed Conc.	QC - Med 2560 (fmol/punch) Observed Conc.	QC - High 5120 (fmol/punch) Observed Conc.	QC - DIL 11000 (fmol/punch) Observed Conc.
Validation 1	1	53.1	143	2150	5080	2570
	2	50.8	143	2550	5090	2300
	3	59.5	154	2570	5500	2410
	4	51.4	157	2550	5250	2310
	5	58.6	156	2560	5270	2270
	6	52.1	155	2700	5130	2440
Validation 2	1	63.7	146	2720	5180	
	2	56.8	159	2650	5290	
	3	65.6	171	2660	5060	
	4	46.2	148	2610	4980	
	5	40.7	173	2690	5510	
	6	62.1	153	2700	5330	
Validation 3	1	52.4	147	2460	4890	
	2	58.4	161	2420	4900	
	3	54.8	152	2470	4970	
	4	57.2	155	2530	5110	
	5	56.5	153	2450	4890	
	6	62.7	149	2450	4940	
	n	18	18	18	18	6
	Average	55.7	154.	2549	5132	2383
	STDEV	6.30	8.26	139	194	113
	CV(%)	11.3	5.4	5.5	3.8	4.7
	%Accuracy	111.4	102.8	99.6	100.2	21.7

5.7. Calibration Range

Results from the validation assays above indicate a valid calibration range of 50–6400 fmol/punch for TFV-DP in extracted DBS. The LLOQ was set at the concentration of the lowest validated STD for TFV-DP, namely 50 fmol/punch.

5.8. Quantification Method

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

5.9. Stability Assessment

Various stability experiments were performed to show that all the necessary precautions were taken to ensure that the analyte concentrations were not affected by the assay procedure or associated conditions. These stability measures are used to dictate how the storage and collection of samples should be conducted. The stock solution, working solution, freeze thaw, and benchtop stability of TFV-DP was not tested, since these stability experiments were previously performed for the indirect method.

5.9.1. Reinjection Reproducibility

Reinjection reproducibility is evaluated to determine if an analytical run can be reanalysed by reinjection in the case of instrument interruptions. Following the injection of the first validation run (Validation batch 1), the extracted samples (96-well plate) remained in the autosampler at the method-defined temperature (~8˚C) for the following 24 hours and were subsequently reinjected. Summaries of the ~24-hour reinjected STD and QC results are shown in Tables 5.13 and 5.14, respectively. The extracted samples (96-well plate) were also reinjected after being stored at ~4˚C for the following six days, thus the samples were reinjected seven days after the extraction was completed. This long storage period was chosen due to stringent instrument time constrains, which would not easily allow the reinjection of the samples within 24 hours. Therefore, the longer storage period mimics the conditions that would most likely be seen in practice. The summaries of the seven-day reinjection results for the STD and QC are shown in Tables 5.15 and 5.16, respectively.

Acceptance criteria: The acceptance criteria for this experiment requires that the accuracy remain within 15% of the nominal value (i.e. %Accuracy should be between 85–115%) and the precision (%CV) remain below 15% for all STDs and QCs, except for the LLOQ. The %Accuracy for the LLOQ must remain within 20%, whereas the precision must remain below 20%.

Discussion: These requirements were met for the reinjection of the extracted samples after 24 hours; however, these requirements were not met with the reinjection of the same samples after 7 days. The 7-day reinjection failed due to the %Accuracy of the LLOQ, which was not within 20%, Table 5.16. As has been mentioned before, there are stringent instrument time constraints and it would be ideal to extend the pre-reinjection storage time as far as possible in the case of instrument interruptions. In order to do so the samples from the third validation batch (Validation batch 3, day 3) were stored at 4˚C after injection and were reinjected after 5 days. The summaries of the STD and QC reinjection results of the third validation batch are shown in Tables 5.16 and 5.17, respectively.

The reinjection of the third validation batch complied with all the acceptance requirements, as can be seen in Tables 5.16 and 5.17. Therefore, samples can be stored at 4˚C for up to 5 days if the batch has stopped due to instrument interruptions.

Table 5.13: Summary of the results of the STDs reinjected after ~24 hours

Sample ID	Nominal concentration (fmol/punch)	Mean Observed concentration (fmol/punch)	Standard deviation	CV(%)	%Accuracy	n
S8	50.0	59.1	0.57	1.0	118.2	2 of 2
S7	100	96.2	2.29	2.4	96.2	2 of 2
S6	200	190	1.09	0.6	94.8	2 of 2
S5	400	372	4.12	1.1	92.9	2 of 2
S4	800	736	6.49	0.9	92.0	2 of 2
S3	1600	1672	23.1	1.4	104.5	2 of 2
S2	3200	3259	47.3	1.5	101.9	2 of 2
S1	6400	6364	369	5.8	99.4	2 of 2

Table 5.14: Summary of the results of the QCs reinjected after ~24 hours

Sample ID	Nominal concentration (fmol/punch)	Mean Observed concentration (fmol/punch)	Standard deviation	CV(%)	%Accuracy	n
QC LLOQ	50.0	60.0	4.03	6.7	120.0	6 of 6
QC L	150	152	9.64	6.3	101.6	6 of 6
QC M	2560	2453	168	6.8	95.8	6 of 6
QC H	5120	5058	116	2.3	98.8	6 of 6

Table 5.15: Summary of the STDs reinjected after approximately 7 days

Sample ID	Nominal concentration (fmol/punch)	Mean Observed concentration (fmol/punch)	Standard deviation	CV(%)	%Accuracy	n
S8	50.0	59.9	1.13	1.9	119.8	2 of 2
S7	100	98.2	1.21	1.2	98.2	2 of 2
S6	200	186	18.5	10.0	92.9	2 of 2
S5	400	363	11.3	3.1	90.8	2 of 2
S4	800	736	5.11	0.7	92.0	2 of 2
S3	1600	1670	17.5	1.0	104.4	2 of 2
S2	3200	3280	24.5	0.7	102.5	2 of 2
S1	6400	6355	388	6.1	99.3	2 of 2

Table 5.16: Summary of the QCs reinjected after approximately 7 days

Sample ID	Nominal concentration (fmol/punch)	Mean Observed concentration (fmol/punch)	Standard deviation	CV(%)	%Accuracy	n
QC LLOQ	50.0	61.7	2.39	3.9	123.4	6 of 6
QC L	150	153	9.08	6.0	101.7	6 of 6
QC M	2560	2451	167	6.8	95.8	6 of 6
QC H	5120	5070	110	2.2	99.0	6 of 6

Table 5.17: Summary of the STDs reinjected after approximately 5 days

Sample ID	Nominal concentration (fmol/punch)	Mean Observed concentration (fmol/punch)	Standard deviation	CV(%)	%Accuracy	n
S8	50.0	55.3	2.55	4.6	110.7	2 of 2
S7	100	97.2	3.76	3.9	97.2	2 of 2
S6	200	195	1.28	0.7	97.4	2 of 2
S5	400	377	10.7	2.8	94.2	2 of 2
S4	800	779	9.53	1.2	97.3	2 of 2
S3	1600	1652	62.9	3.8	103.2	2 of 2
S2	3200	3207	53.8	1.7	100.2	2 of 2
S1	6400	6389	84.9	1.3	99.8	2 of 2

Table 5.18: Summary of the QCs reinjected after approximately 5 days

Sample ID	Nominal concentration (fmol/punch)	Mean Observed concentration (fmol/punch)	Standard deviation	CV(%)	%Accuracy	n
QC LLOQ	50.0	54.0	7.62	14.1	108.0	6 of 6
QC L	150	155	4.09	2.6	103.2	6 of 6
QC M	2560	2503	31.5	1.3	97.8	6 of 6
QC H	5120	5147	113	2.2	100.5	6 of 6

5.9.2. Autosampler Stability

In order to assess autosampler stability, the first validation run was reinjected after ~24 hours at the designated autosampler temperature, namely ~8˚C. The reinjected high and low QC peak area ratios were compared to those obtained during the first injection. This provided an estimation of absolute autosampler stability over ~24 hours. A summary of the results of the ~24-hour autosampler stability at the high and low concentrations are shown in Tables 5.19 and 5.20, respectively.

Table 5.19: Summary of high concentration sample autosampler stability

High Concentration			
Validation 1, Batch Start	**Peak area**	**ISTD peak area**	**Ratio**
Injection 1	546000	341000	1.60
Injection 2	514000	321000	1.60
Injection 3	552000	318000	1.74
Injection 4	527000	318000	1.66
Injection 5	519000	312000	1.66
Injection 6	522000	323000	1.62
Average	530000	322167	1.65
STDEV	15427	9948	0.0518
CV(%)	2.9	3.1	3.1
Validation 1 Reinjection End	**Peak area**	**ISTD peak area**	**Ratio**
Injection 7	539000	343000	1.57
Injection 8	537000	340000	1.58
Injection 9	567000	338000	1.68
Injection 10	556000	340000	1.64
Injection 11	539000	333000	1.62
Injection 12	540000	337000	1.60
Average	546333	338500	1.61
STDEV	12291	3391	0.0391
CV(%)	2.2	1.0	2.4
%Difference after Validation 1 Reinjection			**-1.9**

Table 5.20: Summary of low concentration sample autosampler stability

Low Concentration			
Validation 1, Batch Start	**Peak area**	**ISTD peak area**	**Ratio**
Injection 1	14000	364000	0.0385
Injection 2	12500	325000	0.0385
Injection 3	13600	327000	0.0416
Injection 4	13800	323000	0.0427
Injection 5	13700	322000	0.0425
Injection 6	13300	317000	0.0420
Average	13483	329667	0.0410
STDEV	534	17154	0.00198
CV(%)	4.0	5.2	4.8
Validation 1 Reinjection	**Peak area**	**ISTD peak area**	**Ratio**
Injection 7	13900	365000	0.0381
Injection 8	13000	346000	0.0376
Injection 9	15300	351000	0.0436
Injection 10	14100	329000	0.0429
Injection 11	12700	340000	0.0374
Injection 12	14400	338000	0.0426
Average	13900	344833	0.0403
STDEV	949	12384	0.00296
CV(%)	6.8	3.6	7.3
%Difference after Validation 1 Reinjection			**-1.5**

Acceptance criteria: The autosampler stability acceptance criteria require that the %CV and %difference not exceed 15%, since this would be an indication of autosampler instability.

Discussion: The %CV and %difference for the TFV-DP peak area ratios were reported to be within 15%, indicating that the ISTD compensates well in the assay and that the samples are stable at the set autosampler temperature. Therefore, samples of one batch can be reinjected with samples of a new batch, or part of the batch can be reinjected in the case of instrument failure, provided that the storage period does not exceed 24 hours. If the pre-reinjection storage period exceeds 24 hours, the samples must be placed at 4˚C until they can be reinjected.

5.9.3. Whole Blood Stability

To allow the adequate collection and handling of patient samples at clinical sites, whole blood stability must be determined. Whole blood stability would dictate the amount of time TFV-DP can remain in whole blood prior to spotting onto collection paper. Patient samples were used, due to the uncertainty of TFV-DP state when it is spiked directly into whole blood (extracellular fluid). The phlebotomist collected the whole blood in K3EDTA tubes, which were kept at room temperature until they were spotted onto Whatman Protein Saver 903 Cards at the respective timepoints, namely 0 hours, 6 hours, 24 hours, and 48 hours. The time zero samples were spotted at the clinical site as soon as the whole blood collection was completed. The whole blood was kept at room temperature and subsequently spotted onto the Whatman Protein Saver 903 Cards at each of the following time points: 6 hours, 24 hours, and 48 hours. The whole blood of two patients, with varying adherence levels, were used. The one patient adhered well to the regimen, whereas the other adhered poorly. This resulted in the one patient having high TFV-DP levels, while the other had low TFV-DP levels. Six replicates of each patient were analysed, and a summary of the results are shown in Table 5.21.

Table 5.21: Summary of whole blood stability result over a period of ~48 hours

	Low Concentration (fmol/punch)				High concentration (fmol/punch)			
	Donor 1				Donor 2			
	Storage: 0 hours Peak Area	**Storage: 6 hours Peak Area**	**Storage: 24 hours Peak Area**	**Storage: 48 hours Peak Area**	**Storage: 0 hours Peak Area**	**Storage: 6 hours Peak Area**	**Storage: 24 hours Peak Area**	**Storage: 48 hours Peak Area**
Sample 1	799	660	644	544	2690	2140	1980	2390
Sample 2	788	664	620	640	2630	2200	2000	2380
Sample 3	789	674	659	543	2420	2370	2170	2390
Sample 4	861	661	634	510	2490	2380	2100	2400
Sample 5	817	667	671	593	2810	2410	2230	2310
Sample 6	883	726	626	538	2590	2280	2110	2370
Average	823	675	642	561	2605	2297	2098	2373
STDEV	40.1	25.3	19.7	46.9	140	109	96.2	32.7
CV(%)	4.9	3.8	3.1	8.4	5.4	4.7	4.6	1.4
%Difference		-17.9	-21.9	-31.8		-11.8	-19.4	-8.9

**The percentage difference for each time point is calculated using T0 (0 hours) and the respective Time point.*

Acceptance criteria: The acceptance criteria for the whole blood stability assay requires the %Difference remain below 15%, otherwise it is an indication of whole blood instability.

Discussion: The %Difference for all the low concentration time points failed to comply with the requirements, whereas only one of the high concentration timepoints failed, as can be seen in Table 5.21. Therefore, TFV-DP is not stable in whole blood and must be spotted onto the Whatman Protein Saver 903 Cards immediately after the blood has been collected.

5.10. Specificity

The very high specificity of the LC-MS/MS assay procedure prevents the detection of any compounds that do not possess the capability to produce the specific parent ion followed by formation of the specific product ion produced and monitored in the mass spectrometer. A representative chromatogram of the first STD (6400 fmol/punch) is shown in Figure 5.4.

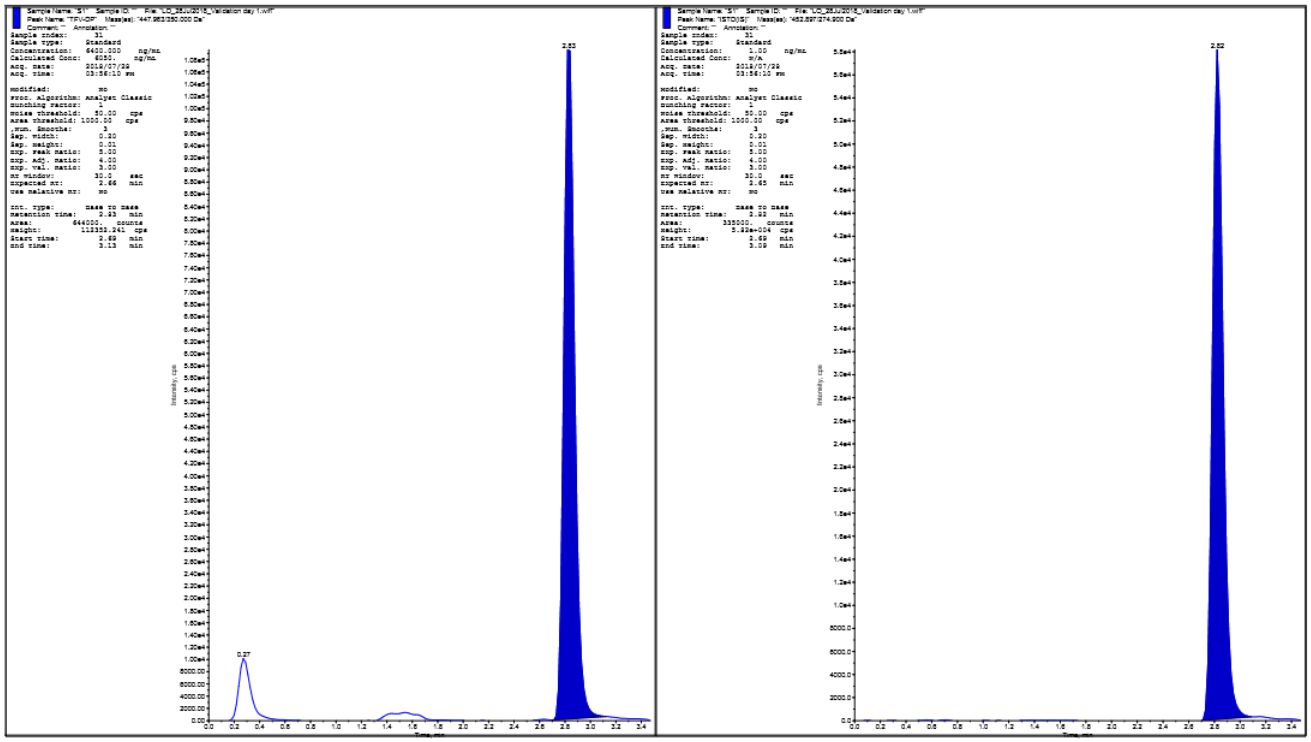


Figure 5.4: Representative chromatogram of STD 1: TFV-DP and ISTD.

5.11. Carry-Over

A double blank sample (without analyte and ISTD) was positioned in the injection sequence immediately after the highest STD to assess possible carry-over effects. A chromatogram of a double blank sample is presented in Figure 5.5.

A blank sample (without analyte) was also included to determine the possible contamination of the analyte by the ISTD without an additional carry-over effect. A chromatogram of a blank sample is presented in Figure 5.6.

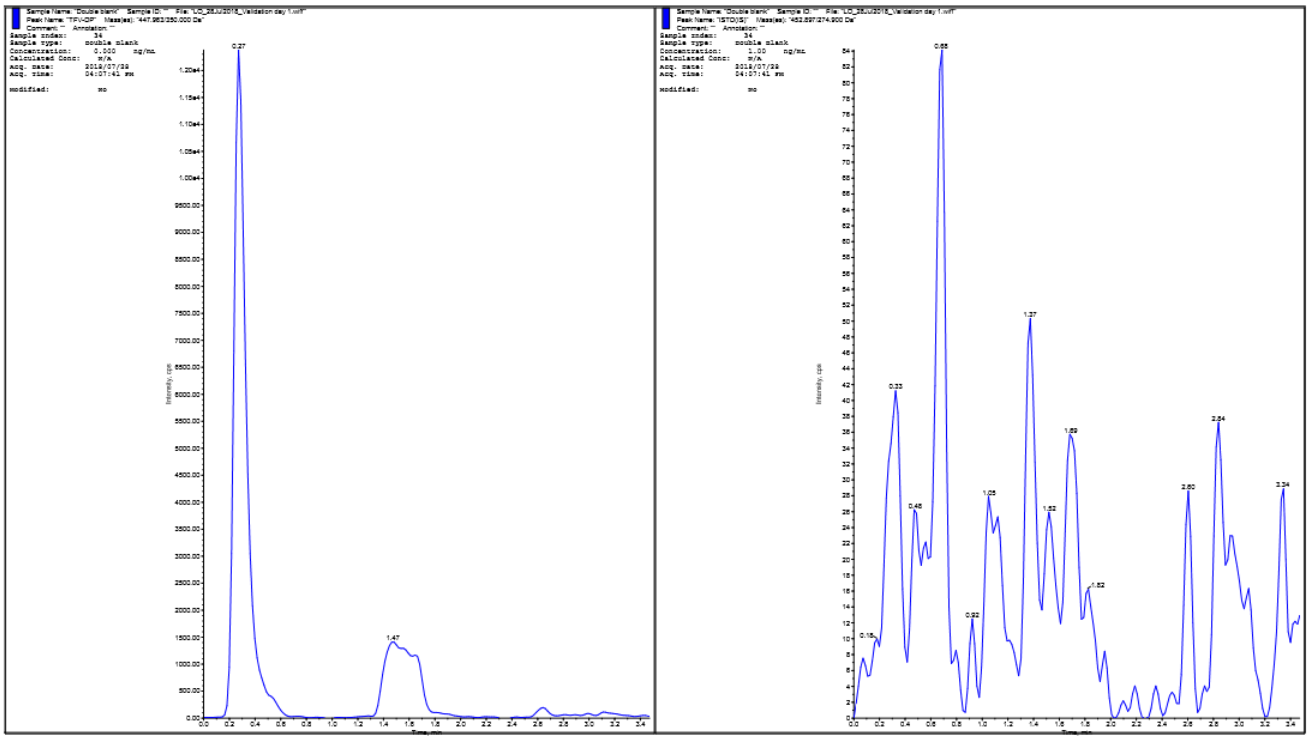


Figure 5.5: Chromatogram of an extracted double blank DBS sample.

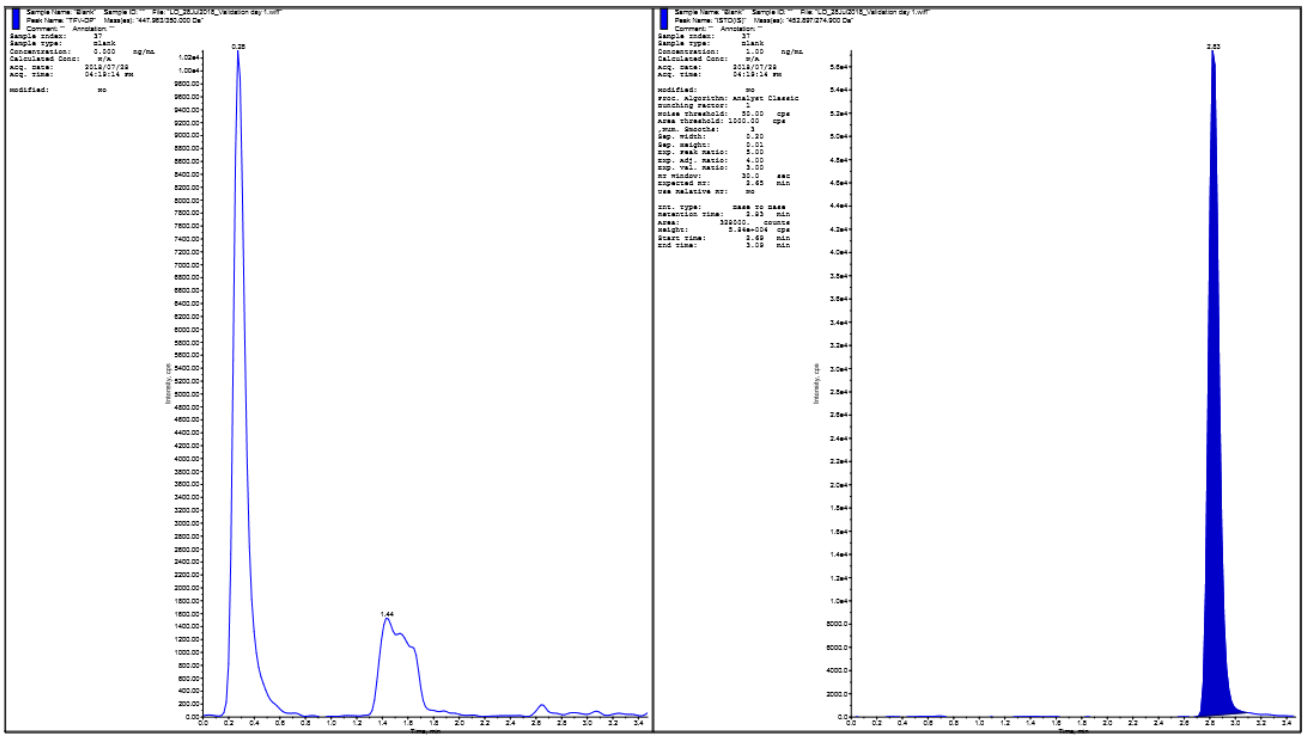


Figure 5.6: Chromatogram of an extracted blank DBS sample.

Acceptance criteria:

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

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

Discussion: No analyte peaks were seen at the retention time and mass transition of TFV-DP in the double blank and blank samples.

5.12. Sensitivity

The LLOQ of this method is 50 fmol/punch, Figure 5.7. Six different lots of matrix were prepared at the LLOQ concentration and extracted, to determine the average signal-to-noise ratio at LLOQ, Figures 5.8–5.13.

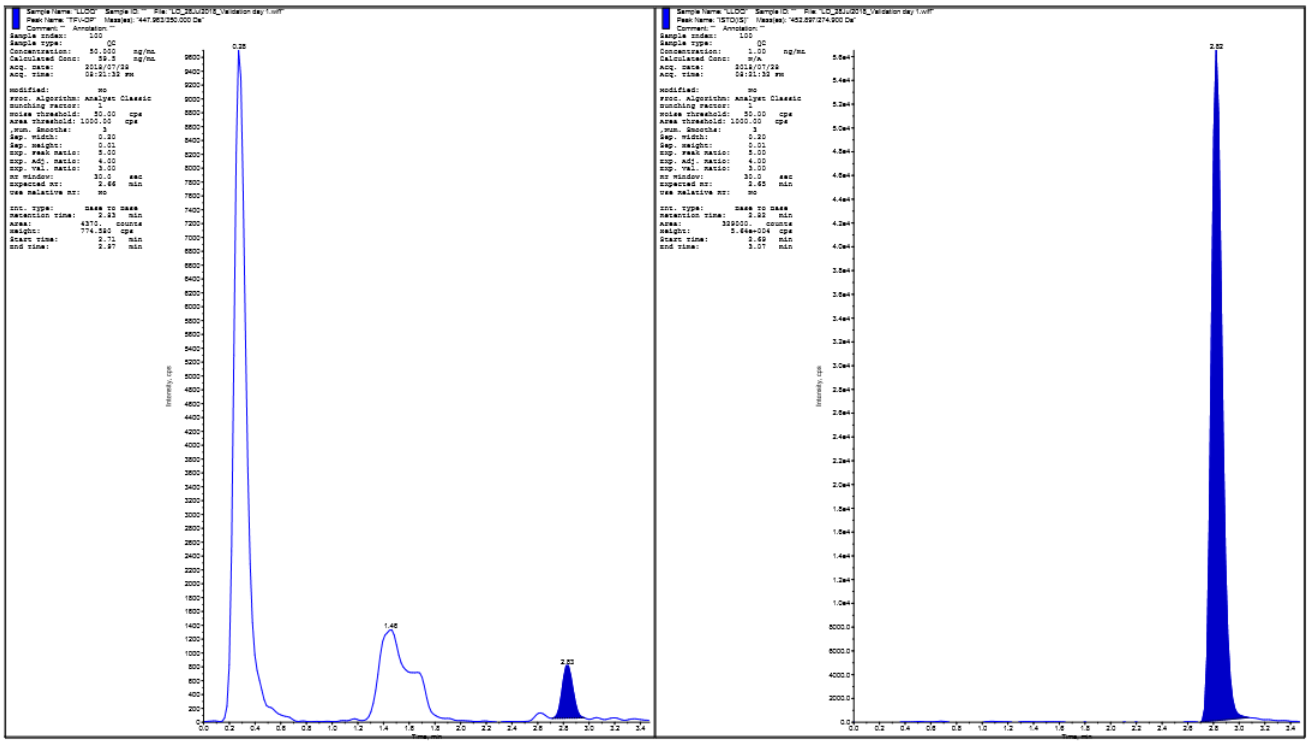


Figure 5.7: Representative chromatogram of a TFV-DP LLOQ sample.

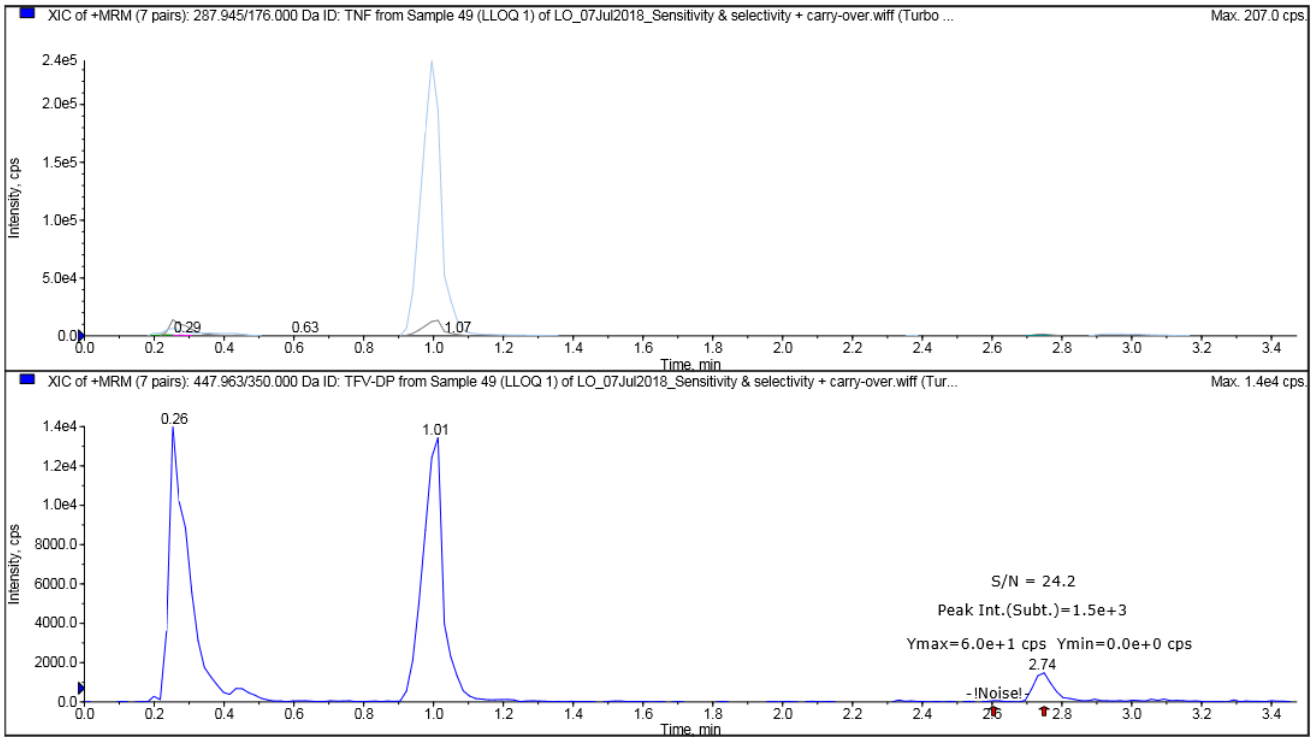


Figure 5.8: Raw chromatogram: The 1st sample spiked at LLOQ.

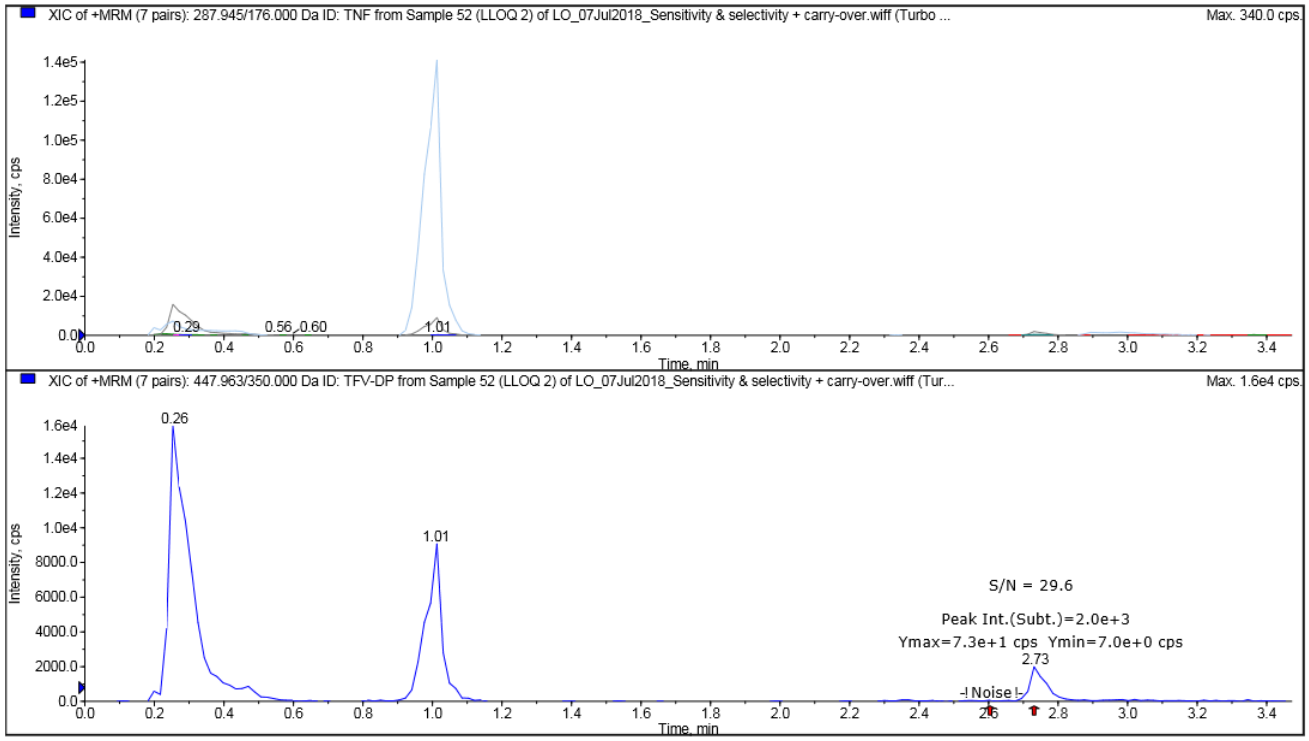


Figure 5.9: Raw chromatogram: The 2nd sample spiked at LLOQ.

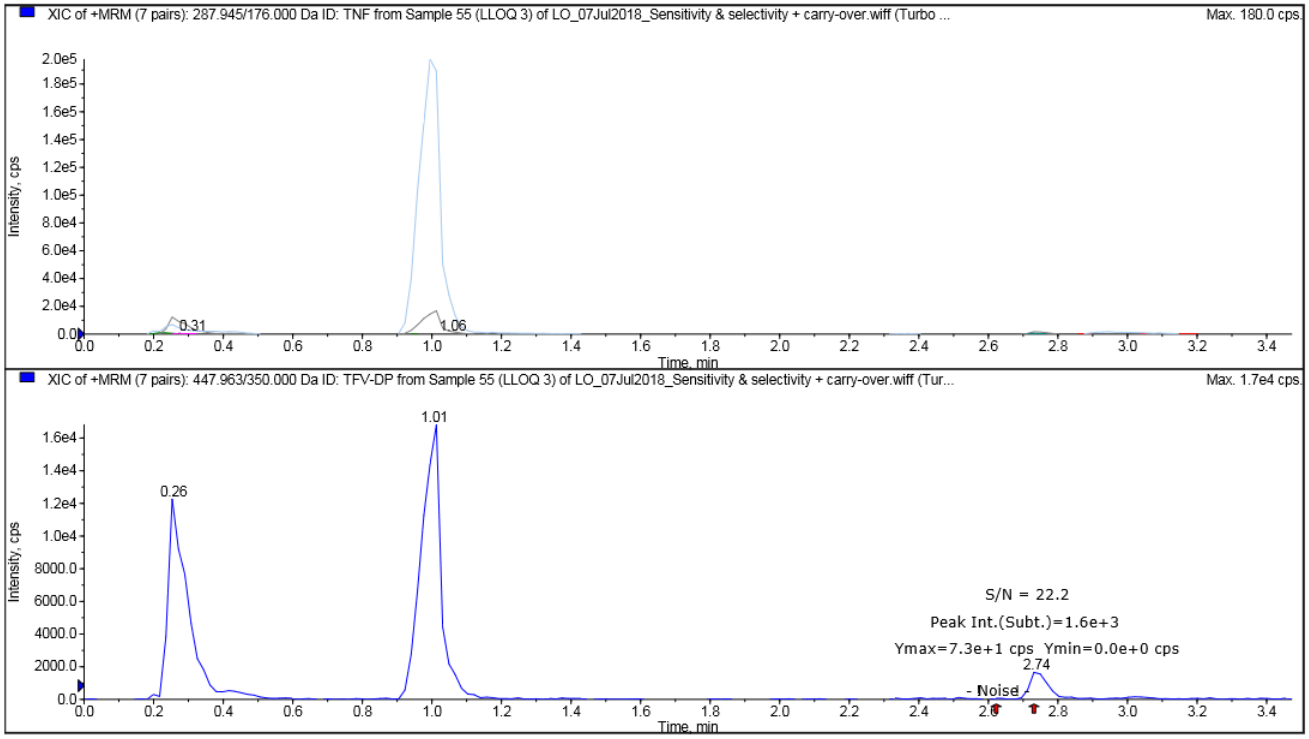


Figure 5.10: Raw chromatogram: The 3rd sample spiked at LLOQ.

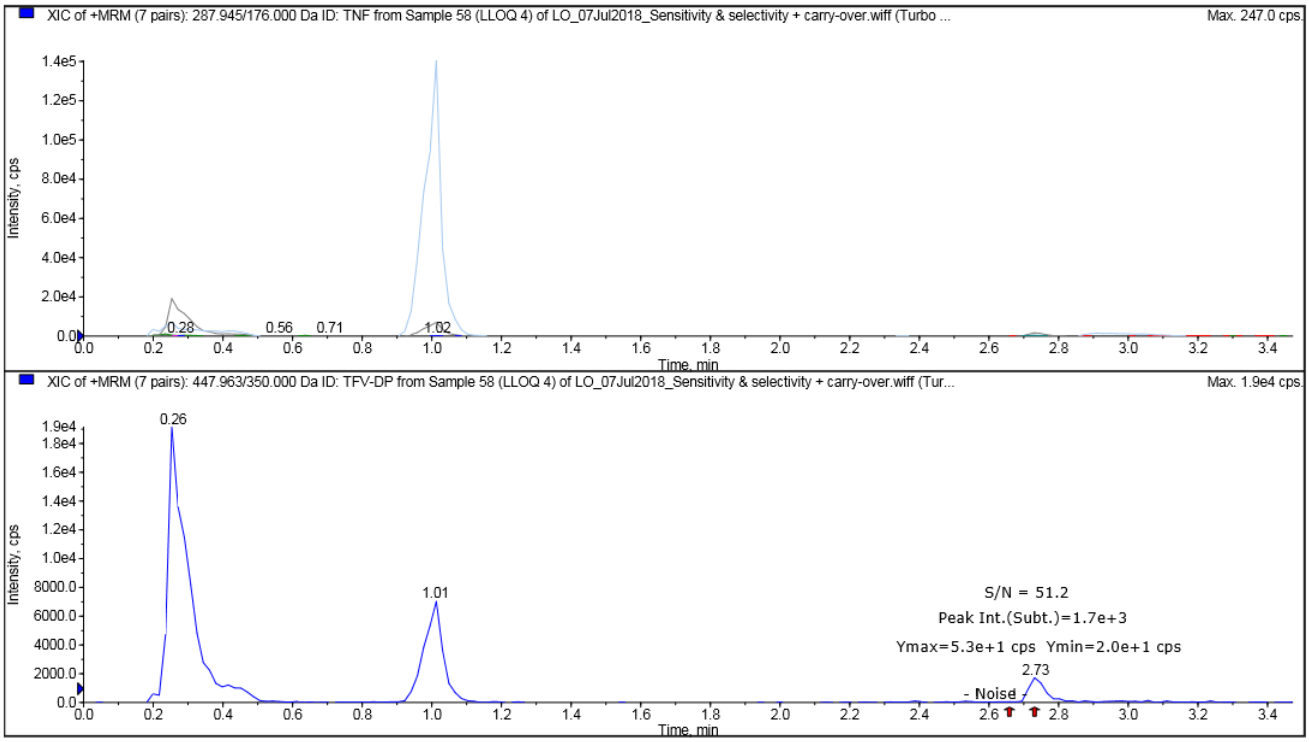


Figure 5.11: Raw chromatogram: The 4th sample spiked at LLOQ.

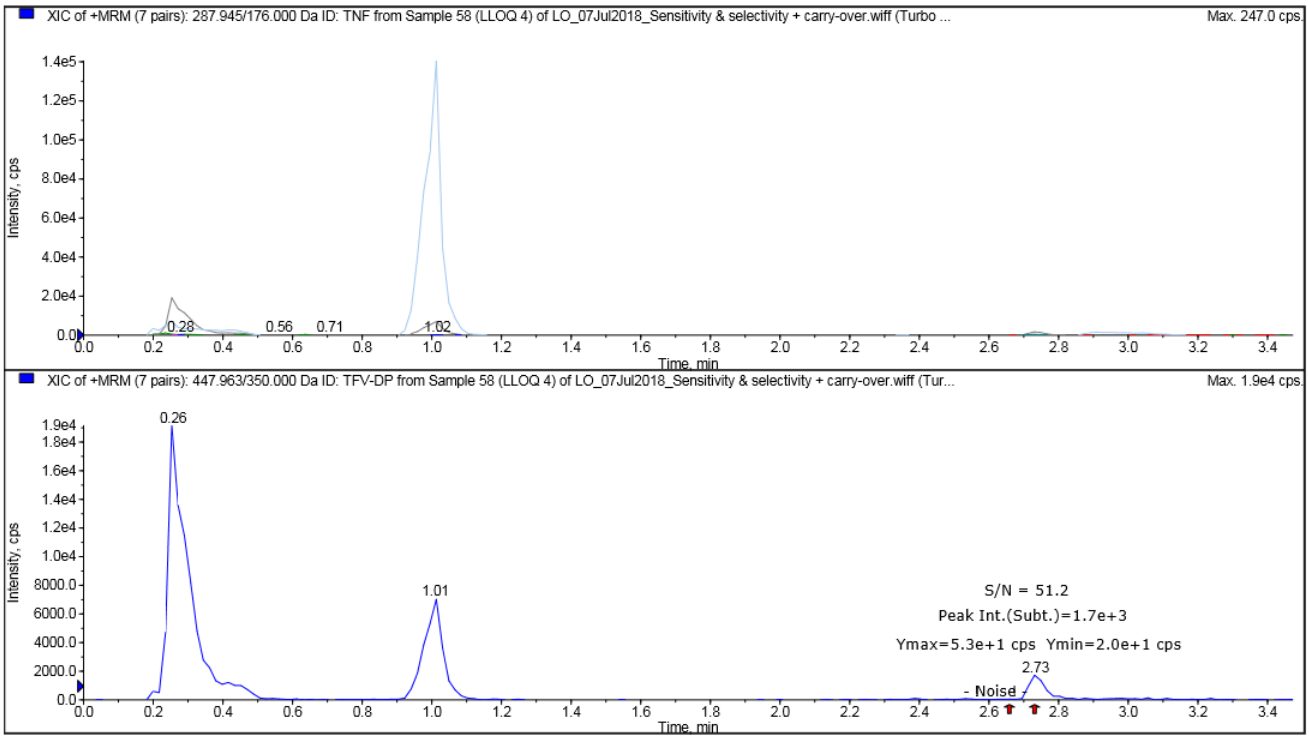


Figure 5.12: Raw chromatogram: The 5th sample spiked at LLOQ.

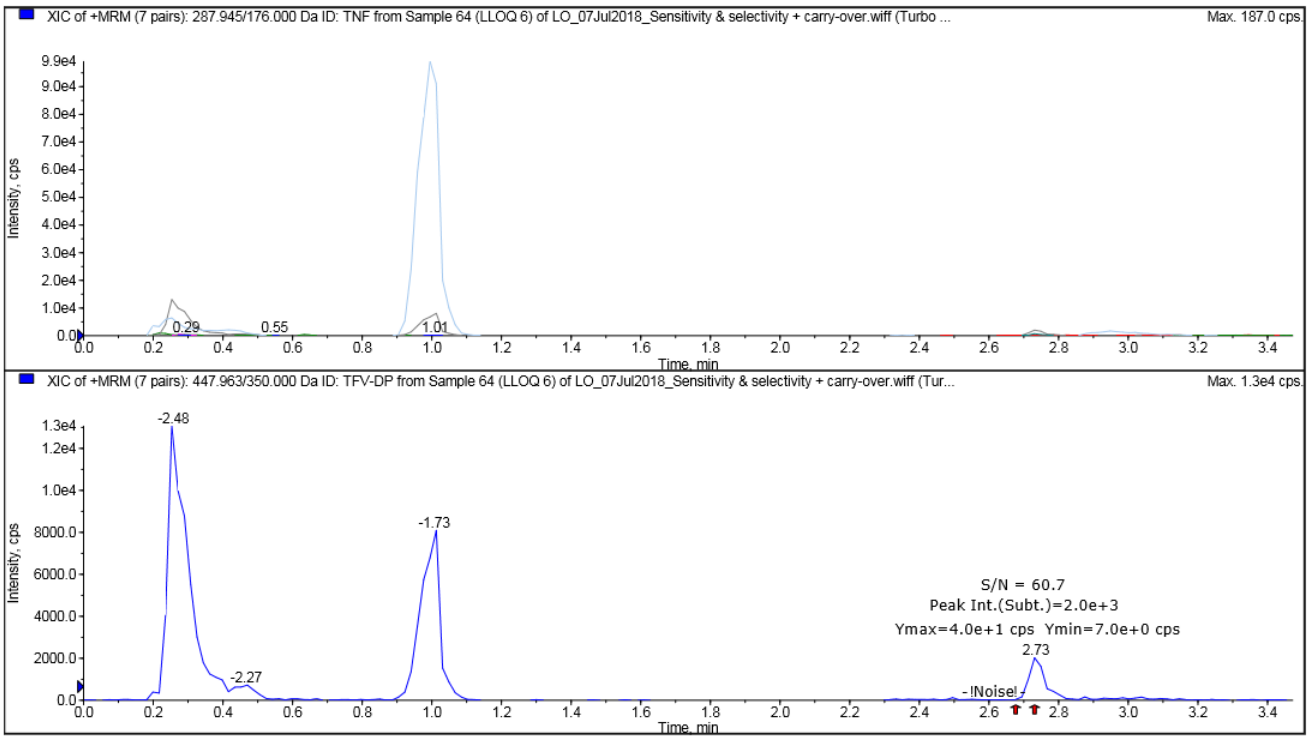


Figure 5.13: Raw chromatogram: The 6th sample spiked at LLOQ.

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

Discussion: The raw LLOQ sample chromatograms presented acceptable intensities for the analyte with an average signal-to-noise ratio of 39.7 (n=6), calculated from six replicates of LLOQ samples prepared from DBS obtained from six different sources.

5.13. Recovery

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

a. Extracted (test) samples: A minimum of 6 QCs at each concentration level in 6 different lots of matrix (low, medium and high) are extracted as per the analytical method describe in Chapter 4.

b. Theoretical samples: Samples are spiked at each concentration level (relative to the final concentration of the corresponding extracted QC level) in six-fold using extracted blank matrix from 6 different lots of matrix.

The analyte peak areas found after extraction are compared to the theoretical peak area expressed as a percentage recovery. A summary of the results is shown in Table 5.22.

Table 5.22: Summary of the recovery results for TFV-DP

	High Concentration (5120 fmol/punch)		Medium Concentration (2560 fmol/punch)		Low Concentration (150 fmol/punch)	
	Pre-extraction spiked: Peak Area	**Post-extraction spiked: Peak Area**	**Pre-extraction spiked: Peak Area**	**Post-extraction spiked: Peak Area**	**Pre-extraction spiked: Peak Area**	**Post extraction spiked: Peak Area**
Sample 1	810000	1110000	349000	520000	19900	27900
Sample 2	793000	1090000	348000	489000	19300	27500
Sample 3	841000	985000	363000	513000	19100	27300
Sample 4	920000	1380000	429000	624000	24500	36100
Sample 5	779000	1080000	372000	519000	19700	29700
Sample 6	960000	1340000	465000	610000	23600	32700
Average	850500	1164167	387667	545833	21017	30200
STDEV	73443	158190	48190	56432	2384	3530
CV(%)	8.6	13.6	12.4	10.3	11.3	11.7
%Recovery		73.1		71.0		69.6
				Average %Recovery		71.2
				Average CV(%)		2.4

Acceptance Criteria: The mean recovery of a bioanalytical method should be consistent and the precision of the measured recovery expressed as CV(%) should not exceed 15% for any particular concentration of the analyte at which it is determined. Recovery reproducibility between concentration levels should not be > 15%.

Discussion: The mean recovery of TFV-DP from six different lots of DBS over the calibration range is 71.2% with a CV(%) of 2.4% and is within acceptable limits.

5.14. Matrix Effects

In biological analyses, matrix refers to the endogenous (or non-endogenous) components of a sample other than the analyte being studied. Matrix effects are of particular importance in LC-MS/MS analyses, and may only become evident once unknown clinical samples are analysed. The presence of background matrix components may have an effect on analyte and ISTD ionization. Having an appropriate ISTD which adequately follows the analyte, compensates for matrix effects. Appropriate steps should be taken to minimize the influence of matrix components. The Matuszewski method attempts to quantify the effect across the calibration range of the assay using different matrix sources [99,100,115].

A minimum of 6 different blank sources of the appropriate biological matrix were extracted (without ISTD). Each individual matrix sample was spiked at low, medium and high concentration levels (taking into account any calculations for dilutions in the analytical method), and at one concentration of the ISTD. The results are presented in Table 5.23 and the overall CV(%)'s of the regression slopes calculated is shown in Table 5.24.

Table 5.23: TFV-DP and ISTD peak areas

	High Concentration		Medium Concentration		Low Concentration	
	Analyte Peak Area	**ISTD Peak Area**	**Analyte Peak Area**	**ISTD Peak Area**	**Analyte Peak Area**	**ISTD Peak Area**
Sample 1	1110000	707000	520000	724000	27900	711000
Sample 2	1090000	711000	489000	666000	27500	619000
Sample 3	985000	649000	513000	669000	27300	713000
Sample 4	1380000	914000	624000	831000	36100	905000
Sample 5	1080000	712000	519000	692000	29700	735000
Sample 6	1340000	852000	610000	849000	32700	873000

Table 5.24: Regression results from different matrices

	High concentration (5120 fmol/punch) Peak Area Ratio	Medium concentration (2560 fmol/punch) Peak Area Ratio	Low Concentration (150 fmol/punch) Peak Area Ratio	Area Ratio v Conc. Regression Slope
Sample 1	1.57	0.72	0.0392	0.000308
Sample 2	1.53	0.73	0.0444	0.000300
Sample 3	1.52	0.77	0.0383	0.000298
Sample 4	1.51	0.75	0.0399	0.000296
Sample 5	1.52	0.75	0.0404	0.000297
Sample 6	1.57	0.72	0.0375	0.000309
Average	1.54	0.74	0.0400	0.000301
STDEV	0.0279	0.0195	0.00244	0.00000591
CV(%)	1.8	2.6	6.1	2.0

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

Discussion: The slope variability (CV(%)) for six different K3EDTA DBS samples is 2.0% for TFV-DP, which indicates that matrix effects do not adversely influence the precision of the assay.

5.15. Process Efficiency

Process efficiency is a comparison between the instrument response from ‘extracted samples’ and the instrument response obtained from ‘unextracted neat samples’ (analyte spiked into injection solvent without any matrix). Process efficiency assesses the combined effect of both extraction recovery and matrix on analyte quantitation.

The pre-extraction spiked samples are prepared in six different lots of matrix at the low, medium and high QC levels and extracted as per the method described in Chapter 4. The ISTD is spiked at the working concentration of the method.

The neat, un-extracted samples are prepared in triplicate in the injection solution (water) at low, medium, and high QC levels (considering any calculations for dilutions in the analytical method). The ISTD is spiked into the samples at the working concentration of the method. A volume of 150 µl of each sample is subsequently added to a 96-well plate.

The analyte peak areas observed after extraction are compared to the peak areas of the neat samples and expressed as percentage process efficiency. The results of the process efficiency assessment are summarised in Tables 5.25.

Table 5.25: Process efficiency for the extraction of TFV-DP from DBS

	High Concentration (5120 fmol/punch)		**Medium Concentration (2560 fmol/punch)**		**Low Concentration (150 fmol/punch)**	
	Extracted solution: Peak Area	**Neat solution: Peak Area**	**Extracted solution: Peak Area**	**Neat solution: Peak Area**	**Extracted solution: Peak Area**	**Neat solution: Peak Area**
Sample 1	810000	1480000	349000	771000	19900	42500
Sample 2	793000	1530000	348000	830000	19300	42300
Sample 3	841000	1630000	363000	824000	19100	43800
Sample 4	920000		429000		24500	
Sample 5	779000		372000		19700	
Sample 6	960000		465000		23600	
Average	850500	1546667	387667	808333	21017	42867
STDEV	73443	76376	48190	32470	2384	814
CV(%)	8.6	4.9	12.4	4.0	11.3	1.9
%Recovery		55.0		48.0		49.0
				Average %Process efficiency		50.7
				Average CV(%)		7.5

Acceptance criteria: Process efficiency represents the combined effect of matrix presence and extraction efficiency on the analyte. The mean process efficiency of a quantitative drug assay method should be consistent. The precision of the measured process efficiency, expressed as CV(%), should not exceed 15% for any particular concentration of the analyte at which it is determined. Process efficiency reproducibility between concentration levels should not be > 15%.

Discussion: The mean process efficiency of TFV-DP from six different lots of DBS over the calibration range is 50.7% with a CV(%) of 7.5% and is within acceptable limits.

5.16. Dilutions

To determine if samples originally reported as above the upper limit of quantitation (ALQ) of the standard curve may be diluted to within the calibration range with accuracy and precision, six extra high QC Dilute samples were prepared at a concentration more than twice the ULOQ (11000 fmol/punch) for TFV-DP. These were subsequently diluted 1:4 with blank extracted samples. The concentration was determined and compared with the nominal concentration to determine the percentage accuracy. The results are shown in Table 5.26.

Table 5.26: Summary of the dilution sample results for TFV-DP

	1:4 Dilution	
	Nominal concentration (fmol/punch)	**Calculated concentration (fmol/punch)**
Sample 1	2200	2570
Sample 2		2300
Sample 3		2410
Sample 4		2310
Sample 5		2270
Sample 6		2440
Average	2200	2383
STDEV		113
CV(%)		4.7
%Accuracy		108.3

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

Discussion: The resulted precision and accuracy fall within the accepted limits and, therefore, concentration reported above the upper limit of the validated calibration curve may be diluted 1:4 and repeated.

5.17. Anticoagulant Matrix Effects

K3EDTA was selected as the analytical methods anticoagulant of choice; however, there is a possibility that samples may be collected at the clinical site using other anticoagulant tubes. Therefore, the matrix anticoagulant effects of K2EDTA also had to be determined and compared to those of the K3EDTA samples. This would thus determine if samples that have been collected using an alternative anticoagulant could be analysed.

QC high, medium, and low samples were prepared to determine the effect of an alternative anticoagulant on the analytical method. Six replicates from different blank matrix sources were analysed at each concentration level. The samples were subsequently extracted and quantified and the result are shown in Table 5.27.

Table 5.27: Summary of the effect of K2EDTA on the assay

	High Concentration		Medium Concentration		Low Concentration	
	K3EDTA Peak Area	K2EDTA Peak Area	K3EDTA Peak Area	K2EDTA Peak Area	K3EDTA Peak Area	K2EDTA Peak Area
Sample 1	4890	5000	2460	2440	147	160
Sample 2	4900	5180	2420	2440	161	148
Sample 3	4970	4940	2470	2440	152	166
Sample 4	5110	4860	2530	2270	155	152
Sample 5	4890	5130	2450	2470	153	156
Sample 6	4940	5100	2450	2500	149	155
Average	4950	5035	2463	2426	152	156
STDEV	84.6	123	36.7	80.5	4.92	6.27
CV(%)	1.7	2.4	1.5	3.3	3.2	4.0
%Difference		1.7		-1.5		2.2

Acceptance criteria: The accuracy of the extracted K2EDTA samples is required to be less than 15% (i.e. %Accuracy should be between 85–115%) and the precision is required to be less than 15% (i.e. CV(%) should be less than 15%).

Discussion: The resulted precision and accuracy fall within the accepted limits. Therefore, the assay can be used to analyse samples that have mistakenly been collected with K2EDTA as anticoagulant.

5.18. Direct vs Indirect Method

To determine the correlation between the previous indirect and the newly developed direct method, a set of 30 patient samples were analysed using both methods. The samples used formed part of the Pluspills study. Approval to use the DBS samples was obtained from the Human Research Ethics Committee of the Faculty of Health Sciences, University of Cape Town (Reference number: 260/2014).

While working with the patient samples, standard good laboratory practises (GLP) were followed as outlined in the laboratory's standard operating procedures (SOP). This requires lab personnel to wear adequate personal protection when generating or working with waste. The required personal protection

includes a laboratory coat, disposable latex gloves and eye protection. All waste generated during the analytical process was discarded in the appropriate demarcated receptacles. Medical waste was discarded in a clearly demarcated red bin, which contained medical waste without any sharps or glassware. The content of the red bins are incinerated after collection. Sharps and glassware were discarded in separate yellow bins (sharps bin) in order to prevent the puncture of the red bin bags, which may lead to medical waste leakage. Therefore, adequate measures were taken to ensure that medical waste was handled and discarded safely.

The same samples were analysed with the two different methods. The Pearson's correlation coefficient was calculated as an indication of the degree of correlation between the current indirect method and the developed direct method. A regression analysis was also completed as an indication of the correlation of the two methods. The formula for the Pearson's correlation coefficient is shown below:

$$r = \frac{n(\sum xy) - (\sum x)(\sum y)}{\sqrt{[n\sum x^2 - (\sum x)^2]\left[n\sum y^2 - (\sum y)^2\right]}}$$

The same samples were analysed using both methods and the results of the two methods are summarised in Table 5.29. A scatterplot presenting the data is shown in Figure 5.14, which gives a visual representation of the data.

Table 5.28: Summary of the results obtained from the two analytical methods, namely UCT direct and the UCT indirect

	UCT direct method (fmol/punch)	Indirect method (fmol/punch)
TPC0029	2520	1743
TPC0030	2460	1389
TPC0032	899	714
TPC0034	2760	2018
TPC0036	1560	1155
TPC0038	2270	1608
TPC0040	1360	1173
TPC0042	501	380
TPC0044	1930	946
TPC0046	1400	1006
TPC0048	365	357
TPC0050	1900	1003
TPC0052	1970	1192
TPC0054	3370	1813
TPC0056	1290	801
TPC0058	1900	1305
TPC0060	2400	1168
TPC0062	889	553
TPC0064	1160	755
TPC0066	2100	1073
TPC0068	977	632
TPC0070	1960	1152
TPC0072	874	833
TPC0074	2730	1503
TPC0075	2120	1170
TPC0076	3820	2523
TPC0077	2320	1374
TPC0078	2160	1411
TPC0079	1320	833
TPC0080	2050	1477
Average	1845	1169
STDEV	8045	480
CV(%)	43.6	41.0
%Difference		-36.6

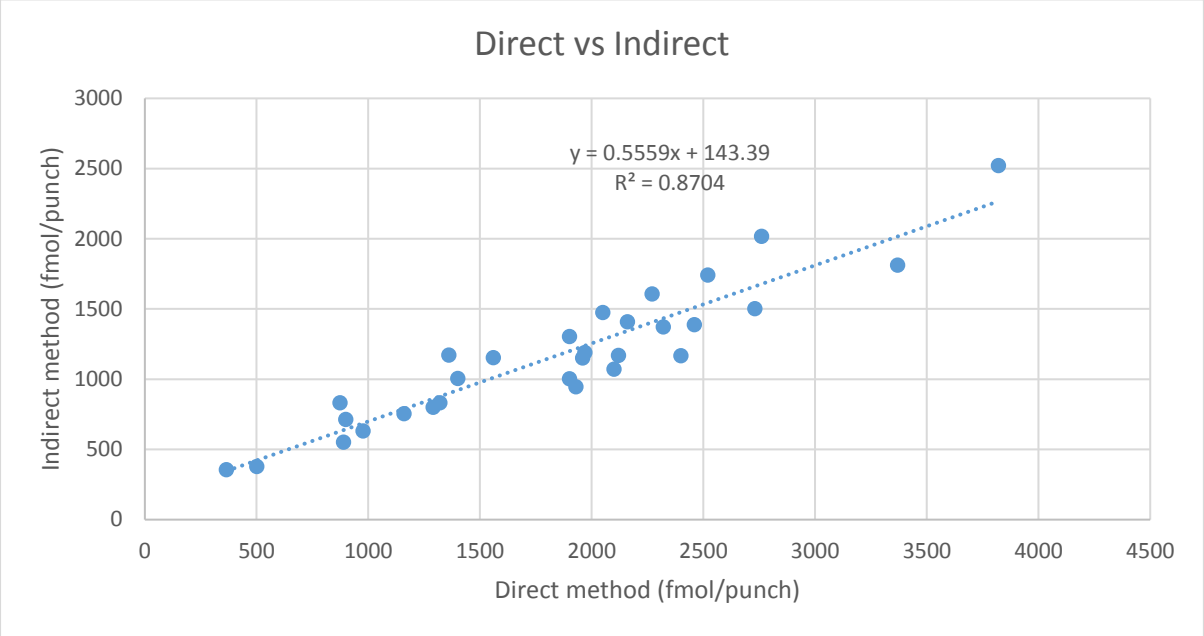


Figure 5.14: A scatterplot with a trendline of the relationship of the results obtained using the developed direct method and the indirect method used by UCT. The r^2 value is 0.8704.

As can be seen in Figure 5.14, a linear relationship can be observed between the results of the direct and indirect methods, and this is backed up by the coefficient of determination (r^2) that approaches one. The Pearson's correlation coefficient was 0.933, which indicates a strong positive correlation between the data obtained by the developed direct and indirect methods. The correlation was statistically significant ($P \leq 0.0001$). A regression analysis was also completed, and the r^2 value was 0.870, which also implies a strong correlation.

On average, the results obtained using the direct method were 36.6 % higher than those obtained using the indirect method. This may be as a result of the indirect method that takes the reference standard purity indirectly into account, whereas the direct method assumes the purity of the reference standard is exactly as stated on the certificate of analysis. However, the correlation from relatively low to high concentrations is strong and therefore a correction factor could be used to adjust the data for adherence studies.

Chapter 6: Conclusion

According to the data generated in the current study it was evident that the developed method met the FDA and EMA requirements for a bioanalytical assay. The aim of the study was to develop a method that could be used as an alternative to the existing TFV-DP indirect method. The existing indirect method used a technique which separates the parent drug from the metabolite and then back-converted all metabolites to the parent drug before analysing the samples on LC-MS/MS. The developed direct method avoided these laborious sample preparation steps, which ultimately reduced the time taken for each assay and the associated cost of the consumables. Therefore, the development and validation of the bioanalytical method made it possible for researchers, with limited funding, to accurately determine the TFV-DP levels of patients. The validation experiments were meant to, not only determine the suitability of the method for its intended use, but also to ensure the reproducibility, robustness, and reliability of the method. Therefore, the method could be an adequate alternative to the indirect method currently used. However, when compared to the current method the results of the developed method were higher than those obtained using the current indirect method. Therefore, therefore a correction factor could be used to adjust the data for adherence studies.

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