Department of Chemistry

FACULTY OF SCIENCE

DEVELOPMENT OF A MULTIPLE REACTION MONITORING LIQUID CHROMATOGRAPHY TANDEM MASS SPECTROMETRY METHOD FOR THE SIMULTANEOUS DETERMINATION OF FIVE ANTIRETROVIRAL DRUGS IN HUMAN BLOOD PLASMA - A CONTRAST OF EXTRACTION EFFICIENCIES USING SELECTED SAMPLE PREPARATION TECHNIQUES

BY

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A DISSERTATION SUBMITED IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF MASTER OF SCIENCE IN ANALYTICAL CHEMISTRY

April 2016

DECLARATION

I hereby declare that the dissertation submitted for the degree of Master of Science in Chemistry to the University of Botswana, Gaborone is my original work and has not been submitted to any other institution and any work quoted is indicated and acknowledged by means of a comprehensive list of references.

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ELLIOTT MWANDO JR April 2016

DEDICATIONS

TO

The loving memory my late mother Getrude Eniat Banda-Mwando who instilled in me the principles of hardwork and commitment

ACKNOWLEDGEMENTS

I would particularly like to thank my supervisor Prof Kwenga F. Sichilongo (University of Botswana) for his guidance and advice in making this project a realization. I acknowledge and thank all those colleagues who unstintingly gave their time and expertise to a careful reading of the chapters of this report. I would also like to thank my research advisory committee, Prof Olayinka A.Oyetunji and Dr V.Obuseng for the assistance they provided at all stages of the intricate and time consuming task of the research and producing the report.

Finally I would like to thank the University of Botswana Foundation for sponsoring my final year study.

ABSTRACT

A multiple reaction monitoring liquid chromatography - tandem mass spectrometry (MRM LC-MS/MS) method for the simultaneous determination of antiretroviral drugs (Emtricitabine, Tenofovir, Efavirenz, Lopinavir and Retonavir) in sterilized human blood plasma was developed for therapeutic drug monitoring (TDM) purposes and validated using United States food and drug administration (US FDA) guidelines. A comparison between seven sample extraction techniques was made. These are QuEChERS (a portmanteau for Quick, Easy, Cheap, Efficient and Rugged), solid phase extraction (SPE), liquid-liquid extraction (LLE), protein precipitation (PPT) and mixed modes i.e. QuEChERS- PPT (Q-PPT), QuEChERS-LLE (Q-LLE) and LLE-PPT. Data acquisition was done in MS fullscan and MRM and the two compared. Chromatographic and mass spectrometric parameters were optimised using peak area's as variables.

The analytical performance characteristics that were investigated for method validation were instrument detection limits (IDLs), method detection limits (MDLs), % mean recoveries, precision (% Relative standard deviation) and accuracy. Optimised LC-MS/MS parameters were employed for this purpose. The percent mean recoveries were between 68.8 – 85.6 % for single modes and 52.4 – 70.5 % for mixed mode techniques. Precision of all the techniques investigated was within acceptable range of < 15 % at all concentration levels for all analytes. Accuracy was calculated as a percentage of deviation of the mean value to the true value and the values were between 5.87 – 65.94 % for single mode techniques and between 21.73 – 51.59 % for mixed mode techniques. SPE proved to be more superior than the other techniques as it recorded the highest percentage recoveries and it satisfied all US FDA guidelines. MDLs for the other six sample preparation techniques fell below the

clinically relevant therapeutic range (3 – 8 ppm) therefore all techniques can be employed for routine TDM studies.

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List of Symbols and abbreviations

AC	Alternating current
AIDS	Acquired immunodeficiency syndrome
Amu	atomic mass unit
AOAC	Association of analytical chemists
ARVs	Anti-retroviral drugs
CD-4	Cluster of differentiation 4
CI	Chemical Ionization
CID	Collision induced dissociation
DAD	Diode array detector
DC	Direct current
DNA	Deoxyribonucleic acid
EFV	Efavirenz
EMT	Emtricitabine
ESI	Electrospray ionization
HAART	Highly Active Antiretroviral Therapy
HIV	Human immunodeficiency virus
HPLC-MS	High Performance Liquid Chromatography- Mass Spectrometry
IDL	Instrument Detection Limit
IS	Internal Standard
Kda	kilo Daltons
LC-MS	Liquid chromatography-Mass spectrometry

LLE	Liquid-liquid extraction
LLE-PPT	Liquid liquid extraction – protein precipitation
µL	microliter
LLOQ	Lower limit of quantification
LOD	Limit of detection
LOQ	Limit of quantification
LPV	Lopinavir
m/z	mass to charge ratio
MDL	Method detection limit
MS	Mass spectrometry
MS/MS	Mass spectrometry/Mass spectrometry
NIAID	National institute of allergy and infectious diseases
NNRTIs	Non-nucleoside Reverse Transcriptase inhibitors
NRTIs	Nucleoside Reverse Transcriptase inhibitors
ppb	parts per billion
ppm	parts per million
PPT	Protein precipitation
PSA	Primary and secondary amine
Q	QuEChERS
QIT	Quadrupole Ion Trap
Q-LLE	QuEChERS - Liquid liquid extraction
Q-PPT	QuEChERS - Protein precipitation
RF	Radio-Frequency

RNA	Ribonucleic acid
RSP	Reserpine
RTV	Ritonavir
rpm	revolutions per minute
SIV	Simian immunodeficiency virus
SPE	Solid phase extraction
TDF	Tenofovir
TDM	Therapeutic Drug Monitoring
ULOQ	Upper limit of quantification
US FDA	United States Food and drug administration
WHO	World health Organisation
ZVD	Zidovudine

CHAPTER 1

1.0 An Overview of HIV/AIDS in Botswana

Botswana's first AIDS case was reported in 1985. Botswana has been hit hard by HIV and AIDS. In 2011 there was an estimated 300,000 adults living with HIV or one quarter of the population aged 15 and over. The country has an estimated adult prevalence rate of 23%, the second highest in the world after Swaziland (HIV & AIDS in Botswana AVERT, 2012).

1.1 How the Virus Works

There are two main sub-types of the virus: HIV-1 and HIV-2, the latter being harder to transmit and slower acting. Both originate in Simian (Monkey) Immunodeficiency Virus (SIV) found in Africa. The source of HIV-1 was the common or robust chimpanzee(*Pan troglodytes*) in Central Africa while HIV-2 was Sooty Mangabey monkeys (*Cercocebus atys*) on West Africa.(WANJOHI, 2014)

When HIV enters the Human body, it attaches to a specific type of immune cells called dendritic cells (DCs). These are antigen-presenting cells (also known as accessory cells) of the mammalian immune system. Scientists believe that the dendritic cells transport the virus from site of infection to the lymph nodes where HIV can infect other immune system cells.

HIV targets the CD4 lymphocyte cells also knowns as T-cells or CD-4 cells. The virus will use these cells to reproduce. This is illustrated in Figure 1.1. The replication process of HIV can be summarised as follows:

1.**Binding and Fusion**: This is when HIV attaches to a specific type of CD-4 receptor and co-receptor on the surface of the CD-4 cell.

2. **Reverse transcription**: An enzyme called *reverse transcriptase* changes the genetic material of the virus, so it can be integrated into the host DNA.

3. **Integration**: The new genetic material of the virus then goes into the nucleus of the CD4 cells. An enzyme called *integrase* is used to integrate the virus into our own genetic material where it may stay inactive for several years.

4. **Transcription**: At this stage, the virus replicates using our enzymes and creates a lot more of its genetic material.

5. **Assembly**: Longer HIV proteins are cut into shorter individual HIV proteins by the help of a special enzyme called *protease.* A new virus is assembled when the virus' genetic material comes together with the shorter individual HIV proteins.

6. **Budding**: This is the last stage of the virus' life cycle. The virus goes out of the host cell enveloped in its membrane. At this stage it contains all necessary structures to bind to a new CD4 cell and the process begins again. These steps are important to know because the medication used to control HIV infections act to interrupt this replication cycle. (nam Aidsmap, 2014).

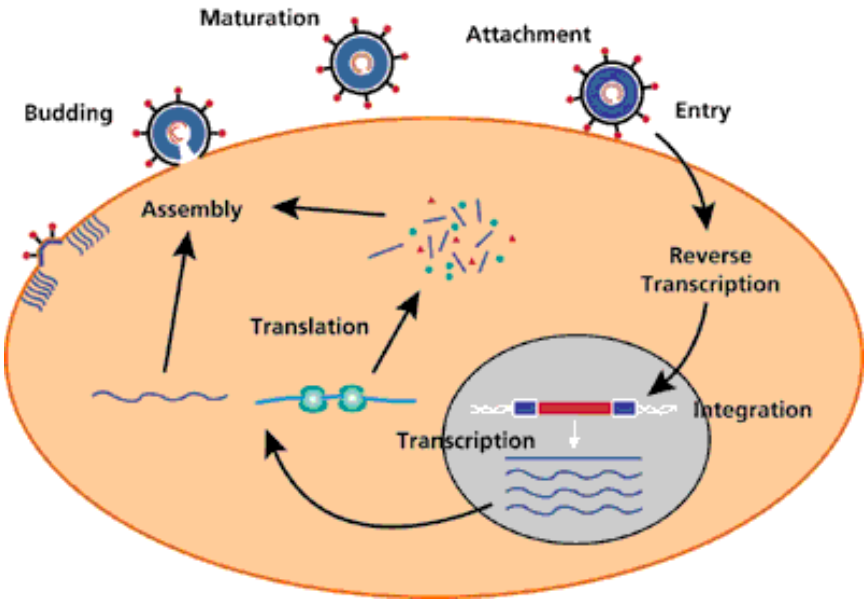

Figure 1.1: Life Cycle of HIV in the cells (nam Aidsmap, 2014)

1.2 Antiretroviral Drugs

Today, there are 31 antiretroviral drugs (ARVs) approved by the Food and Drug Administration to treat HIV infection (Scott & Pharm, 2007). Without treatment, HIV infection slowly destroys the immune system and advances to AIDS. HIV medicines protect the immune system by blocking HIV at different stages of the HIV life cycle. HIV medicines are grouped into different drug classes according to how they fight HIV. Each class of drugs attacks HIV at a different stage of the HIV life cycle (AIDSinfo, 2015).

To prevent strains of HIV from becoming resistant to a type of antiretroviral drug, healthcare providers have recommended that people infected with HIV take a combination of antiretroviral drugs in an approach called highly active antiretroviral therapy (HAART). Developed by NIAID-supported researchers, HAART combines drugs from at least two different classes (Scott & Pharm, 2007). Standard HIV treatment (also called antiretroviral therapy or ART) involves taking a combination of HIV medicines from at least two different HIV drug classes every day. Because HIV medicines in different drug classes block HIV at different stages of the HIV life cycle, ART is highly effective at preventing HIV from multiplying. Having less HIV in the body protects the immune system and prevents HIV from advancing to AIDS. ART also reduces the risk of HIV drug resistance (AIDSinfo, 2015). In settings with poor resources the combination of drugs usually offered as a first line treatment include two from the Non-nucleoside reverse transcriptase inhibitors (NNRTIs) class and one from the Nucleoside reverse transcriptase inhibitor (NRTI) class. There is evidence that first line therapy provides about five years of healthy life before resistance develops. When the resistance develops a second line of treatment must be considered to prolong life. WHO recommends second line therapy include two from NRTIs and a third class of drugs called protease inhibitors (PIs) (Alan Whitesid, 2008).

There are three main antiretroviral drug classes currently used to construct first-line treatment regimens, these include:

1) Non-Nucleoside reverse transcriptase Inhibitors **(NNRTI)**

2) Nucleoside Reverse transcriptase Inhibitors **(NRTI)**

3) Protease Inhibitors **(PI)**

1.2.1 Non-Nucleoside reverse Transcriptase Inhibitors (NNRTI)

When HIV enters the CD4 cells of a human, it programmes the cell to create new copies of it. HIV's genetic material is in the RNA form. For it to infect other CD4 cells, it first has to convert the RNA to DNA with the help of a special enzyme called *reverse transcriptase*.

NNRTIs, also known as "non-nucleosides" attach themselves to *reverse transcriptase* to prevent the enzyme from converting RNA to DNA. In turn, HIV's genetic material cannot be incorporated into the healthy genetic material of the cell, and prevents the cell from producing new virus (De Clercq, 1994). The NNRTI's studied in this group are Emtricitabine (EMT) and Tenoforvir (TFV). The structures of these two are given below.

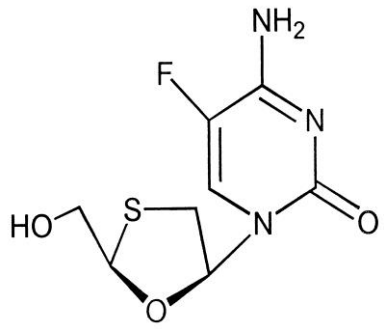

Figure 1.2: The structure of EMT

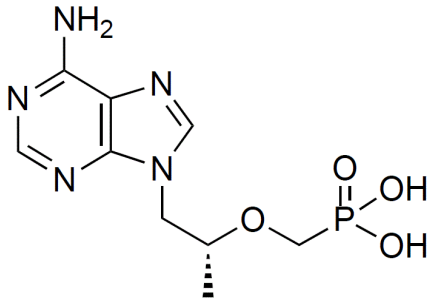

Figure 1.3: The structure of TDF

1.2.2 Nucleoside Reverse Transcriptase Inhibitors (NRTI)

These contain defective versions of nucleotides (building blocks) used by reverse transcriptase to convert RNA to DNA. When reverse transcriptase uses these defective building blocks, the new DNA cannot be built accurately. This means HIV's genetic material cannot be incorporated into the healthy genetic material of the cell and prevents the cell from producing new virus.(De Clercq, 1994). The NRTI used in this study was Efavirenz (EFZ). The structure of EFZ is given below.

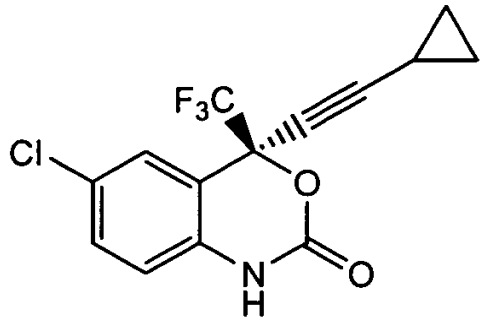

Figure 1.4: The structure of EFZ

1.2.3 Protease Inhibitors (PI)

PI's impede the protease enzyme and inhibit the cell from generating new viruses. They are usually used in combination with at least two other HIV drugs to treat HIV infection. The PI's that were studied in this group were Lopinavir (LPV) and Ritonavir (RTV). The structure of these two are given below.

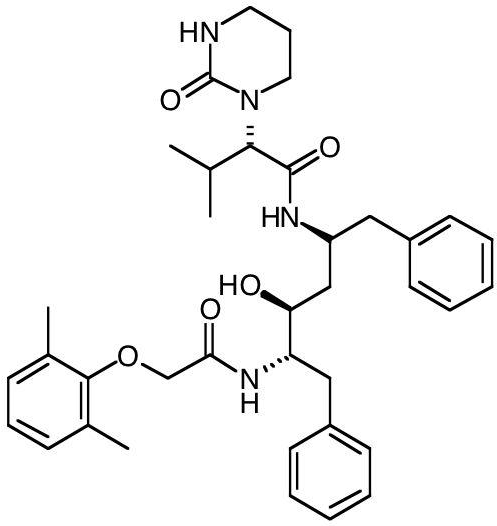

Figure 1.5: The structure of LPV

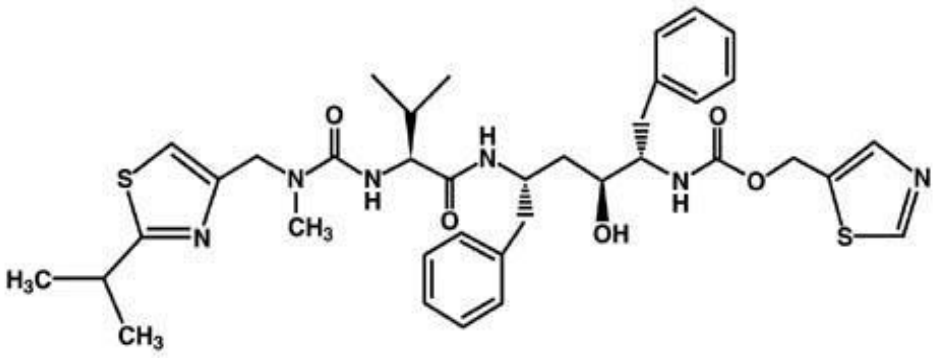

Figure 1.6: The structure of RTV

1.4 Analytical Methods for the Determination of ARVs in this Study

Various analytical methods have been employed in the determination of ARVs. These include HPLC coupled with UV, florescence or MS(/MS) detection, UPLC-MS(/MS), Enzyme immunoassay (EIA) and MALDI based methods. The use of MS detection has been reported in recent publications while UV detection, though widely used is becoming obsolete (DiFrancesco *et.al.*,2013). In this study, HPLC coupled with MS(/MS) detection was employed because of the high sensitivity and accuracy it offers. ARVs are biomolecules and they might degrade if analysed at high temperatures, they also have high molecular weights as observed from their structures above hence HPLC-MS was the method of choice.

1.5 High Performance Liquid Chromatography- (HPLC)

HPLC is the most dominant of all chromatographic techniques. Separation in HPLC is achieved by the relative distribution ratios of the solutes between two phases, the stationary and mobile phase. Molecules in the sample interact different with the stationary phase depending on their polarity leading to their separation. Molecules which interact stronger with the stationary phase will stay longer in the column i.e move more slowly than molecules with weaker interactions. Different compounds are separated in this way as they move through the column. Another important parameter in HPLC is the mobile phase, it has a major effect of the retention of the analytes. A number of factors have to be considered in proper selection of the mobile phase. These include polarity of the mobile phase, the selected mobile phase must have the ability to dissolve the analytes of interest up to a required level. When using MS detection, the mobile phase must be volatile so that it easily vaporises.

The apparatus consists of an HPLC pump, an injector, a column, stationary and mobile phases, connecting tubing and fittings, an HPLC detector and a recorder (computer).

HPLC is very efficient, it yields excellent separations in a short time. The inventors of modern chromatography, Martin and Synge, were aware as far back as 1941 that, in theory, the stationary phase requires very small particles and hence a high pressure is essential for forcing the mobile phase through the column. As a result, HPLC is sometimes referred to as High Pressure Liquid Chromatography (Meyer, 2010). A number of HPLC methods have been developed for the analysis of ARV drugs in biological matrices (Önal, 2006). C 18 columns were most often used to separate the ARVs. The number of ARV drugs to be analysed determine if isocratic or gradient elution is to be used. The growing number of antiretroviral drugs and drug combinations has made developing new HPLC methods very useful. The developed methods should be convenient for the clinical laboratories responsible for the therapeutic drug monitoring of the ARVs (Önal, 2006).

1.6 MASS SPECTROMETRY (MS)

In 1913, J.J. Thompson first used MS to demonstrate that neon gas consisted of a mixture of non-radioactive isotopes. In the 1940s, the first commercial mass spectrometers were developed for petroleum analysis (Robinson, 2005). Over the last two decades (20 years), mass spectrometry has undergone tremendous technological developments allowing for its application to proteins, drugs and many other biologically relevant molecules (Siuzdak, 2004). Due to ionization sources such as electrospray ionization and matrix-assisted laser desorption ionization (MALDI), mass spectrometry has become an irreplaceable tool in the biological sciences (Kang, 2012).

Mass spectrometry is based on the separation of ions or charged particles on the basis of mass to charge ratio (*m/z*). Ions are then moved into the mass analyser where they are separated according to their *m/z* ratios and are then detected. The result of molecular ionization, ion separation, and ion detection is a spectrum that can provide molecular mass and even structural information (Kang, 2012).

1.6.1 Electrospray ionisation

The first electrospray experiments were carried out by Chapman in the late 1930s and the practical development of electrospray ionization for mass spectrometry was accomplished by Malcolm Dole in 1968 (Pramanik *et.al.*,2002). The electrospray ionization (ESI) technique was first reported by Masamichi Yamashita and John Fenn in 1984 (Yamashita, 1984) while not new, it has been rejuvenated with its recent application to biomolecules.

The ESI technique utilizes electrical energy to aid in the transfer of ions from solution into the gaseous phase before they can be analysed in the mass spectrometer. Ionic species in solution are favoured because of better sensitivity when using ESI. Compounds which are neutral can also be studied in ESI-MS by converting them to ionic species in gaseous phase or solution phase. There are three major processes that an analyte undergoes when its transferred from solution to gas phase namely; 1) Dispersal of a fine spray of charged droplets (with analyte) from the high voltage capillary tip. 2) Continual solvent evaporation from the charged droplet. The charged droplet fragments resulting in very small charged droplets which are able to produce the charged analyte. 3) Finally there is ion ejection from the highly charged droplet. The mechanism for the third process is not well understood. A number of models have been proposed which try to explain how an ion is ejected from the highly charged droplet. These models include the Charge residue model (CRM), the ion

evaporation model (IEM) and the Fenn's model of ion formation which is an extension of the IEM.

In the charge residue model (CRM) which was proposed by Dole and Co-workers, an extremely small charged droplet (Appox. 1 nm) which contains only one analyte is formed by continual solvent evaporation and columbic fission. Desolvation of this charged droplet causes its charges which are on the surface to land on the analyte molecule. The residual droplet charge is retained by the analyte molecule in the gas phase hence this model is called the charge residue model.

In the ion evaporation model (IEM) proposed by Iribarne and Thomson, after continual solvent evaporation and Coulomb fission, there is a decrease in the radii size of the charged droplets. They decrease to a size that results in an electric field that is strong enough to cause direct emission of the solvated ions. When the size of the radii goes below 10 um, ion emission dominates over Rayleigh fission (Banerjee & Mazumdar, 2012; Ho *et al*., 2003)

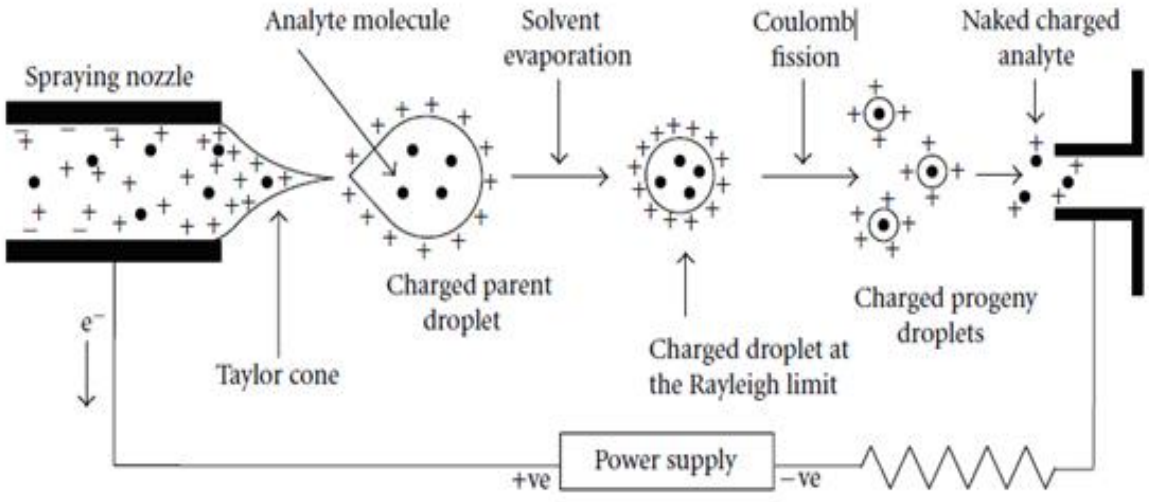

Figure 1.7: Schematic Diagram of the Electro-spray Ionisation (ESI) process (Banerjee & Mazumdar, 2012)

1.6.2 THE QUADROPOLE ION TRAP (QIT) MASS ANALYSER

The quadrupole ion trap (QIT) was first described by W.Paul and E.Fischer (Todd & March, 1999). It's a remarkable device that can operate as an ion store and as a mass spectrometer of considerable mass range. Basically a Quadrupole can be defined as a rectangular array of conducting rods to which variable voltages can be applied.

The QIT consists of two hyperbolic metal electrodes, the top and bottom endcaps and a hyperbolic ring electrode positioned halfway between the other two electrodes. The top endcap has a narrow opening, an aperture which is used to gate ions into the trap while the bottom end cap has small multiple apertures which ions use when leaving the trap to the detector. The ions are trapped in the space between these three electrodes by imposing AC and DC voltages creating electric fields which trap ions with mass/charge ratio of interest. The AC is oscillating while the DC is static. To achieve a perfect electric field in the quadrupole, the poles should ideally have a hyperbolic cross section. In practice most instruments have poles that are circular in cross section (the latter being much less expensive to make, as well as easier to manufacture to an accurate shape).

The conventions are that DC voltage is symbolised as U and AC is symbolised as *V*. In most cases, U = 0 V and the AC i.e radiofrequency (RF) voltage is applied to the ring electrode thus allowing ions to be trapped. Ions can be created within the quadrupole field or externally. Ion ejection is achieved by increasing the amplitude of the RF potential. This is called Mass Selective Instability Scan. Increasing the RF potential makes the trajectories of the ions unstable as ion axial excursions exceed the dimensions of the ion trap. These ions escape the trapping field through the multiple apertures of the bottom endcap and impinge on the detector. The detected ion current signal intensity as a function of the time corresponds to a mass spectrum of the ions that were trapped initially.

Ions in a quadrupole field experience some restoring forces that drive them back towards the center of the trap. The motion of the ions in the field can be described by solutions to the Mathieu equation. This is a six parameter differential equation that balances the equation for the motion of ionised particles in a quadrupole mass analyser to the electrical potentials (Hao & March, 2001; March, 1997, 2000; Todd & March, 1999).

The differential form of the Mathieu equation is given in Equation 1 below:

$$\frac{d^2u}{d\varepsilon^2} + (a_u - 2q_u \cos \varepsilon)\mu = 0 \quad (1)$$

Where μ are the Cartesian coordinates x, y and z, ε^2 is a dimensionless parameter equal to Ωt/2 and Ω is frequency at time t. a_u and q_u are also dimensionless parameters called trapping parameters.

Solutions to equation 1 are of two types i) periodic but unstable and ii) periodic and stable. On the stability diagram in Figure 1.8, solutions of type i) form the boundaries of unstable region and solutions to type ii) determine the motion of ions in an ion trap. We are interested in solutions to equation 1 in the axial direction.

$$a_z = \frac{-16eU}{m(r_o^2+2z_o^2)\Omega^2} \quad (2)$$

$$q_z = \frac{8eV}{m(r_o^2+2z_o^2)\Omega^2} \quad (3)$$

Equations 2 and 3 contain the radial frequency (Ω), the size of the ion trap (r_o) and the amplitude V of the r.f potential and the *m/z* ratio of a given ion. Those are the only parameters required in understanding the operation of the ion trap (March, 1997).

The stability diagram displays regions where axial and radial stability overlaps theoretically. A given ion of *m/z* ratio has a_z and q_z values (parameters defined above), the ion will only be

trapped if these values fall within the boundaries of the stability diagram, if not, the ions will hit the electrode and be lost. All this depends on the amplitude of the voltage placed on the ring electrode. Commercial ion traps are designed to work along the line $a_z = 0$.

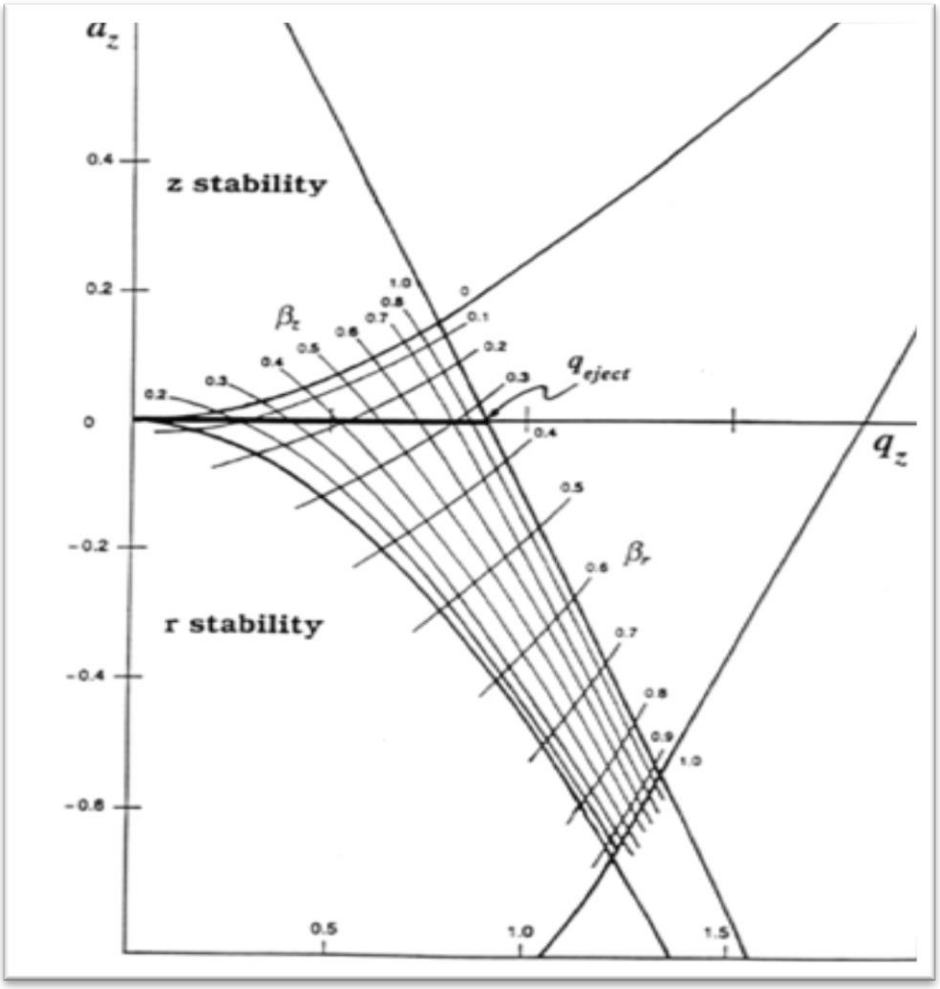

Figure 1.8: Typical stability diagram for a quadrupole ion trap

(Source: proteomicsresource.washington.edu)

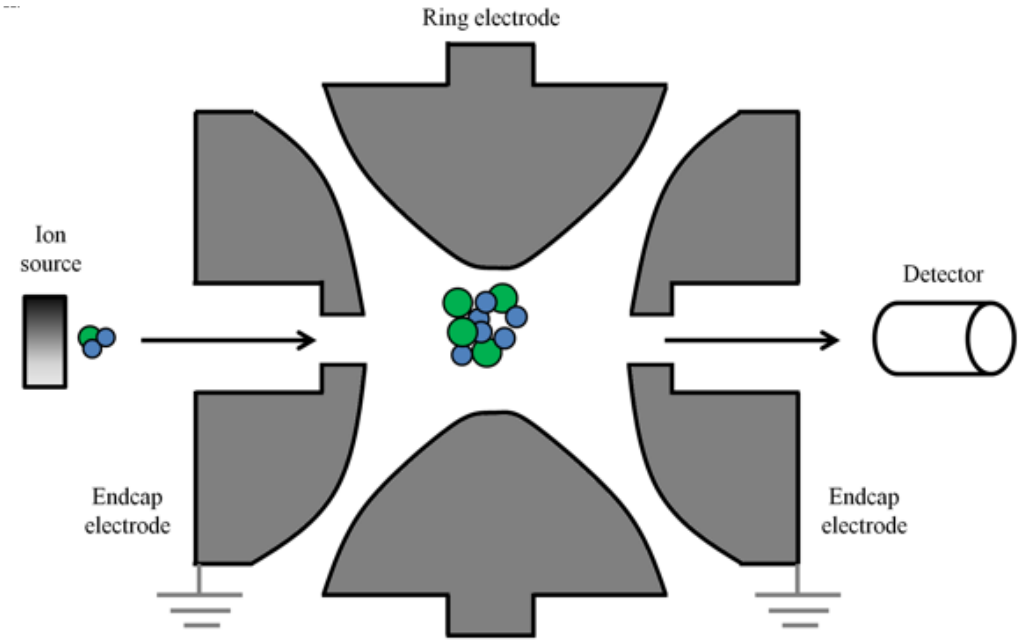

Figure 1.9: A diagrammatic presentation of the Quadrupole Ion Trap (QIT) ("Spectroscopic Techniques," 2015)

1.6.3 TANDEM MASS SPECTROMETRY (MS/MS)

Tandem (Latin :at length) mass spectrometry (MS/MS or MS^2) can be defined as the practice of performing one mass-selective operation after another (March, 1997). MS^2 can be performed in time or in space. In time the separation is accomplished with ions trapped in the same space by carrying out one mass selective operation after another in the quadrupole ion trap. In space is when you place one mass spectrometer after another. The parent ion is isolated in the first mass selective operation and the second mass selective operation determines the *m/z* ratio of the fragment ions created or formed by CID of the parent ion. MS^2 carried out using a Quadrupole ion trap has numerous advantages, namely:

i) Can perform Tandem Mass Spectrometry to the *n*th degree

ii) Mass-selected ions can be accumulated over time because the ion trap operates in a pulsed mode in contrast to sector and triple stage quadrupole which operate in continuous mode.

iii) All isolated ions can be dissociated (March, 1997, 2000).

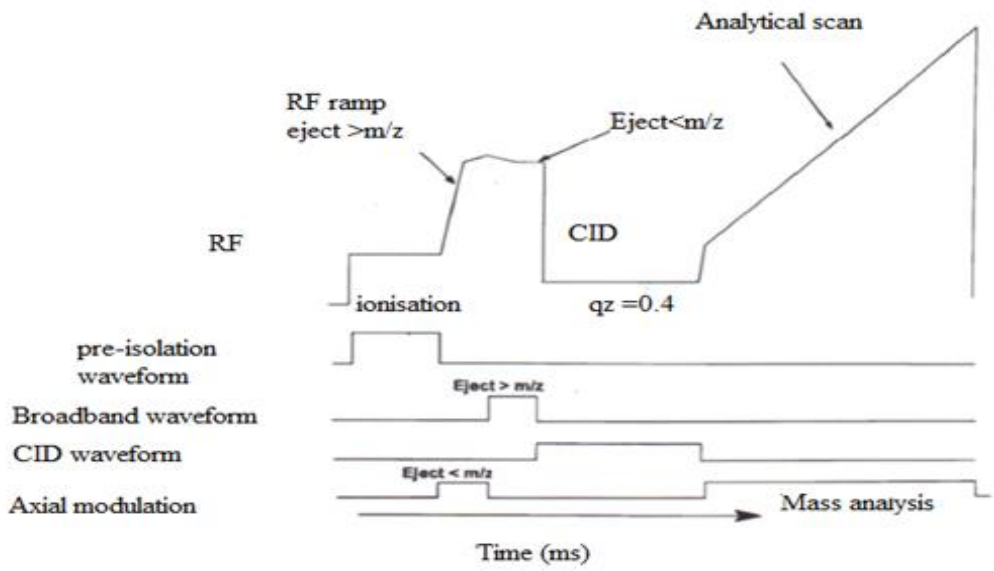

Figure 1.10: Tandem Mass Spectrometry Scan functions

Figure 1.10 above summarises the Tandem Mass Spectrometry stages. Axial modulation ejects ions with *m/z* values lower than the parent ion. Broadband Isolation waveform (BBISO) ejects ions with *m/z* higher than the parent ion.

1.7 Multiple Reaction Monitoring (MRM)

Multiple reaction monitoring a form of selected reaction monitoring, can be described as the selected ion monitoring (SIM) of product ions after the MS/MS of a precursor ion (Murray et al, 2013). In an ion trap, the scan function shown in Figure 1.10 for MS/MS is invoked for a precursor ion up until the CID waveform has been applied and excitation of the precursor ion

is accomplished. The full scan mass analysis is then replaced with a SIM supplementary waveform that allows detection and monitoring of selected *m/z* ratios. Thus, instead of observing a full scan mass spectrum of product ions, the resultant mass spectrum is a collection of only the *m/z* ratios that are allowed to be scanned. In this study, full scan MS/MS were done as a prelude for all the analytes. This was followed by selection of three most intense product ions to monitor in the MRM experiments again for each of the analyte precursor ions.

Multiple reaction monitiring (MRM) has many advantages as compared to other data aquisition methods. It has the ability to detect low level analytes in complex matrix, can detect multiple transitions on the order of 10 msec per transition, it is highly reproducible and sesitive (Anderson, 2005).

A combination of LC and MS has been used in therapeutic drug monitoring (TDM) of drugs including ARVs that were selected in this study**.**

1.8 THERAPEUTIC DRUG MONITORING (TDM)

Gerber and Edwards (2002) define Therapeutic drug monitoring (TDM) as a strategy by which the dosing regimen for a patient is guided by repeated measurements of plasma drug concentrations. He further states that if the concentration is not within a predefined target range, the dose is adjusted to bring this level within this target range (Gerber & Edward, 2002). TDM is used to improve therapeutic efficacy and to avoid drug toxicity. Its main focus is on drugs with a narrow therapeutic range. Precise and accurate assays are available for all the classes of ARV's using LC-MS, however analysis of NRTI's is challenging because they circulate in the plasma as inactive pro drugs and require intracellular metabolism to the active triphosphate derivate. This means that NRTI's may not be a good candidate for TDM (Gerber & Edward, 2002). However, Takahashi *et.al* (2005) has reported the quantitative

determination of NRTI EFV in human plasma using HPLC (Takahashi *et al*., 2005). In 2006, 10 years after the introduction of HAART, therapeutic needs in HIV are different from what they were a decade ago (Saliba & Yeni, 2006). Relatively little has been published in the field of TDM despite recent changes in dosing (Mallon *et.al*.,2003). A study carried out in the United States of America (USA) revealed that the increasing frequency of resistant HIV strains is a major challenge. This study led to considerable therapeutic advances (Saliba & Yeni, 2006). TDM studies for ARVs using LC-MS have been reported in the literature (Fayet *et al*., 2009; Jung *et.al*., 2007; Koal *et al*., 2005; Sichilongo *et.al*., 2014). As long as the appropriate sample preparation strategies are employed, LC-MS is a method of choice for TDM.

1.9 LITERATURE REVIEW OF SAMPLE PREAPARTION TECHNIQUES EMPLOYED IN THE TDM OF ARVs

1.10 SAMPLE PREPARATION

Sample preparation in Analytical chemistry can be defined as the use of various chemical and separation methods to prepare samples for analytical measurement of different components (Agilent, 2013b). This is how a sample is treated prior to its analysis. Sample preparation has often been over looked but in the last 20 years, the analyst's predominant task has shifted from assay measurement to sample preparation (McDonald, 2001). The importance of proper sample preparation cannot be over emphasised because even the best analytical techniques cannot correct problems created by a lax sample pre-treatment process. Winefordner states that sample preparation is often the bottleneck in a measurement process, as they tend to be slow and labour-intensive (Winefordner *et.al*., 2003).

Ramos states that it is estimated that 60% to 80% of the work activity and operating costs in the analytical laboratories is spent in preparing samples for introduction into the analytical system selected for instrument determination, he further states that it is also estimated that this part of the analytical process is responsible for more than 50 % of the error associated to the final reported data (Ramos, 2012). The goal of sample preparation is enrichment, clean-up and signal enhancement of an analyte in a sample (Winefordner, 2003).

This study focuses on sample preparation for deactivated human blood plasma because plasma is readily available and is the most widely tested biological fluid in human diagnostic assays but it is also a challenging biological matrix because it is complex (Freue & Borchers, 2012). Sample preparation techniques that were reviewed in this literature review were QuEChERS, protein precipitation (PPT), liquid-liquid extraction (LLE), solid phase extraction (SPE) and the mixed modes which are a combination of any two of the stated sample preparation techniques.

1.11 QuEChERS

QuEChERS (pronounced “catchers”) is a portmanteau word formed from Quick, Easy, Cheap, Effective, Rugged and Safe. This is a sample preparation technique developed by the United States Department of Agriculture in 2003 (USDA, 2015). The technique was developed for pesticide analysis in fruits and vegetables but has in recent times expanded its scope to other areas such as veterinary drugs (Stubbings & Bigwood, 2009), and Antiretroviral drugs (Sichilongo *et al*., 2014). The major official methods for QuEChERS are the AOAC 2007.01 method by the Association of Official Analytical Chemists and its European Equivalent, the EN 15662 2007 which uses a similar methodology (Agilent, 2013a).

1.11.1 Principle of Operation of the QuEChERS technique

The QuEChERS technique has 3 major steps namely: i) Sample Extraction, ii) Sample extract clean-up and iii) Sample Analysis

The QuEChERS technique involves the extraction of the sample using acetonitrile (MeCN) with 1% acetic acid (HAc). Acetonitrile extracts the greatest number of analytes with the least number of inteferences. 1 % acetic acid in acetonitrile, when combined with sodium acetate, prevents base sensitive analytes from breaking down during extraction and again acetic acid works best with LC MS/MS analyses because it improves ionisation. The next process is Liquid-liquid partitioning formed by the addition of anhydrous magnesium sulfate ($MgSO_4$) plus sodium acetate (NaAc). Anhydrous magnesium sulfate ($MgSO_4$) works as a desiccant, plays a major role in partitioning and also improves recoveries of polar analytes. The clean-up step, a dispersive solid-phase extraction (dSPE) step involves using various combinations of porous sorbents and salts to remove interfering substances such as sugars

and fatty acids. The use of porous sorbents such a primary-secondary amine (PSA) and C18 helps to remove a variety of matrix compounds that are co-extracted in the first step.

1.11.2 Benefits and limitations of the QuEChERS technique

The QuEChERS technique has a number of advantages in comparison to other sample preparation techniques. Solvent usage and waste is minimised and no chlorinated solvents are used, not much training or technical skill is required to use the method, little glassware is used, extract clean-up step is done to remove organic acids (method very rugged), solvent exposure to worker is minimal, reagent costs are inexpensive, very high recoveries are achievable, high sample throughput and the results obtained are accurate and precise (Schenck & Hobbs, 2004). Drawbacks for the QuEChERS technique are quite limited and this might be attributed to the fact that its still a very new technique. Sample and solvent amount required in the method, are not "biological friendly" (Agilent _ QuEChERS, 2015).

1.11.3 Applications of QuEChERS in ARV analysis

Literature on applications of QuEChERS in ARV analysis is limited. Majority of methods developed for QuEChERS involve extracting pesticides in fruits and vegetables. In recent times it has expanded its scope to drugs (Schmidt & Snow, 2016). Usui *et.al* has applied QuEChERS extraction technique to the simultaneous determination of forensically important drugs and poisons in human whole blood followed by LC MS/MS (Usui *et.al*., 2012). Sichilongo has applied QuEChERS extraction method to antiretroviral drugs (Sichilongo *et al*., 2014). The work was based on comparative chromatography-mass spectrometry studies of Nevirapine which is a combination of Tenoforvir, Emtricitabine and efavirenz. Using LC-MS, the method detection limits (MDLs) were ranged between 3.14 and 47.1 ug/L. The limit of quantitation ranged between 2.85 and 90.0 ug/L respectively. The % mean recovery was 87 % and the precision (RSD) was 2.8.

1.12 Solid Phase Extraction (SPE)

Solid-phase extraction (SPE) is a sample preparation technique by which compounds that are dissolved or suspended in a liquid mixture are isolated from other compounds in the mixture according to their physical and chemical properties. Analytical laboratories use solid phase extraction to concentrate and purify samples for analysis. Solid phase extraction can be used to isolate analytes of interest from a wide variety of matrices, including urine, blood, water, beverages, soil, and animal tissue (Sigma-Aldrich, 1998). A typical SPE device has 50 times more separation power than a simple, single liquid-liquid extraction. SPE's practice is governed by Liquid Chromatography (LC) principles. A sample is introduced into a cartridge device containing a bed of appropriate particles, the stationary phase. A mobile phase (solvent) flows through the bed. By picking an appropriate combination of the mobile and stationary phase, sample components may pass directly through the column bed, or they may be selectively retained.(Rezk *et.al*., 2008). The result is that either the desired analytes of interest or undesired impurities in the sample are retained on the stationary phase.

The stationary phase comes in the form of a packed syringe-shaped cartridge, a 96 well plate or a 47- or 90-mm flat disk, each of which can be mounted on its specific type of extraction manifold equipped with a vacuum port to speed up the extraction process.

Most stationary phases are silica-based. The silica is bonded to a specific functional group. Some of the functional groups include hydrocarbon chains of variable length (for reversed phase SPE), amino groups (for anion exchange), and sulfonic acid orcarboxyl groups (for cation exchange) (Simpson, 2000).

1.12.1 Principle of operation for the Solid Phase Extraction technique

The principle of solid phase extraction (SPE) is based on chromatographic separation. All four modes of chromatographic separation which include adsorption, partition, ion exchange and size exclusion or gel permeation are used in SPE.

Solid Phase extraction involves atleast 4 main steps i) Conditioning

ii) Loading

iii) washing

iv) Elution

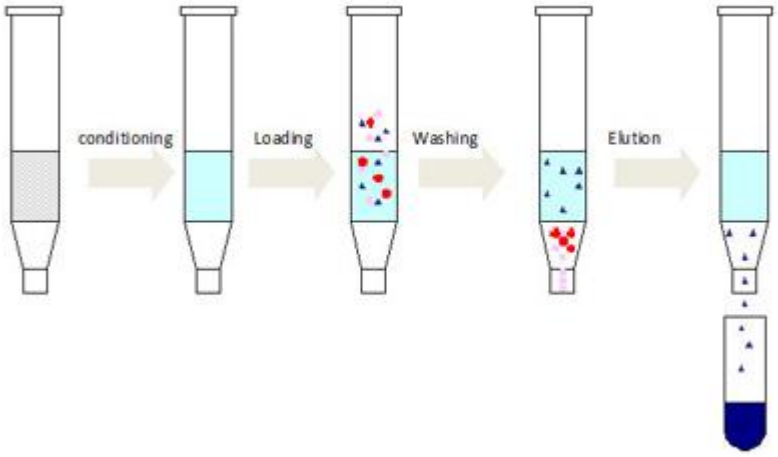

Figure 1.11: Solid Phase Extraction Steps

Firstly, the cartridge is conditioned with an appropriate solvent to wet the sorbent. Then the loading solution containing the analyte is passed through the solid phase slowly. In an ideal situation, the analyte and some impurities are retained on the sorbent. The sorbent is then washed to remove impurities, After removal of impurities, the analyte is collected during the elution step.

1.12.2 Benefits and limitations of the Solid Phase Extraction technique

The advantages of SPE over other sample preparation techniques include: lower cost because it consumes less amounts of solvents. It has higher and more reproducible recoveries and this might be attributed to the minimal sample transfer in the method. There is less exposure to toxic substances. Emulsion problems are minimised because of the few steps that the technique has. There is reduced harm to liable compounds because there is minimal evaporation. SPE is readily automated and has improved throughput. There is reduced waste generation and organic solvent usage (Agilent, 2013). SPE is easy to automate and there is less manual effort, low purity solvents can still give good separations and has a high range of immiscible solvents available. Limitations of SPE include Restricted flow rates and plugging of the top frit, the cross sectional area of SPE cartridges is small therefore sampling processing rates are reduced and there is tolerance to blocking particles, SPE has inadequate packing density, the channelling in SPE reduces the capacity of the cartridge to retain analytes and lastly incomplete reversibility of the sorption of some analytes from active sorbent sites lowers their expected recovery (Płotka-Wasylka *et.al*., 2015).

1.12.3 Applications of SPE in ARV analysis

Checa *et.al* reported the use of SPE for ARV sample pre-treatment using reverse phase liquid chromatography method with spectrophotometric detection to determine antiretroviral drugs in blood plasma for TDM purposes (Checa *et.al*., 2008). Limits of detection were around 5 ng/mL for Emtricitabine and 20 ng/mL for Lopinavir and Ritonavir. Recovery values were around 100 % for all drugs. The limit of detection were 10, 20, 80 ng/mL for Emtricitabine, Ritonavir and Lopinavir respectively. The precision were between 3.7 and 13.6 %. Notari *et. al* has also used spectrophotometric detection to determine 16 ARVs in human plasma using SPE (Notari *et.al*., 2006). The ARVs determined were Lopinavir, Retonavir, Emtricitabine

and Efavirenz. The recovery ranged between 88 and 120 %. The precision ranged between 0.6 to 1.0 %. The limit of quantitation were 0.005 ug/mL for Lopinavir and Retonavir. 0.025 ug/mL for Efavirenz and Emtricitabine. Rezk *et.al* reported the quantitation of protease inhibitors (Retonavir and Lopinavir), NNRTI (Efavirenz) and other ARVs in human plasma using RP - LC method with UV-diode array detection (DAD). The recoveries of all analytes were > 90 % with the exception of Lopinavir and Retonavir which were > 74 %. The lower limit of quantitation for Efavirenz was 10 ng/mL and 0.025 ug/mL for all other analytes. The upper limit of quantitation was 10 ug/mL for all analytes Retonavir and Lopinavir which was 5 ug/mL. Precision was between 1.7 and 5.8 %. D'Avolio et al determined plasmatic concentrations of Tenofovir and Emtricitabine in HIV infected patients (D'Avolio *et.al*., 2008) using LC-MS and SPE as the sample preparation technique. Detection of the analytes was achieved using ESI-MS. The limits of detection were 2 ng/mL for Tenoforvir and 1.5 ng/mL for Emtricitabine. Limits of quantitation were 15.6 ng/mL and 11.7 ng/mL for Tenoforvir and Emtricitabine respectively. Mean recoveries were 46.5 % (RSD: 8.8 %) for Tenoforvir and 88.8 % (RSD: 1.0 %) for Emtricitabine. Mishara *et.al* has reported simultaneous determination of Ritonavir and other protease inhibitors in human plasma by ultra performance liquid chromatography tandem mass spectrometry (UPLC MS/MS), sample pre treatment was by SPE (Mishra & Shrivastav, 2014). Detection and quantitation of the analytes were carried out using multiple reaction monitoring (MRM). Accuracy and precision (% RSD) for Ritonavir were 98.28 % to 103.00 % and 0.61 to 5.92 respectively. The limit of detection and lower limit of quantitation were 0.35 and 1.0 ng/mL. Mean recoveries were around 100 %.

1.13 Protein Precipitation

Protein Precipitation is a process in which proteins are concentrated and purified from from various contaminants. There are a number of different methods of precipitation available, these include salt induced precipitation also known as salting out. When high concentrations of salt are present, proteins tend to aggregate and precipitate out of solution. Factors such as temperature, protein purity and pH are important in determining the salting out point of a particular protein. Ammonium sulfate is usually the salt of choice because it has many useful features such as pH versatility, high solubility and low heat of solution (DNA Extraction 1,2015)

There is also precipitation with organic solvents. This type of precipitation has been used in this study. It involves the use of organic solvents such as acetone, tricholoroacetic acid (TCA), ethanol, ammonium acetate and acetonitrile. Organic solvents cause precipitation of proteins largely by changing the solvation of the protein with water. Most proteins larger than 15 kDa precipitate with 50% organic solvent ("DNA Extraction 1," 2015) Other methods of protein precipitation are Isoelectic, thermal, polyethylene glycol (non-ionic polmer) precipitation (Burgess, 2009).

1.13.1 Principle of operation for the Protein Precipitation technique

The principle of operation for Protein Precipitation when using organic solvents relies on the reduction in water activity. A medium decrease in the dielectric constant when adding an organic solvent leads to a decrease in the solvating power of water, protein solubility decreases and precipitation occurs.

This can further be explained by the following empirical formula;

$$\text{Log } S = A / \varepsilon^2 + \log S_0 \qquad (4)$$

Where S is solubility in presence of a solvent, S_0 is the original solubility, A is a constant which depends on the protein employed and the temperature and ε is the dielectric constant which depends on the type of solvent used (Kaul & Mattiasson, 2003).

1.13.2 Benefits and limitations of the Protein Precipitation technique

The major benefit of using protein precipitation is the relative ease of use and also precipitating agents can be chosen that provide a more stable product than found in the soluble form (Dixon, 1988). The organic solvents used in protein precipitation are relatively inexpensive and the method uses limited amount of equipment . This technique comes with a number of disadvantages especially at industrial scale. Some precipitants are highly flammable and hence dangerous to work with. Most organic solvents must be disposed carefully after use. Low recoveries are likely to be recorded if the initial protein concentration is low because precipitation under such conditions is inefficient when using organic solvents.

1.13.3 Applications of Protein Precipitation in ARV analysis

Dickinson et al reported the simultaneous determination of protease inhibitors (Lopinavir and Retonavir) in human plasma using high performance liquid chromatography tandem mass spectrometry (HPLC MS/MS). The method was used for pharmacokinetic studies and routine therapeutic drug monitoring (Dickinson *et.al*., 2005). Sample preparation was by protein precipitation followed by addition of ammonium formate buffer. The recovery was about 87 % and precision was below 11 %. The lower limit of quantitation (LLOQ), upper limit of quantification (ULOQ) and limit of detection (LOD) were 95, 15.584 and 3.9 ng/mL for LPV. 25, 4941 and 1.2 ng/mL for RTV. Djerada *et.al* has reported the validation of a method for the quantitative analysis of Tenoforvir, Lopinavir, Retonavir, Efavirenz and other ARVs in human plasma using UPLC MS/MS technology for TDM purposes (Djerada et al., 2013). Mean recovery for all drugs was higher than 85 %. Precision (%RSD) was 7.8, 1.1, 6.3, 7.9

% for Efavirenz, Lopinavir, Retonavir and Tenoforvir respectively. The LODs were 8.00, 10.00, 0.50, 2.00 ng/mL and LLOQ 31.25, 78, 7.81, 6.00 ng/mL for Efavirenz, Lopinavir, Retonavir and Tenoforvir respectively.

1.14 Liquid Extraction

In separation science, two phase systems can be exploited for the extraction of desired biomolecules. Liquid extraction (LE) is a technique used to isolate compounds based on their relative solubilities in two different liquids that are immiscible. LE is a widely used technique in the pharmaceutical industry. It involves the extraction of a substance from one liquid into another liquid phase (Mazzola *et.al.*, 2008)

1.14.1 Principle of operation of the liquid-liquid extraction technique

LLE involves the addition of a solvent to a sample that is immiscible. In the two phases that are formed, selective partitioning of the analytes and contaminants occurs. It is important to use an adequate amount of extracting solvent to retain all the analytes from the original sample. After addition of the extraction solvent, the two are physically mixed (by vortexing or shaking) and allowed to separate. The phase containing the analyte is then removed, dried down and reconstituted with the mobile phase in case of LC-MS. Non-polar organics such as ethyl acetate, hexane and methylene chloride are commonly used for this process because most pharmaceutical samples are aqueous.

1.14.2 Benefits and limitations of the liquid-liquid extraction technique

Benefits of LLE include simplicity, it is fairly simple to use and does not require much skill. It comes at a low cost because the chemicals used cost lower. It can easily be scaled up. It has shorter phase separation time. It has reasonable selectivity and has a rapid method development process. The major draw back in LLE is the formation of emulsions. This is a suspension of very tiny droplets of one solvent mixed in the other. To avoid formation of

emulsions, shake the mixture gently. Although LLE might have a reasonable selectivity but it is also limited. Lastly, LLE is difficult to automate (Mazzola *et al*., 2008)

1.14.3 Applications of liquid extraction in ARV analysis

Avery *et.al* has reported the use of liquid liquid extraction in the quantification of protein free and bound efavirenz in human seminal and blood plasma. Ultra-performance liquid chromatography – tandem mass spectrometry (UPLC – MS/MS) was used for this work. Multiple reaction monitoring data acquisition mode was employed. Accuracy (% dev) of 5.2 – 8.0% and precision of < 8 % were reported. Detection limits reported were 15 – 20 femtomole (Avery *et.al* 2010). Takahashi *et al* reported the simultaneous determination of lopinavir, retonavir and efavirenz in human plasma using HPLC and an ultraviolet detection at a single wavelength of 205 nm. The average accuracy ranged between 97.2 – 106.8 %. Precision was less than 8.5 % (Takahashi *et al.*, 2005).

1.15 Applications of mixed modes extraction techniques in ARV analysis

There are a few mixed modes techniques reported in literature for the extraction of ARVs. Jung *et.al* has reported the simultaneous determination of 17 antiretroviral drugs in human plasma with LC MS/MS (Jung *et.al*., 2007). A combination of liquid extraction and protein precipitation was used to extract the analytes which were monitored in multiple reaction monitoring mode. The analytes monitored were Tenoforvir, Efavirenz, Lopinavir and Ritonavir. The lower limit of quantitation was 5 ng/mL for all the analytes mentioned above. The recoveries were 72.9, 103, 108 and 81.0 % for Tenofovir, Lopinavir, Ritonavir and Efavirenz respectively. Accuracy (%) and precision (% RSD) values at a concentration of

500 ng/mL are as follows: 104.6 (7.8 %), 111.5 (14.1), 92.3 (7.7), 100.3 (2.9) for Tenofovir, Lopinavir, Ritonavir and Efavirenz respectively.

1.16 OBJECTIVES OF THE STUDY

The main objective of this study was to develop and validate an LC-MS method for the determination of selected ARVs i.e. efavirenz, emtricitabine, ritonavir, lopinavir and tenofovir which in turn would be used to compare the efficiency of seven different sample preparation strategies in human blood plasma.

The **specific objectives** of the study are:

1) To optimize mass spectral parameters for the detection of the selected ARVs.
2) To optimise chromatographic parameters for the separation of the selected ARVs.
3) To assemble a method for the determination of the selected ARVs.
4) To validate the method using Food and Drug Administration (FDA) guidelines.
5) To compare the extraction efficiencies of the drugs in human blood plasma using QuEChERS (a portmanteau for Quick, Easy, Cheap, Efficient and Rugged), solid phase extraction (SPE), Liquid - liquid extraction (LLE), protein precipitation (PPT), and mixed modes i.e. QuEChERS- PPT (Q-PPT), QuEChERS-LLE (Q-LLE) and LLE-PPT for the selected ARVs.

CHAPTER 2

2.0 EXPERIMENTAL SECTION

2.1 Reagents and Materials

Standards of emtricitabine (EMT), tenofovir (TFV), lopinavir (LPV), ritonavir (RTV) and efavirenz,(EFZ) were purchased from U.S Pharmacopeia Convention (Rockville, USA). The internal standard (I.S), Reserpine was purchased from Sigma - Aldrich, Germany. HPLC grade acetonitrile, methanol (MeOH) and tetrahydrofuran (THF) with 99.9 % purity were purchased from Sigma - Aldrich Co, St Louis (USA). Formic acid of 85 % purity was purchased from Sigma - Aldrich (Saarchem, Muldersdrift, RSA). The formic acid was triple distilled to increase its purity. Acetic acid (glacial) of 99.8 % purity was purchased from Rochelle Chemicals, Gauteng, South Africa. Sodium Carbonate (99.5 %), n-hexane (95.0 %) and ethyl acetate (99.9 %) were purchased from Associated Chemical Enterprises Pty (Ltd), Johannesburg, South Africa. Ultra high purity (99.999 %) nitrogen and helium gas were purchase from Afrox (Gaborone, Botswana). All extracts were filtered through 0.45 µm Acrodisc Syringe filter (Thomas Scientific-Swedesboro.,USA) prior to HPLC-MS analysis. Commercially sterilized human plasma from HLL Life Care Ltd (Kerala, India) was donated by the African Institute of Biomedical Science and Technology (AiBST), Harare, Zimbabwe. The calibration standards for the pH meter were prepared from buffer tablets pH 7.0 and pH 4 supplied by Merck (Gauteng, RSA). Mass spectrometry calibration solutions Ultramark 1621b was from Alfa Aeser, Johnson Mathey Company, Haysham, Lancaster, Met-Arg-Phe-Ala, MRFA 98.5% (Barnegat, N. J., U.S.A) and caffeine (99%) was obtained from Sigma Aldrich(St. Louis, MO, U.S.A). The SPE cartridges, Waters Sep-Pak® Vac 6cc (C18/1g) were purchased from Waters Corporation, Massachusetts, USA. The QuEChERS kits, the EN buffered extraction kits, and the SPE dispersive kits for sample clean up were obtained from Agilent Technologies (Milford, CA USA).

2.2 Apparatus

The MilliQ Ultrapure Ionex Gradient A10 purification system (Millipore Cop., Bedford, MA, USA) was used to process high purity water. The Visiprep™ SPE vacuum 12 port manifold (Sigma Aldrich, USA) coupled with a KNF Neuberger vacuum pump (Oxfordshire, Great Britain) were used in this work for solid hase extraction (SPE) experiments. A Heraeus® Sepatech® Labofuge 200 Centrifuge by Thermo Scientific (USA) was used for experiments that required centrifuging. All calibration standards were weighed on a Sartorius super microbalance GmbH (Goettingen, Germany). The pH of the mobile phases was checked using a pH meter from HANNA instruments (Hanna instruments Inc, Romania). A Vortex Genie 2 from Scientific Industries,Inc (New York, USA) was used in vortexing.

2.3 Stock solutions

1000 ppm individual stock standard solutions of EMT, LPV, RTV, EFZ and reserpine i.e the internal standard were prepared separately by diluting 10 mg of each pure compound in 10 mL of water and methanol (50:50) and stored in an ARTIKO refrigerator of permitted range -75 °C to -85 °C (Arctiko Int., Lammefjordsyej, Denmark). TFV was prepared by diluting 10mg in 10mL of water and methanol at 90:10. This was done because it did not completely dissolve at 50:50 (Water:Methanol). Working standard solutions were prepared by serial dilutions of the stock solutions in water and methanol every day prior to analysis. All working standard solutions were stored at 4 ºC in amber vials to protect them from light.

2.4 Instrumentation

2.5 Liquid Chromatography-Mass Spectrometry (LC-MS)

The HPLC system consisted of an Agilent model 1100 series High Performance Liquid Chromatography HPLC (Agilent technologies, Palo Alto, CA USA). The Agilent 1100 series has a quaternary pump, solvent compartment, vacuum degasser, thermostarted column compartment, autosampler and diode array detector. The LC was hyphenated to a Thermo Scientific LCQ-DECA Quadrupole ion trap mass spectrometer (QIT) with an electrospray ionisation (ESI) source (Thermo Scientific, San Jose, CA, USA).

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2.5.1 Liquid Chromatography

An ACE C18 analytical column with dimensions 50 X 3.0 mm; 3 μm particle size was used for all chromatographic separations (Mac-mod,PA, USA). The mobile phase was composed of water and tetrahydrofuran (THF) filtered through a 0.45 μm filter paper then degassed ultrasonically for 10 minutes with a Scientech Ultrasonic cleaner (Labotech, Midrand,South Africa). In the gradient program that was used, the water content of the mobile phase was kept constant at 40 % for the first 2.50 minutes then increased linearly to 80 % from 2.56 minutes to 7.00 minutes. The water content was then returned to 40 % from 7.00 minutes to 9.00 minutes. Chromatographic analysis was performed at ambient temperature because of the explosive nature of THF. LC-MS control and spectral processing was done using Thermo/Finnigan Xcalibur software, version 2.0 (Thermo Scientific, San Jose, CA, USA). The mobile phase was delivered at a flow rate of 0.2 ml/min and an injection volume of 20 μL.

2.5.2 Mass Spectrometry

The Thermo scientific Finnigan LCQ-DECA Quadrupole ion trap mass spectrometer (QIT) with an electrospray ionisation (ESI) source was operated in positive ionisation mode for all the analytes and the internal standard except for EFZ when it was switched to negative ionisation mode. The mass spectrometer was operated in MS/MS: SRM mode for MRM experiments. The capillary temperature was at 300 ºC. Ultra high purity (99.999%) nitrogen was used as sheath and auxiliary gas at a pressure of 100 psi. Ultra high purity (99.999%) helium gas was used as a damping gas. 5 ppm solutions of each analyte were used to tune parameters of the instrument using a built-in syringe pump of the instrument. Tune Plus version 2.0 (ThermoElectron Corp 1998-2006) was used as the control software for direct infusion experiments. The acquisition software was installed on a Microsoft Windows XP operating system, service pack 3 with 992 MB of RAM and 160 GB of Hard disk.

2.5.2.1 Fullscan and multiple reaction monitoring (MRM) experiments

Fullscan and multiple reaction monitoring experiments were performed in evaluating instrument detection limits (IDLs) and method detection limits (MDLs). The procedures for these are fully described in sections 2.7.1 and 2.7.2 respectively. The results are given in Chapter 3.

2.6 SAMPLE PREPARATION

Seven sample preparation techniques were selected to be evaluated in this study. These were SPE, PPT, LLE, QuEChERS abbreviated Q in the text that follows and the mixed modes i.e. Q-PPT, Q-LLE and LLE-PPT.

2.6.1 Sample Storage

The commercially sterilized human blood plasma was stored frozen at – 10 ºC and was only allowed to liquefy when it was being used.

2.6.2 Sample Extraction

Four single mode extraction techniques and three mixed mode extraction techniques were used for sample extraction. The single mode techniques used were solid phase extraction (SPE), protein precipitation (PPT), liquid-liquid extraction (LLE) and QuEChERS symbolized Q for simplification purposes herein. The mixed mode techniques employed were Q-PPT, Q-LLE and LLE-PPT. Spiking of the sample was before and after extraction for all the seven methods studied. This enabled percent recoveries to be calculated. For all techniques described below, the commercially sterilized human plasma was allowed to thaw for an hour before being subjected to the spike and extract procedures.

2.6.2.1 Solid Phase Extraction

The sample extraction procedure for SPE was adopted from D'Avolio *et.al* (2008) and slightly modified to suit the needs (D'Avolio *et. al*., 2008). 1 mL of plasma was diluted with 1 mL of a solution of HPLC-grade water with 0.6% of formic Acid (FA). The samples were vortexed for 10 seconds. SPE cartridges (C-18) were placed on a vacuum elution manifold Visiprep™ (Sigma Aldrich, USA) coupled with a KNF Neuberger vacuum pump (Oxfordshire, Great Britain) and activated with 1 mL of methanol, followed by 1 mL of

HPLC-grade water with 0.6 % of FA before loading of the samples. Gravity was used to load the samples to the cartridges. Then the cartridges were washed with 2.5 mL of HPLC-grade water with 0.6 % of FA, and elution was carried out using 5 mL of methanol and acetonitrile solution (90:10, v/v). The eluted solutions were collected into glass tubes, dried to dryness at room temperature using Nitrogen gas. Each extract was reconstituted with 2 mL of water–THF (50:50) solution. The extract was filtered through 0.4 5 µm Acrodisc Syringe filter then introduced to the HPLC-MS for analysis.

2.6.2.2 Protein Precipitation

The method for protein precipitation was adopted from Chi *et.al* and slightly modified (Chi *et.al*., 2002). 1.5 mL of plasma from deactivated study samples was placed in 15 mL centrifuge tubes, and mixed gently. 6 mL of acetonitrile was added to each tube and vortexed for 20 s at high speed. The tube was centrifuged at 5,500 rpm for 10 min to pellet the precipitated proteins and give a clear supernatant. The extract was filtered through 0.45 µm Acrodisc Syringe filter then introduced to the HPLC-MS for analysis.

2.6.2.3 Liquid Extraction

The Liquid Extraction procedure used in this study was adopted from Takahashi *et.al* (2005) and slightly modified (Takahashi *et al*., 2005). 4 mL of ethyl acetate/nhexane (50 : 50, v/v) and 2 mL of 0.5 M sodium carbonate were added to a 1 mL plasma sample. The mixture was vortexed and then centrifuged at 2500rpm for 5 min. The organic layer was separated and evaporated to dryness using nitrogen gas at room temperature. The dried material was then dissolved in 1mL of a mobile phase solution (water: THF-50:50) and centrifuged at 5800 rpm for 6 min. The extract was filtered through 0.45 µm Acrodisc Syringe filter then introduced to the HPLC-MS for analysis.

2.6.2.4 QuEChERS

The QuEChERS extraction technique used was adopted from Sichilongo *et.al* (2014) *(Sichilongo et al.,* 2014). This method was slightly modified due to the limited amount of the commercially sterilized human blood plasma that was available. Bond Elut QuEChERS Extraction kits EN Method were employed for this procedure.

5 mL of plasma sample was placed in a 50 mL fluorinated ethylene propylene (FEP) centrifuge tube. The sample was hand shaken for a minute and allowed to stand for 30 minutes to allow dispersion and interaction of the analyte with the matrix. Extraction and partitioning was performed by adding 5 mL of 0.1% acetic acid in acetonitrile followed by addition of 2.16 g of extraction salts. The sample was immediately shaken for 4 minutes to prevent agglomerates forming during $MgSO_4$ hydration and centrifuged at 4500 rpm for 5 minutes. For sample cleanup, the supernatant was transferred into a centrifuge tube containing $MgSO_4$ (150 mg), C18 and PSA. The sample extract was shaken vigorously for 2 minutes and centrifuged at 4500 rpm for 2 minutes. The supernatant was filtered through 0.45 μm acrodisc syringe filters prior to HPLC-MS analysis.

2.6.2.5 Mixed Modes

In the mixed modes extraction techniques, one extraction technique was performed after another. i.e QuEChERS-Protein Precipitation, QuEChERS - Liquid liquid Extraction and Liquid liquid Extraction-Protein Precipitation.

2.7 Method Validation

The method was validated using guidelines by the United States Food and Drug Administration (U.S FDA). The analytical performance characteristics that were investigated

were instrument detection limits (IDLs), method detection limits (MDLs), % mean recoveries which define the accuracy, % relative standard deviations (% RSDs) which define the precision.. Optimised LC-MS parameters were employed for this purpose.

2.7.1 Instrument detection limits (IDLs)

The IDL is a measure of the slightest quantity of an analyte that can be detected by the instrument in the absence of matrix effects. The IDLs were determined by constructing a five point calibration curve. Five different concentrations at 1, 2, 3, 4 and 5 ppm of the analytes were prepared. Reserpine (RSP) was added as an internal standard so that its final concentration was 3 ppm to all the five concentrations of the analytes. Five determinations per concentration were made in MS fullscan and MRM acquisition mode. The results of the two acquisition modes, MS fullscan and MRM were compared. Peak area ratios of analyte to internal standard at the 5 concentration levels for each analyte were tabulated against their corresponding concentrations and a calibration curve constructed. The LOD, LLOQ and ULOQ were calculated using equations 8, 9 and 10 below. Results for this experiment are given in table 3.7 & 3.8 in Chapter 3.

2.7.2 Method detection limits (MDLs)

MDL is the minimum concentration of an analyte that can be detected by the instrument in the presence of matrix effects. Six different concentrations at 10, 30, 40, 60, 80 and 100 ppb were prepared. RSP was added at a final concentration of 60 ppb to all the vials containing the analyte standard solutions above. 15 mL of sterilized human blood plasma was extracted using the QuEChERS technique adopted from Sichilongo *et.al* (2014). The technique is fully described in the sample extraction in Section 1.11 above (Sichilongo *et al*., 2014**).** The technique is fully described in the sample extraction section above. The extract from the QuEChERS technique was used as a diluent for the standards to generate matrix matched

standards. The extracted diluent was called post extraction matrix spike (PoMES). Five determinations per concentration were made in MS/MS fullscan and MRM acquisition modes and the results were compared. Results for this experiment are given in table 3.9 & 3.10 in Chapter 3. Since the MDLs using the MRM acquisition mode were much lower compared to the MS/MS fullscan MDLs and the limited amount of the sterilized human blood plasma, the subsequent experiments for generating calibration curves for the remaining six sample preparation techniques were done using the MRM acquisition mode. Peak area ratios of analyte to internal standard at the 6 concentration levels for each analyte were tabulated against their corresponding concentrations and a calibration curves constructed. The LOD, LLOQ and ULOQ were calculated using equations 8, 9 and 10 below.

2.7.3 Percent Recoveries

Recovery studies involved spiking the plasma at three concentrations i.e the LOD, LLOQ and ULOQ. These spikes were called Pre-extracted Matrix Spikes (PrEMS). The extraction techniques under study were then used to process the samples. Five determinations per concentration were made in MRM acquisition mode.

The percent recoveries were calculated using equation 5 below.

$$\% \text{ Recovery Extraction} = \frac{RESPONSE - extracted\ sample}{RESPONSE - Post\ extracted\ Spiked\ samples} \times 100\% \qquad (5)$$

Percent recoveries are tabulated in Table 11 in Chapter 3.

2.7.4 Accuracy and Precision studies

Accuracy and precision studies involved spiking the plasma at three concentrations i.e the LOD, LLOQ and ULOQ of the MDLs then applying the extraction techniques under study.

Seven determinations per concentration were made in MRM acquisition mode. Accuracy was calculated as a percentage of deviation of the mean value to the true value. Equation 6 below was used in calculating % deviation.

$$\% \text{ deviation} = \frac{/True\ value - mean\ value/}{True\ value} \times 100\% \quad (6)$$

The % deviation should not exceed 20 % at the LLOQ and 15 % at the ULOQ according to the FDA guidelines.

2.7.5 Precision

Precision was calculated as the relative standard deviation (RSD) or coefficient of variation (CV).

$$\text{RSD} = \frac{100S}{\ddot{x}} \quad (7)$$

Where S is standard deviation and ẍ is the mean of all values in the data set. According to the FDA guidelines, the precision i.e. % RSD should not exceed 20 % at the LLOQ and 15 % at the ULOQ.

2.7.6 Linearity

The R squared (R^2) value is the coefficient of determination and a measure of linearity. It indicates how well the data fits a statistical model. It ranges between 0 and 1. A value close to 1 indicates that the data fits the statistical model well and the reverse is true.

2.7.7 Detection and quantitation limits

2.7.7.1 The limit of detection

The Limit of detection (LOD) is lowest concentration level that can be determined to be statistically different from a blank at a specified confidence level .

The LOD was calculated using the following equation:

$$LOD = \frac{3Sx/y}{m} \quad (8)$$

Where Sx/y is the STEYX function: A measure of the amount of error in the prediction of y for an individual x. m is the slope of the line of best fit or line of regression of y on x.

m: The gradient of the line of best fit.

2.7.7.2 The limit of quantitation

The limit of quantitation (LOQ) is the level above which quantitative results may be obtained with a specified degree of confidence (Wisconsin Department of Natural Resources, 1996). With the LOQ, there is the Lower limit of Quantitation (LLOQ) and the Upper limit of Quantitation (ULOQ). There are various ways of calculating these limits.

2.7.7.3 The lower limit of quantitation

The LLOQ were calculated using equation 9 below.

$$\text{LLOQ} = \frac{6Sx/y}{m} \quad (9)$$

2.7.7.4 The upper limit of quantitation

The ULOQ were calculated using equation 10 below.

$$\text{ULOQ} = \frac{10Sx/y}{m} \quad (10)$$

2.8 Statistical evaluation of data

Statistical evaluation of data was done using Microsoft Excel 2010 and MINITAB® Release 14 Statistical Software.

CHAPTER 3

3.0 RESULTS AND DISCUSSION

3.1 LC-MS method development

In this study, development of the LC-MS/MS method took at least three phases or stages. These were optimization of parameters to afford multiple reaction monitoring (MRM) in mass spectrometry, optimization of separation parameters for liquid chromatography and followed by method validation. Eventually the optimum parameters were assembled into one package called the method and used to compare sample preparation techniques for ARV determination in human plasma. The biggest challenge in LC method development is the selection of an analytical column that has the required selectivity and suitable reproducibility in terms of retention of the compounds of interest. A good understanding of the paramount features of analytical columns can present quick solutions to chromatographic problems such as poor resolution or bad peak shape (Ahuja & Rasmussen, 2007; Ayrton, 2008)

In this study, MRM-MS parameters were optimised first, flowed by the LC separation parameters then method validation followed by comparison of sample preparation techniques.

3.2 Optimisation of MRM-MS parameters

In the optimisation of MRM parameters, the first thing was to discern the MS/MS patterns by direct infusion of all the ARV drugs including the internal standard (IS). This was done in order to establish the fragmentation pattern of the precursor ions. The most intense product ions where selected and monitored. Table 3.1 gives the precursor ion of each analyte and the transitions that were monitored.

Table 3.1: Precursor ion and product ion that were monitored

Analyte	**Transition *(m/z values)***
LPV	651→633; 651→439; 651→325
RTV	743→628; 743→325
EMT	270→152; 270→169
TDF	288→270; 288→176; 288→206
EFV	314→244; 314→298
RSP (I.S)	609→448; 609→397; 609→577

During preliminary experiments it was also established that positive polarity provided the best response for all the ARVs and the internal standard except for EFV which was more responsive in the negative mode.

In order to induce fragmentation in the ion trap, the ion-source collision induced dissociation (CID) had to be optimised.

3.3 Optimisation of the ion-source Collision Induced Dissociation (CID)

Ion source collision-induced dissociation (CID) is used to fragment ions in an atmospheric pressure ionization (API) source. Collisions take place between the analyte ions and the background gas in the case of the LCQ DECA, nitrogen. When these collisions take place, the ion's internal energy increases and it undergoes dissociation. Adduct ions between the solvent molecules and the analyte ions are formed in the region from the API region to the region of the octapole ion guides in the ion optics. Adduct formation is favoured when the pressure is high in this region. Formation of adduct ions appreciably decrease the number of sample analyte ions that can reach the analyser thus diminishing sentivity of analysis. Thus,

efficient dissociation of the adduct ions at optimal source CID voltages, can improve the sample ion current and the sensitivity of the mass spectrometer. In addition the following features have been observed by carefully using optimal source CID voltage:

- Source CID also increases the translational energy of the ion at the skimmer. This is a figure of merit performance wise. Higher translational energy early in the beam improves detection efficiency.
- Has been observed to improve performance by knocking solvent adducts off of the ion thus placing more of the analyte at the precursor *m/z*.
- It can also in exceptional instances be used to break weak bonds in preparation for breaking stronger ones in the ion trap mass analyser.

Due to this reason, source CID voltages were optimized for each of the ARVs in this study. This was done by incrementing the source CID voltage by 5 V starting from 0 V and observing the ion currents of the selected ions. Graphical presentation of the data was done in the end.

Figure 3.1 illustrates the results of source CID of *m/z* 288 as the precursor ion for TDF while Table 3.2 gives results for optimization of source CID for all other ARVs.

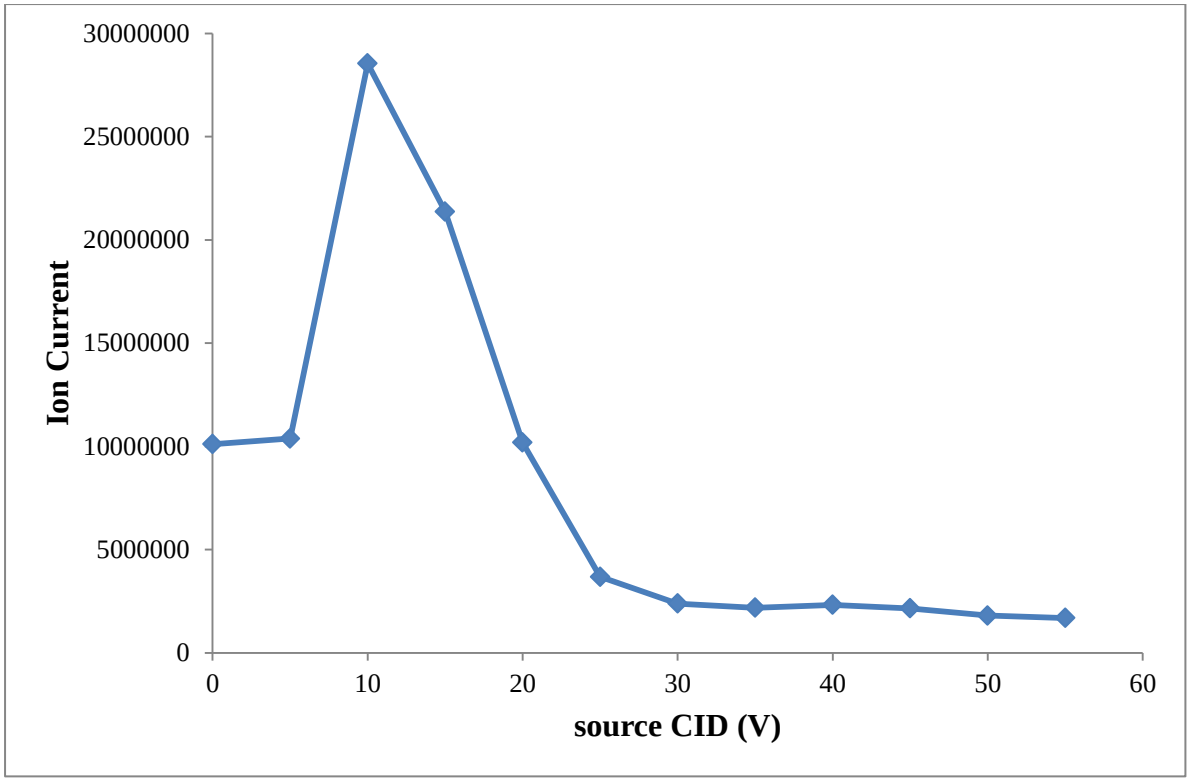

Figure 3.1: Ion Current versus source CID Voltage (V) for TDF 288

From Figure 3.1, the optimum source CID voltage of the ion at *m/z* 288 from TDF was 10 V. From Table 3.2, the trend appeared to be consistently the same for all the analytes where a peak was observed in the region between 5 and 15 volts except for EFZ shown in Figure 3.2 which was the opposite.

Table 3.2: Optimal ion source CID voltages for the precursor ions in the study

Analyte	**Precursor ion (*m/z*)**	**Optimum source CID (V)**
EFZ	314	0
EMT	270	5
TFV	288	10
LPV	651	5
RTV	743	5
RSP (IS)	609	5

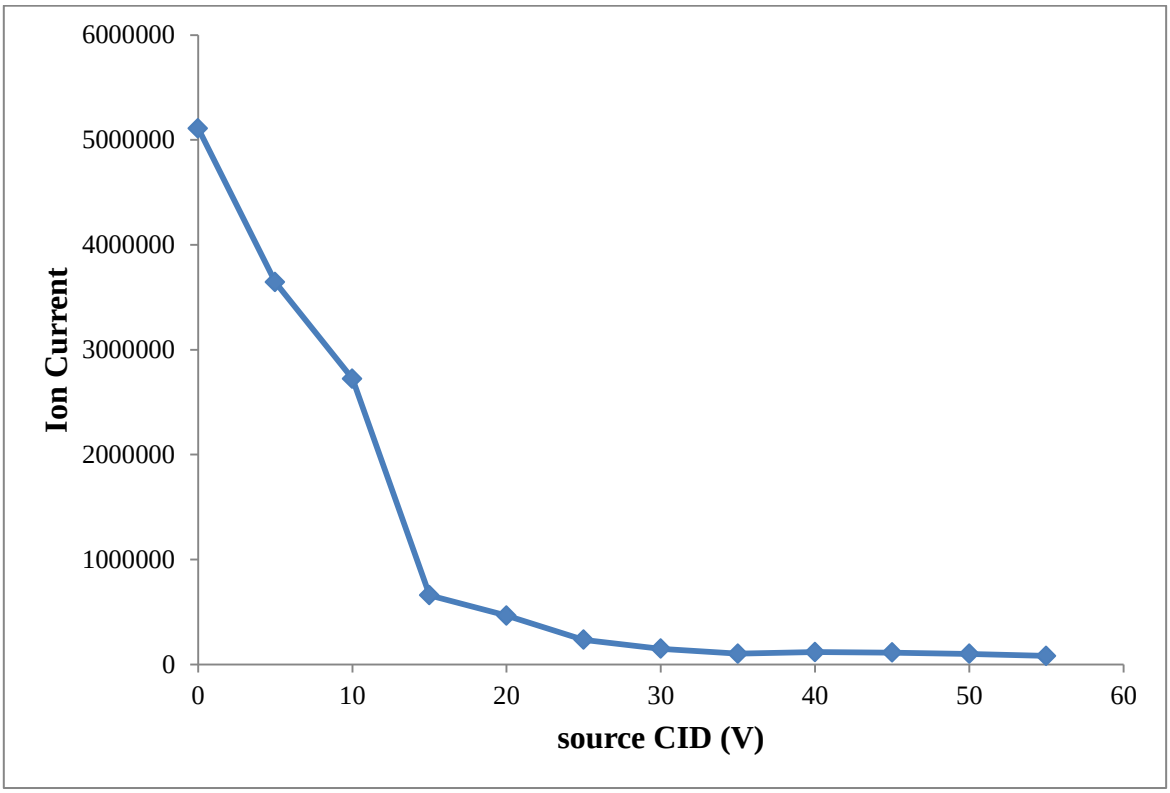

Figure 3.2: Ion current versus CID Voltage (V) for EFZ *m/z* 314

As stated, *m/z* 314 from EFZ as seen in Figure 3.2 behaved differently from the other precursor ions. It was speculated that perhaps this was because this was done in the negative mode thus yielding negative ions at [M-H]- with *m/z* 314. However, The same behaviour was observed when EFZ was run in positive mode thus yielding [M+H]+ ions at *m/z* 316 as seen in Figure 3.3 below.

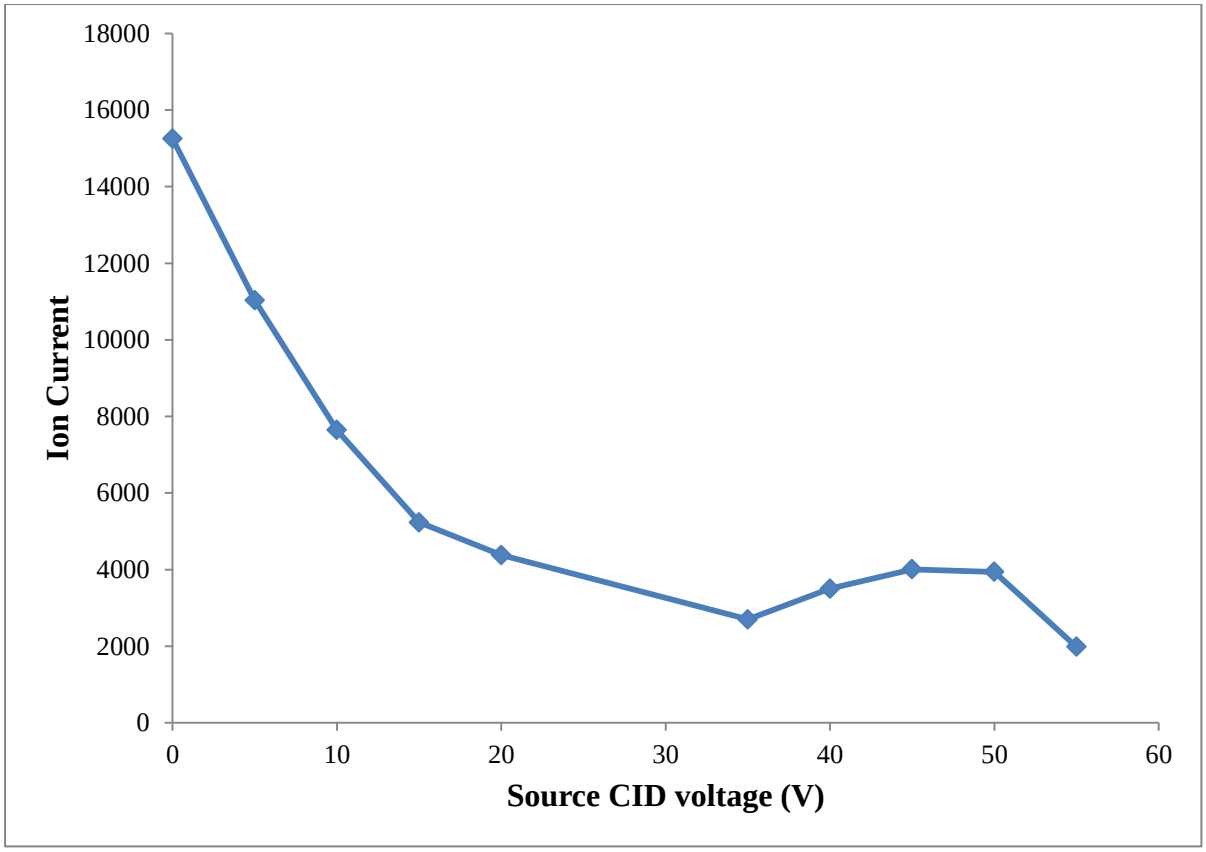

Figure 3.3: Ion current versus source CID voltage(V) for EFZ *m/z* 316

It was further speculated that both the positive and negative ions at *m/z* 316 and 314 respectively were very efficiently created by ESI that desolvation was almost complete before the region where source CID occurs thus rendering invocation of source CID redundant. The only difference observed between the negative and positive ions of EFV was that the negative mode produced extremely high ion intensities compared to the positive ones e.g. at a source CID voltage of 0 V, the ratio of negative to positive ions under the same conditions was 335.

Interaction between the ion source CID and the tube lens voltage (TLV) was noted. In order to follow this interaction, 3 dimensional (3D) experiments between source CID, TLV and intensity were carried out.

The following sub-section outlines the outcomes of these experiments.

3.4 Optimisation of the Tube Lens Voltage

The link between the LC and MS is the atmospheric pressure ionisation (API) source which consists of two assemblies namely, the API stack and the API probe assembly. The API stack is made up of the API source components that are held under vacuum, these include the heated capillary and the tube lens. The heated capillary aids in the desolvating of ions produced by ESI/APCI probe. Ions leaving the heated capillary enter the tube lens or the tube gate which helps in creating descret packets of ions that are introduced into the mass analyser during ion detection. The tube lens has a potential applied to it to help in focusing ions towards the opening of the skimmer. There is a background gas present in the capillary-skimmer region. An additional potential termed the tube lens offset voltage can be applied to the tube lens to speed up the ions to the background gas. Collisions of the ions with the background gas increases sensitivity and also assists in the desolvation process. If the collisions are too energetic resulting from setting the tube lens offset voltage too high, then the ions will fragment, decreasing sensitivity. When tuning the LCQ DECA instrument, the tube lens offset voltage is adjusted to maximize sensitivity by balancing desolvation with fragmentation hence it is important to optimise the tube lens offset voltage (LCQ DECA Manual, 1999).

The data for TLV was collected by varying it at constant CID and noting the intensity. Once this was done, the CID would be moved to a higher notch then the TLV varied at that set CID voltage while noting the intensity. The three dimensional plots for the TLV and CID and

intensity are given below. The results though not deviating from the two dimensional source CID optimization results, gave TLVs that were seen to be interacting with the source CID.

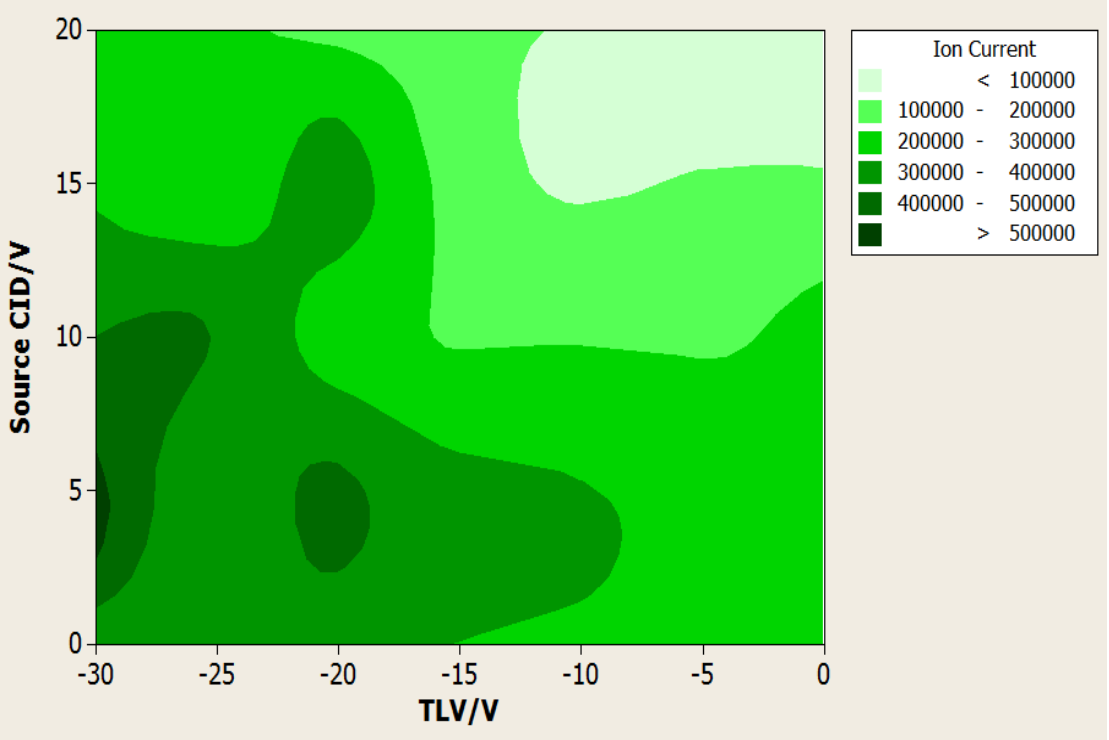

Figure 3.4: Contour Plot of ion current vs source CID, TLV for EMT *m/z* 270

Fig 3.4 above shows the optimised Source CIDs and TLVs for EMT *m/z* 270. The most intensely coloured region of the graph gives the optimum conditions. With reference to Figure 3.4, the optimum conditions for EMT *m/z* 270 were 5 V for source CID and - 30 V for TLV. Optimised conditions for the remaining analytes and the IS are given in table 3.3 below.

Table 3.3: Optimal values of the Source CID/V, TLV/V and their Ion Current/V

Analyte	**Source CID/V**	**TLV/V**	**Ion Counts**
EMT	5	-30	500 000
EFV	0	25	1 200 000
LPV	5	-30	40 000 000
RTV	5	-40	75 000 000
TDF	10	-20	12 000 000
RSP (IS)	5	-40	15 000 000

The TLVs ranged between 25 V and ± 40V for all analytes. EFV had a positive TLV because the ions are deprotonated and negatively charged. LPV are RTV had relatively high ion currents compared to EMT and EFV. This was ascribed to the high molecular weights that LPV and RTV have. Speculation arose that the lighter ions i.e. with lower ionic masses move very fast through the nitrogen gas pressurized region and are more prone to undergo CID than the higher ionic masses ions from RTV and LPV. Thus the higher ionic mass ions reach the detector without undergoing extensive CID and are therefore more intense. The contour plot of LPV was slightly different as compared to the other analytes. It had a wider range of TLV which span from around - 20V to - 40V as shown in Figure 3.5 below.

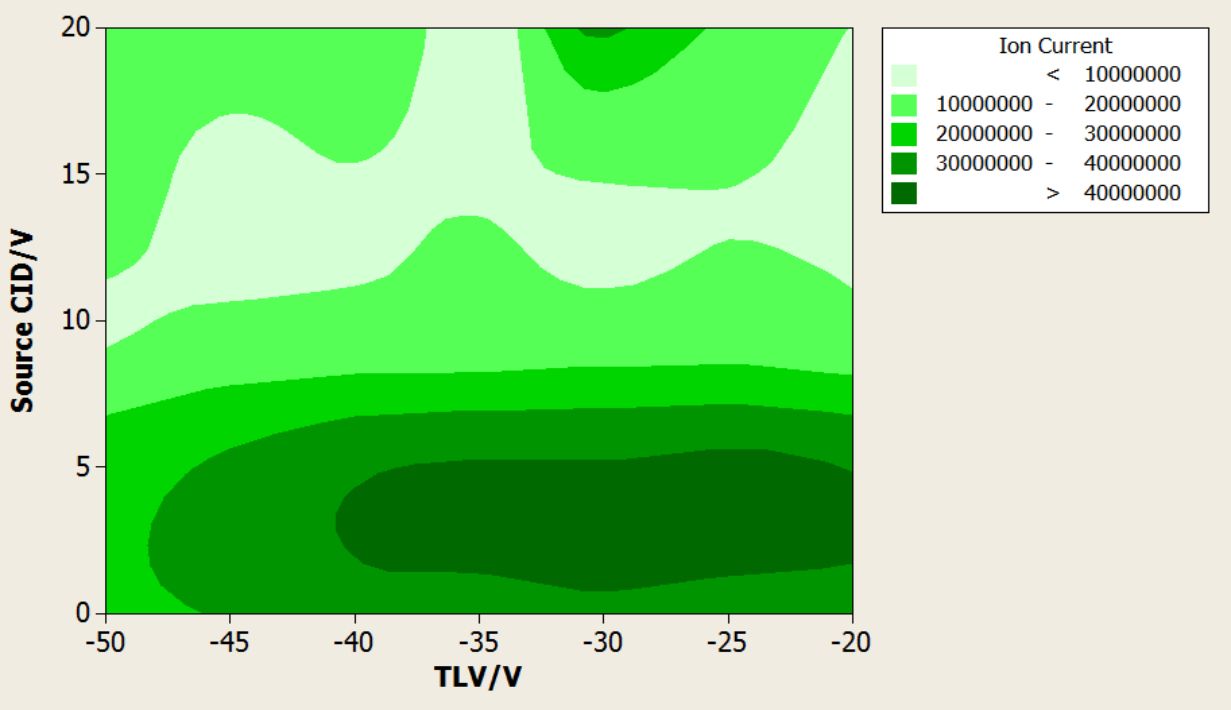

Figure: 3.5 Contour Plot of ion current vs source CID, TLV and intensity for LPV *m/z* 651

A wider range of TLV for LPV i.e. *m/z* 651 is indicative of its stability to undergo CID over the range of TLVs spanning from - 40 to - 20 and source CIDs spanning from 1 - 5 V. This aspect needs to be investigated further in relation to other analytes in this study.

Once the source CID and TLVs were optimised, the amplitude of the excitation wave forms for CID were optimized in order to perform MS/MS in both full scan and MRM at optimal levels.

3.5 Optimisation of the CID waveform amplitudes

The optimum CID waveform amplitude, i.e. given as the normalized collision energy (NCE) in (%) is the value that gives 10 % of the original ion intensity of the precursor ion. It is based on a scale of 0 to 5 V full scale corresponding to 0 to 100 %. CID was done using the resonant excitation mode i.e. using a pre-loaded scan function that matched a supplemental waveform of a calculated frequency that matched the secular frequency of the precursor ion. When resonance occurred, the precursor ion underwent fragmentation to form product ions. In this particular experiment, the amplitude of the supplemental CID waveform was incremented as required and the intensities of three product ions were recorded. These were plotted as functions of the amplitudes of the CID supplemental waveform.

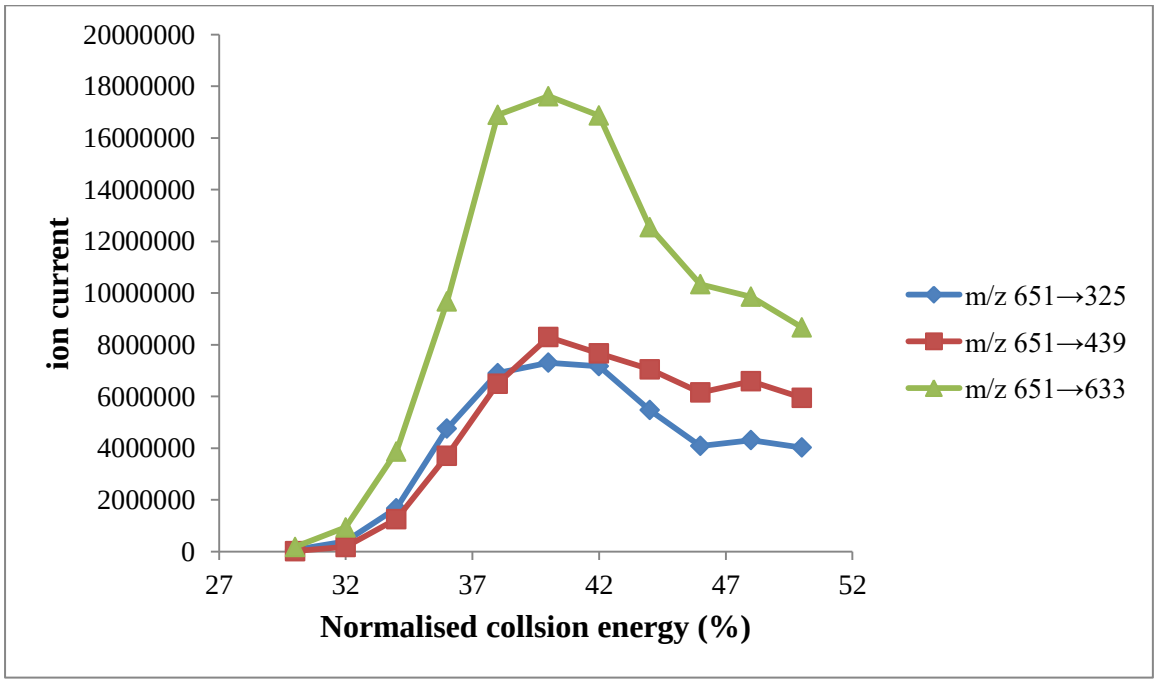

Figure 3.6: Ion current vs normalised collision energy (%) for LPV m/z 651

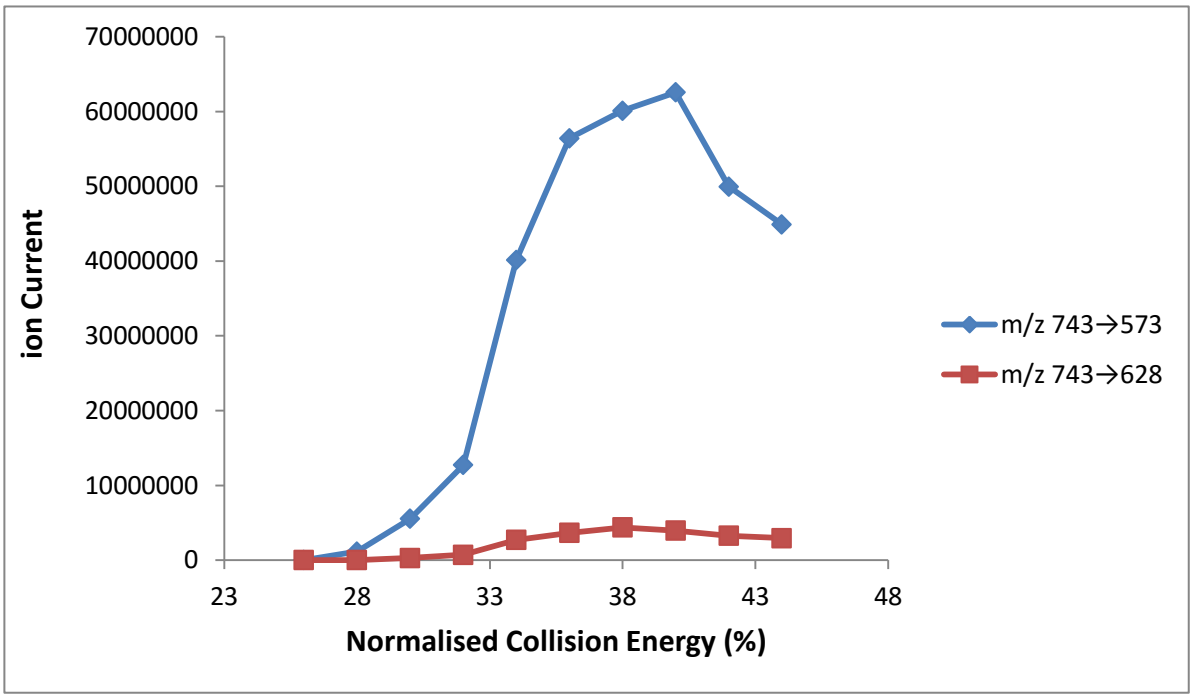

Figure 3.7: Ion current versus normalised collision energy (%) for RTV *m/z* 743

Figure 3.6 and Figure 3.7 above give the optimum values of the amplitudes of the CID waveform for LPV and RTV respectively. The optimum amplitude of the CID waveform for both LPV and RTV were 40 V. Values for other analytes are tabulated in Table 3.4.

Table 3.4: Optimal values for the normalised collision energies of analytes in this study.

Analyte	Normalised collision energy (%)
EFV	38
EMT	31
TDF	32
RTV	40
LPV	40
RSP (IS)	38

RTV and LPV had the highest NCE. This was attributed to the high molecular weights and stabilities of the molecules. The CID waveform amplitude has been reported to be mass dependent as well as other factors such as the stability of the precursor ion. The high CID waveform amplitudes for EFV and RSP were speculated to be due to the stability of the precursor ions rather than the molecular masses.

Other optimum mass spectral parameters for the ARVs in this study were adopted from previous studies on the same in this laboratory. The major parameter that was adopted was the isolation window for each of the five ARVs as shown in Table 3.5.

Table 3.5: Summary of optimized MS parameters for all the standards and internal standard

Analyte	TLV (V)	NCE (%)	MS/MS isolation window	In-source CID (V)
EFV	25	38	3	5
EMT	-30	31	2	5
TDF	-20	32	3	10
RTV	-40	40	3	5
LPV	-30	40	4	5
RSP (I.S)	-40	38	4	5

Results of the HPLC optimization experiments are given in the next sub-section.

3.6 Optimization of HPLC parameters

A variety of columns were employed in order to determine one which gave good selectivity of the ARVs. These include X-terra MS C_{18} (2.1*50 mm, 3.5 µm), Zorbax Eclipse plus C_{18} (4.6*100 mm, 3.5 µm) and Supelcosil C_{18} (4.6*100 mm, 5 µm). All these columns exhibited multiple co-elusions of the analytes. The ACE C_{18} analytical column with dimensions 50 X 3.0 mm; 3 µm exhibited only one co-elusion between LPV m/z 651 and RTV m/z 743 and therefore was selected. Quantification of the two analytes was based on the capability of the acquisition software to distinguish the ion currents of co-eluting species.

3.6.1 Optimum gradient used in the study

After an extensive literature search and a series of repetitive experiments, the mobile phase was chosen after several trials with isopropyl alcohol (ISP), acetonitrile (ACN), Tetrahydrofuran (THF) and water in various proportions at different pH values. A mobile phase consisting of THF and water was selected because it gave good separations as illustrated in Figure 3.8 below

Table 3.6: Optimum gradient program

Time (Min)	**Water (%)**	**Tetrahdrofuran (THF) (%)**	**Flow rate (ml/min)**
0.00	40	60	0.20
2.50	40	60	0.20
2.55	70	30	0.20
7.00	70	30	0.20
9.00	40	60	0.20

3.6.2 Optimisation of flow rate

The Flow rate influences the height and amount of theoretical plates and hence must be optimised but often it is over looked (Kaiser, 1996). At optimum flow rate, the plate height is smallest which translates to the number of theoretical plates being largest. Resolution reaches a maximum and the peaks are narrow because they are eluted with the largest possible height (Meyer, 2010). Flow rates of between 0.1 mL/min to 0.9 mL/min were investigated in this study while observing the resolution between the peaks. As the flow rate increased from 0.1 mL/min to 0.9 mL/min, co-elution between TDF and EMT was observed in addition to the LPV and RTV co-elution. A flow rate of 0.2 mL/min gave the best possible resolution and was adopted as the optimum flow rate.

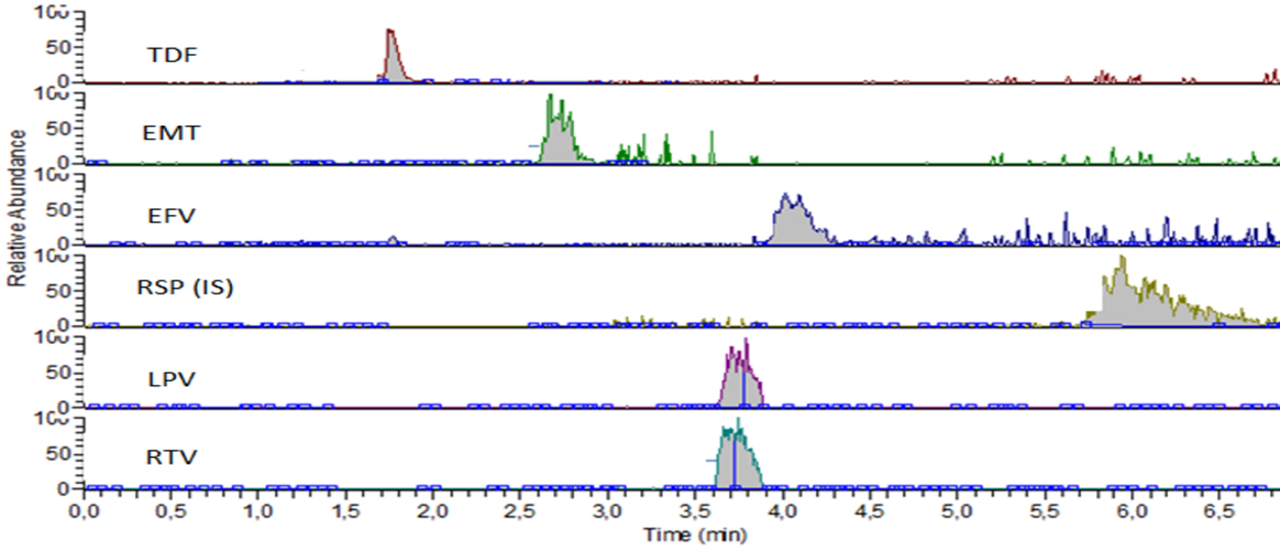

Figure 3.8: Reconstructed ion chromatogram obtained from spiked human blood plasma at 100 ppb

3.7 METHOD VALIDATION

The first thing done in method validation was to determine the instrument detection limits (IDL). Five point calibration curves were constructed in order to determine these.

3.7.1 Five Point calibration Curves for IDLs

The IDLs provide information regarding the state of the instrument at that particular time. They provide data relating to how sensitive the instrument is. Agilent technologies state that IDLs are a better measure of performance than signal-to-noise (S/N) ratio (Agilent _ Instrument Detection Limit (IDL), 2016). A comparison of IDLs in MS fullscan and MRM was done. Results for these experiments are given in the Table 3.7 and 3.8 below

Table 3.7: Instrument detection limits in MS fullscan acquisition mode.

COMPOUND	REGRESSION EQUATION	R^2	LOD (ppm)	LLOQ (ppm)	ULOQ (ppm)
TDF	y=0,0199x-0,0026	0,9928	0.5	1.1	1.8
EFV	y= 0.0204x + 0.0025	0,9987	0.1	0.2	0.4
EMT	y=0,0523x-0,0167	0,9885	0.6	1.3	2.3
LPV	y=2,0789x-1,149	0,9942	0.4	0.9	1.6
RTV	y=2,4374x-1,4956	0,9942	0.3	0.7	1.2

n = 5

Table 3.8: Instrument detection limits in MRM acquisition mode

COMPOUND	REGRESSION EQUATION	R^2	LOD (ppm) (INSTRUMENT)	LLOQ (ppm)	ULOQ (ppm)
TDF	y=0.002x + 0.0103	0.9935	0.5	1.0	1.6
EFV	y=0.0051x + 0.0091	0.9892	0.6	1.3	2.1
EMT	y=0.0263x + 0.003	0.9956	0.4	0.8	1.3
LPV	y=0.5683x + 0.4452	0.9934	0.4	0.8	1.3
RTV	y=0.5802x + 0.5412	0.9948	0.5	1.0	1.6

n = 5

The IDLs in MS fullscan and MRM were comparable. This was attributed to the fact that it was prior to the introduction of the matrix. They were both low and this suggested that the instrument was in good condition. The LOD range was from 0.1 ppm to 0.6 ppm for MS fullscan and 0.4 ppm to 0.6 ppm for MRM. The R^2 value was greater that 0.9900 for most analytes in both methods except for a few which might be attributed to instrumental errors. After assessing the performance of the instrument with IDLs, the Method detection Limits (MDLs) were determined.

3.7.2 METHOD DETECTION LIMITS (MDLs)

These are statistically determined values that outline how measurements of an analyte by a specific analytical protocol can be differentiated from background noise or blank measurements. MDLs are instrument, analyst and matrix specific (Wisconsin Department of Natural Resources, 1996). Six concentrations were prepared, namely 10 ppb, 30 ppb, 40 ppb, 60 ppb, 80 ppb and 100 ppb in order to evaluate the MDLs. Each validation run incorporated one blank plasma. The MDLs were done in MS fullscan and MRM using the QuEChERS sample preparation technique. The results are given in the Table 3.9 and 3.10 below.

Table 3.9: Method detection limits in MS fullscan acquisition mode using QuEChERS

COMPOUND	**REGRESSION EQUATION**	**R^2**	**LOD (ppm)**	**LLOQ (ppm)**	**ULOQ (ppm)**
TDF	y= 0.0006x-0.0183	0.9919	14.2	28.4	48.8
EFV	y= 0.005x-0.0066	0.9846	23.5	47.1	78.5
EMT	y=0.0025x-0.0871	0.9876	4.2	8.4	14.0
LPV	y=0.0082x-0.2268	0.9709	1.9	3.9	6.5
RTV	y=0.0901x-3.2612	0.9838	4.0	2.6	4.6

n = 5

Table 3.10: Method detection limits in MRM acquisition mode using QuEChERS

COMPOUND	**REGRESSION EQUATION**	**R^2**	**LOD (ppb)**	**LLOQ (ppb)**	**ULOQ (ppb)**
TDF	y=0.0032x-0.0369	0.9822	14.6	29.3	48.8
EFV	y= 0.0009x+0.0105	0.8207	56.2	112.5	187.5
EMT	y=0.0003x+0.0103	0.9746	16.3	32.7	54.5
LPV	y=0.4869x-1.0648	0.9554	29.4	58.8	98.0
RTV	y=0.4765x-1.0648	0.9881	20.9	41.8	69.7

n = 5

MDLs acquired in MRM acquisition mode are much lower and better as compared to those acquired in MS fullscan mode. This might be attributed to the fact that MRM has the ability to detect low level analytes in complex matrix making it very sensitive. Based on these results, experiments for the subsequent sample preparation techniques were done in MRM acquisition mode.

3.7.3 Recovery studies

As part of method validation, recovery studies were conducted on all the seven sample preparation techniques investigated. The US FDA defines recovery as a comparison of the detector response acquired from the analyte added to and extracted from the biological matrix to the detector response of the true concentration (FDA, 2001). Detector response of Extracted samples at 3 concentrations (LLOQ, LOD and ULOQ) were compared to unextracted standards which represented 100 % recovery. Results for recovery are given in Table 3.11.

Table 3.11: Recoveries of different extraction techniques of selected ARVs

Extraction Technique	**Recovery (%)**
QuEChERS	71.3 – 72.1
SPE	81.2 – 85.6
LE	68.8 – 81.2
PPT	70.3 – 74.2
Q-PPT	60.6 – 63.9
Q-LE	60.9 – 70.5
LE-PPT	52.4 – 59.2

Preliminary data analysis conducted by Microsoft Excel (2010) show that SPE has the overall best recoveries as compared to the other sample preparation methods that were investigated. It is then followed by the other three (PPT, LLE & QuEChERS). The mixed modes have the

least recoveries and this might be attributed to the multiple analytical steps that were undertaken. For all techniques, highest recoveries were achieved at ULOQ followed by LLOQ then LOD respectively. This is illustrated by Figure 3.9, 3.10 and 3.11 below.

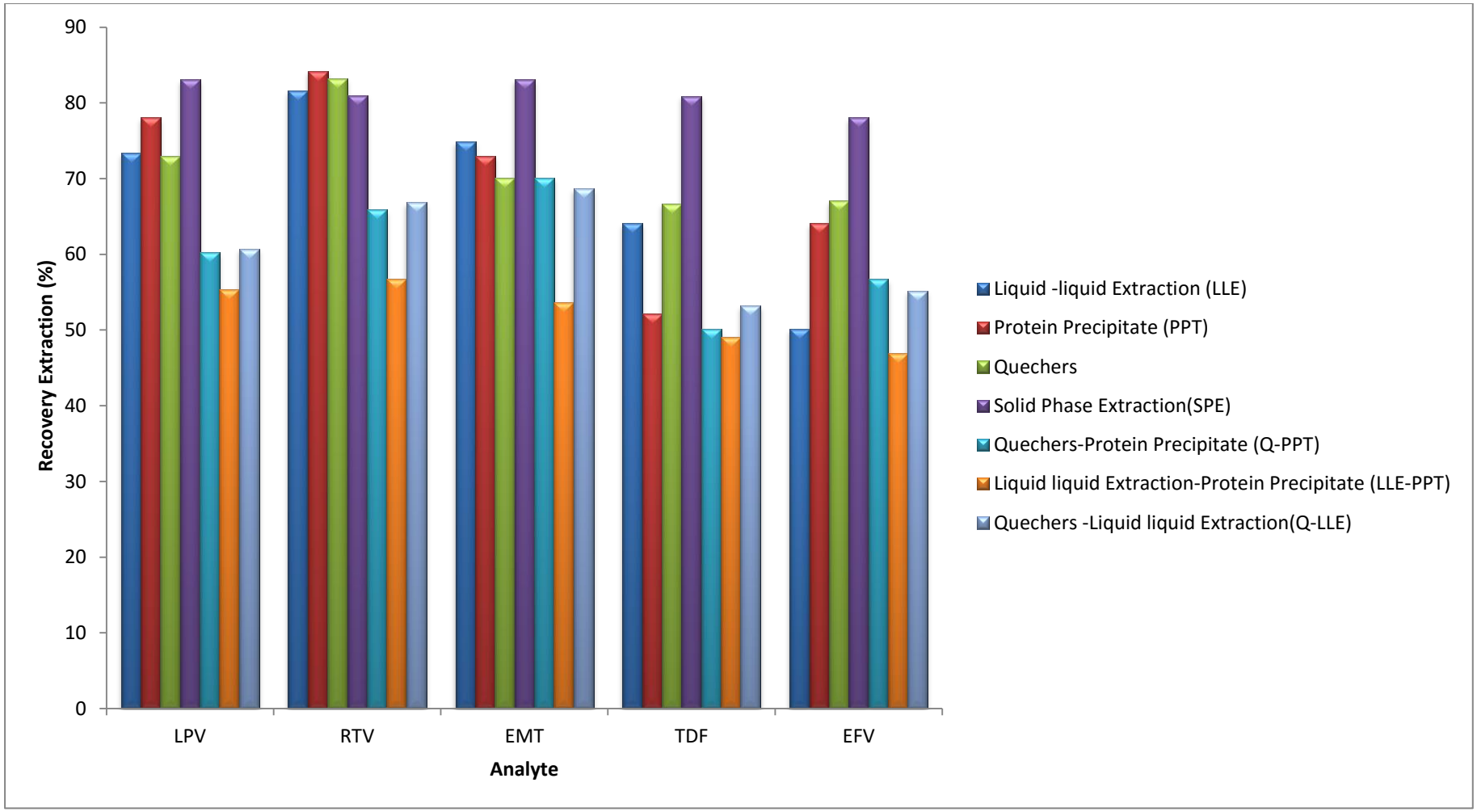

Figure 3.9: Recovery Extraction (%) Against analyte for different extraction methods at LOD

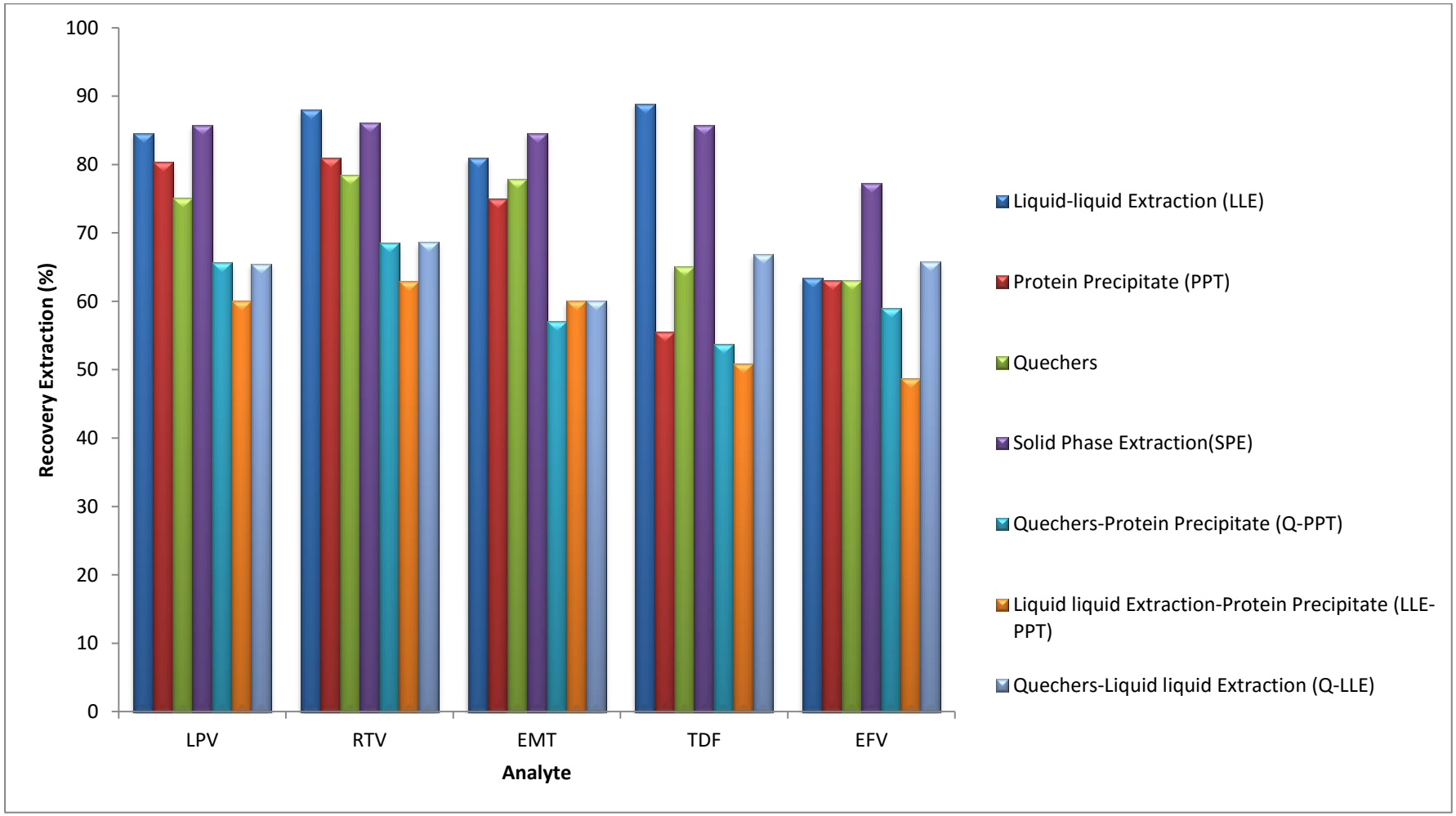

Figure 3.10: Recovery Extraction (%) Against analyte for different extraction methods at LLOQ

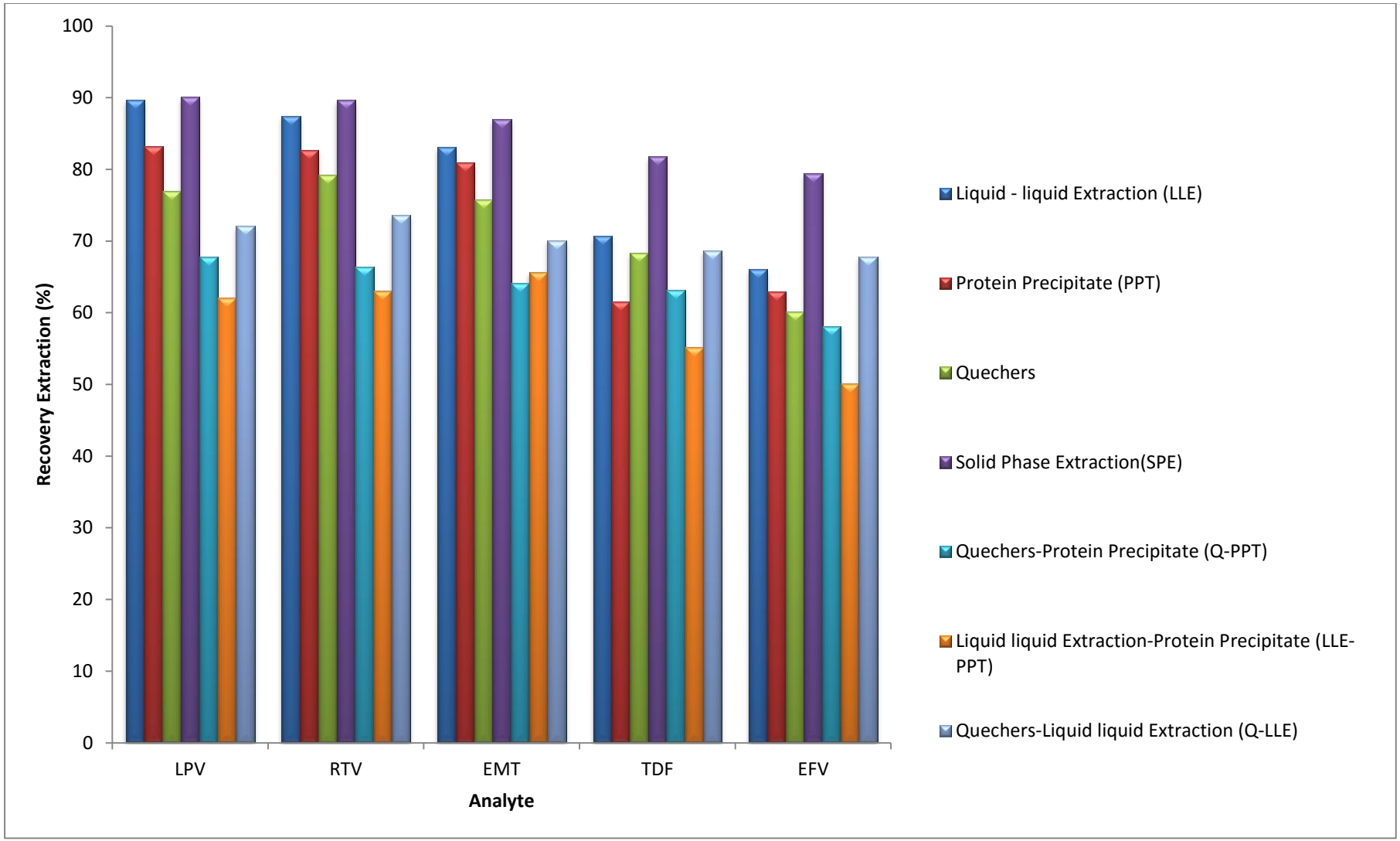

Figure 3.11: Recovery Extraction (%) Against analyte for different extraction methods at ULOQ

An in-depth analysis of the mean recoveries is given in section 3.8.2. Partial least squares was used.

3.7.4 Accuracy and Precision studies

Accuracy is defined as the closeness of mean test results to the true value (concentration). Seven determinations per concentration were made in order to evaluate accuracy. A minimum of three concentrations were used, that is at LOD, LLOQ and ULOQ. Accuracy was calculated as a percentage of deviation of the mean value to the true value. The FDA guidelines recommend that the deviation of the mean value to the true value should be within 15% with an exception of the LLOQ where it should not deviate by 20% (FDA, 2001)

Precision is defined as the degree of scatter between a series of measurements when the analytical procedure is applied recurrently to multiple aliquots of a single homogenous volume of biological matrix. Seven determinations per concentration were made in order to evaluate precision. A minimum of three concentrations were used, that is at LOD, LLOQ and ULOQ. Precision was calculated as the relative standard deviation (RSD) or coefficient of variation (CV). The FDA guidelines recommend that precision should not exceed 15% at each concentration level determined with an exception of the LLOQ where it should not exceed 20% (FDA, 2001).

The percentage of deviation of the mean to the true value (accuracy) for the seven extraction techniques studied are given in Table 3.12 below.

Table 3.12: Accuracy of the Extraction techniques

Extraction Technique	**Deviation of mean value to true value (%)**
QuEChERS	17.97 – 39.92
SPE	5.78 – 23.41
LLE	8.73 – 44.67
PPT	15.59 – 65.94
Q-PPT	21.73 – 50.56
Q-LLE	23.49 – 50.46
LLE-PPT	29.14 – 51.59

SPE had the least deviation from the mean in comparison with other techniques. PPT had the greatest deviation. For all techniques, deviation gradually reduced LOD to ULOQ. i.e LOD had the highest deviation then ULOQ had the lowest deviation.

Deviation at ULOQ was > 15% for most analytes in all techniques except SPE which recorded a deviation of < 15% for all analytes.

Deviation at LLOQ was > 20% for most analytes in all techniques with an exception of SPE which had a deviation of < 20% for all analytes. Deviation at LOD was > 30% for most analytes in all techniques. SPE had an average deviation of 20% at LOD. It can be concluded that the SPE technique is the only technique that managed to satisfy FDA guidelines regarding accuracy.

Table 3.13: Precision of the Extraction techniques

Extraction Technique	**Coefficient of Variation (CV) in %**
QuEChERS	0.33 – 7.70
SPE	0.49 – 4.50
LLE	0.74 – 8.79
PPT	0.73 – 9.41
Q-PPT	0.83 – 7.30
Q-LLE	0.61 – 6.60
LLE-PPT	1.41 – 11.5

Precision of all the techniques was within acceptable range. It was < 15 % at all levels of concentration (LOD, LLOQ and ULOQ). Detailed precision and accuracy tables for each technique can be found in the appendix.

A summary of parameters compared for all sample preparation techniques is given in Table 3.14 below.

Table 3.14: Summary of parameters compared for all sample preparation techniques

Sample preparation technique	Time taken to prepare sample (min)	Recovery (%)	Accuracy (%)	Precision (%)
QuEChERS	45	71.3 – 72.1	17.97 – 39.92	0.33 – 7.70
SPE	65	81.2 – 85.6	5.78 – 23.41	0.49 – 4.50
LLE	25	68.8 – 81.2	8.73 – 44.67	0.74 – 8.79
PPT	20	70.3 – 74.2	15.59 – 65.94	0.73 – 9.41
Q-PPT	70	60.6 – 63.9	21.73 – 50.56	0.83 – 7.30
Q-LLE	75	60.9 – 70.5	23.49 – 50.46	0.61 – 6.60
LLE-PPT	50	52.4 – 59.2	29.14 – 51.59	1.41 – 11.5

3.8 Statistical Evaluation of the data

3.8.1 Partial least squares (PLS) comparison of method detection limits (MDLs)

Figure 3.12 below shows the scores and loading plots of the PLS analysis of method detection limits (MDLs). The data points were mean values of three replicate determinations of the MDLs.

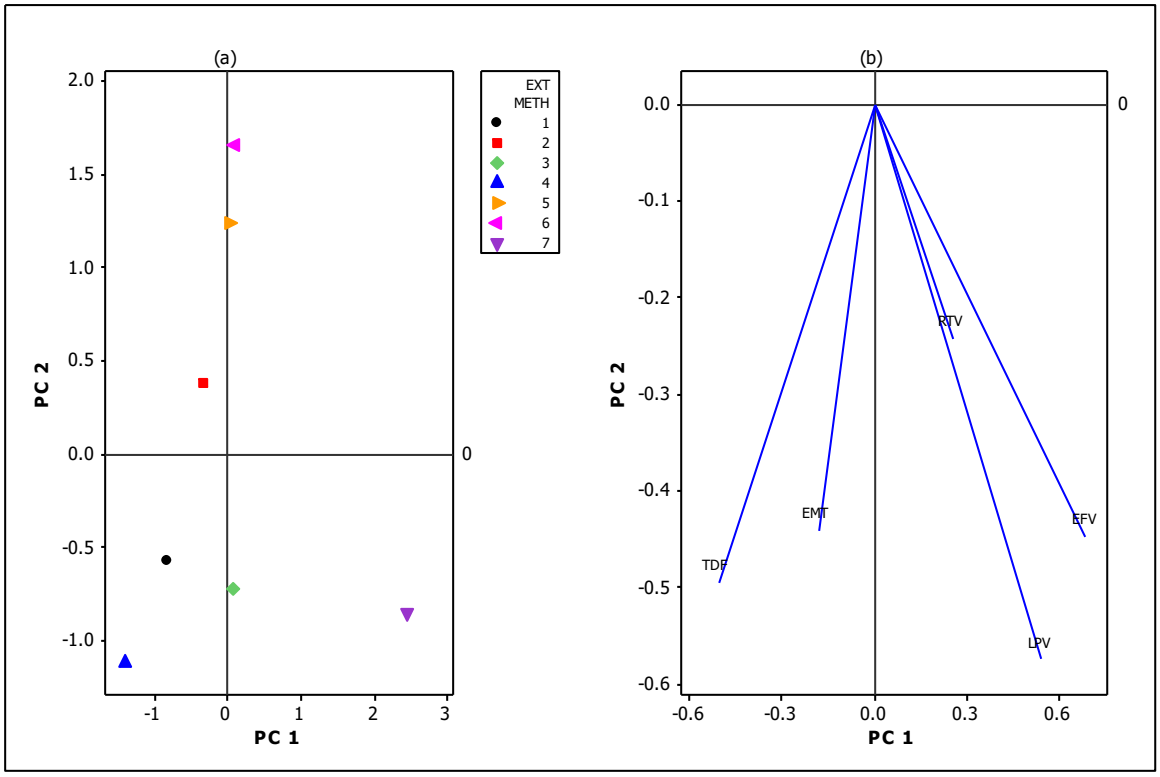

Figure 3.12: PLS scores and loadings plot for the mean MDL values for the five analytes

Data codes: 1 = LLE; 2 = LLE-PPT; 3 = PPT; 4 = QLLE; 5 = QPPT; 6 = SPE; 7 = Q
Q = QuEChERS

From the scores plot in Figure 3.12 (a), QPPT and SPE gave very similar MDLs followed by LLE-PPT. The rest of the methods i.e. LLE and QLLE gave MDLs that were negatively loaded impacting the MDLs of EMT and TDF according to the loadings plot in Figure 3.12 (b). The two analytes were also closely correlated and responded in a similar way to the two extraction procedures. PPT and Q loaded negatively and impacted EFZ, LPV and RTV in a

similar fashion. RTV and LPV were very closely correlated implying they behaved in a similar fashion. Since the LLOQs and ULOQs were estimated from amplifying the MDLs by certain factors, their response to different extraction procedures would be expected to be the same.

3.8.2 Partial least squares (PLS) comparison of the mean recoveries

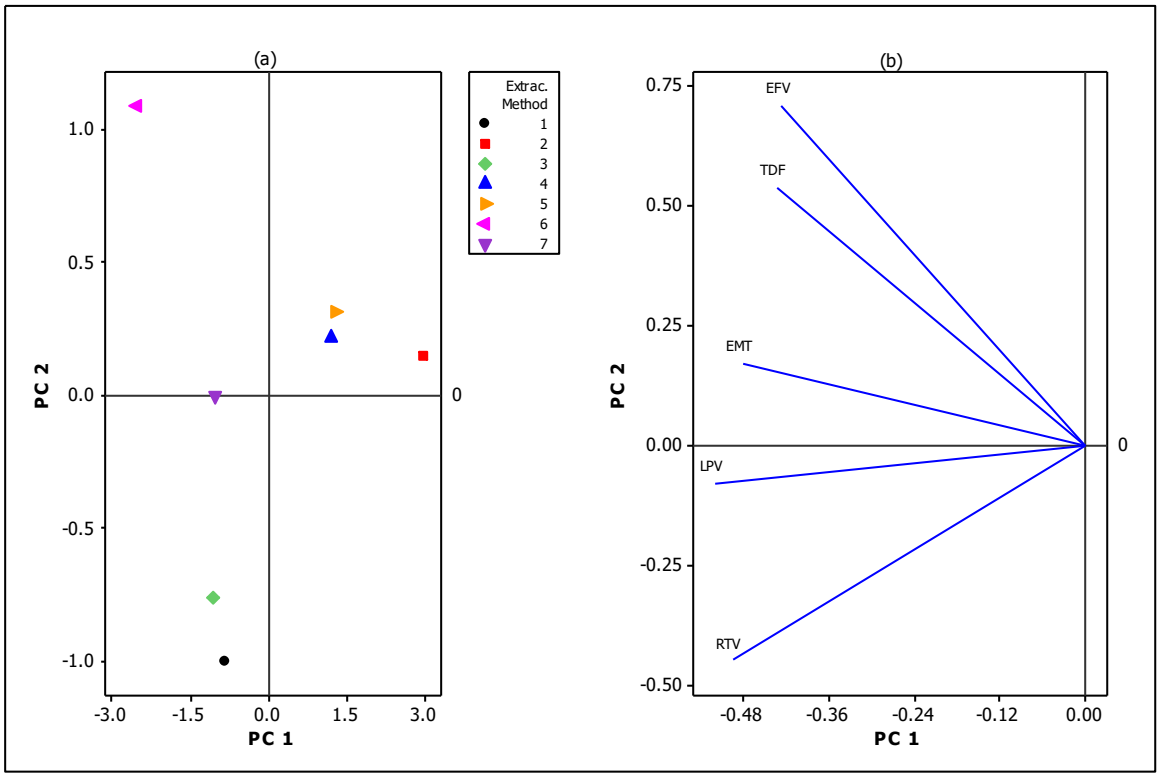

Figure 3.13: PLS scores and loadings plot for the mean percent recoveries of the five analytes spiked at the MDL.

Data codes: 1 = LLE; 2 = LLE-PPT; 3 = PPT; 4 = Q-LLE; 5 = QPPT; 6 = SPE; 7 = Q
Q = QuEChERS

From Figure 3.13, the mean percent recoveries (n = 3) for the blank sterilized human plasma spiked at the MDLs for each were analyte were compared using PLS. The optimum model used two components with a variance of 86.6 % and a predicted R^2 value of 0. 294887. The p value for the model was 0.018 which was much less than 0.05. The scores plot in Figure 3.13

(a) and the loadings plot in Figure 3.13 (b) showed that TDF, EMT and EFZ were correlated and extracted more efficiently using SPE while LPV was extracted more efficiently using QuEChERS while RTV though correlated to LPV was extracted more efficiently using LE and PPT. The mixed modes gave results that were close to QuEChERS extraction.

Using similar PLS data analysis of the percent recoveries of the spiked blank sterilized human plasma at the LLOQ, and a model that carried 82.3 % variance and all the five components, TDF, LPV, EMT and RTV were very highly correlated and were best extracted using LE. SPE on the other hand extracted EFZ more efficiently than all the other methods. QLE, QPPT and LEPPT loaded negatively and registered lower percent recoveries. These are all mixed modes recovery experiments and thus the losses at each individual extraction technique were additive.

The mean percent recoveries (n = 3) were also compared at the ULOQ using PLS and an optimum model with 2 components which had a variance of 33.9 %. The scores and loadings plots (not shown here) showed that LE was the best method for extracting RTV, LPV and EMT while SPE extracted EFZ more efficiently than all the other techniques. TDF was best extracted by Q-LLE. Q-PPT gave results that were similar to Q-LLE. LLE-PPT gave results that were similar to QuEChERS and finally PPT gave results that were similar to LLE.

A notable observation was the dependence of the extraction procedures on the spike concentrations of the analytes in the sterilized commercial human plasma. At the MDL, SPE was superior and at the LLOQ and ULOQ, LE was superior.

CHAPTER 4

4.0 CONCLUSIONS AND RECOMMENDATIONS

4.1 Conclusions

Therapeutic drug monitoring (TDM) is a fairly new area of research however several studies propose that it may contribute significantly to the treatment of HIV infected patients. There are a number of MRM LC-MS methods developed for the determination of ARVs in human blood plasma however there is limited data on the MRM LC-MS method developed for this particular cocktail of ARVs. The method has a short separation time of 9 minutes and the baseline separations were achieved without incorporating buffers which tend to crystallize and clog the separation system if mishandled. Detection limits in MS fullscan and MRM were compared and MRM detection limits were superior than the MS fullscan limits. The percent mean recoveries were between 68.8 – 85.6 % for single modes and 52.4 – 70.5 % for mixed mode techniques. Precision of all the techniques investigated was within acceptable range of < 15 % at all concentration levels for all analytes. Accuracy was calculated as a percentage of deviation of the mean value to the true value and the values were between 5.87 – 65.94 % for single mode techniques and between 21.73 – 51.59 % for mixed mode techniques. SPE proved to be more superior than the other techniques as it recorded the highest percentage recoveries and it satisfied all US FDA guidelines. MDLs for the other six sample preparation techniques fell below the clinically relevant therapeutic range (3 – 8 ppm) therefore all techniques can be employed for routine TDM studies. It was noticed that SPE sample extraction technique coupled to the developed method produced the best results regarding recovery, accuracy and precision. The recovery values for SPE were between 81.2 – 85.6 %, the accuracy values were between 5.78 – 23.41 % and the precision values were between 0.49 – 4.50 %. SPE was the only technique that satisfied the US FDA guidelines. SPE may be expensive and time consuming but the MDLs for the other six sample

preparation techniques fell below the clinically relevant therapeutic range (3 - 8ppm) therefore all techniques can be employed for routine TDM studies. The mixed modes gave slightly lower recoveries but could still be used.

4.2 Recommendations

The MRM LC-MS method with SPE extraction technique can be applied to real samples. Further research work on the stability of LPV over a wide range of TLV value could be done in order to gain an insight as to why it behaves differently to other ARVs. Further work on developing multi-residue routine TDM analytical methods should be done to cover other ARVs which are not coved by this method.

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APPENDICES

APPENDIX A: Recovery data

Appendix A1: LLE recoveries

ANALYTE	LOD (RSD %)	LLOQ (RSD %)	ULOQ (RSD %)
LPV	73.4 (9.1)	84.6 (6.7)	89.7 (5.4)
RTV	81.6 (8.5)	88.0 (8.2)	87.4 (5.5)
EMT	74.9 (6.6)	81.0 (7.1)	83.1 (5.8)
TDF	64.1 (9.9)	88.9 (12.7)	70.7 (8.5)
EFV	50.1 (9.1)	63.4 (7.2)	66.1 (8.3)
AVERAGE	**68.8%**	**81.2%**	**79.4%**

Appendix A2: PPT recoveries

ANALYTE	LOD (RSD %)	LLOQ (RSD %)	ULOQ (RSD%)
LPV	78.1 (4.7)	80.4 (5.2)	83.2 (6.8)
RTV	84.2 (5.8)	81.0 (8.9)	82.7 (7.9)
EMT	73.0 (8.3)	75.0 (5.5)	80.9 (6.3)
TDF	52.1 (10.9)	55.5 (11.0)	61.5 (9.8)
EFV	64.1 (8.4)	63.1 (8.9)	62.9 (10.4)
AVERAGE	**70.3%**	**71.0%**	**74.2%**

Appendix A3: QuEChERS recoveries

ANALYTE	LOD (RSD %)	LLOQ (RSD %)	ULOQ (RSD %)
LPV	73.0 (5.1)	75.1 (8.1)	77.0 (7.5)
RTV	83.2 (7.8)	78.5 (6.7)	79.2 (3.1)
EMT	70.1 (2.1)	77.9 (7.9)	75.8 10.1)
TDF	66.7 (8.2)	65.1 (9.5)	68.3 (8.8)
EFV	67.1 (11.1)	63.0 (9.1)	60.1 (11.7)
AVERAGE	72.0%	71.3%	72.1%

Appendix A4: SPE recoveries

ANALYTE	LOD (RSD %)	LLOQ (RSD %)	ULOQ (RSD %)
LPV	83.1 (3.4)	85.8 (4.7)	90.1 (2.7)
RTV	81.0 (5.1)	86.1 (4.1)	89.7 (5.3)
EMT	83.1 (4.4)	84.6 (5.4)	87.0 (6.1)
TDF	80.9 (7.2)	85.8 (8.1)	81.8 (6.7)
EFV	78.1 (9.2)	77.3 (7.7)	79.5 (8.2)
AVERAGE	**81.2%**	**83.9%**	**85.6%**

Appendix A5: Q-PPT recoveries

ANALYTE	LOD (RSD %)	LLOQ (RSD %)	ULOQ (RSD %)
LPV	60.3 (8.0)	65.7 (7.8)	67.8 (8.8)
RTV	65.9 (8.8)	68.5 (9.7)	66.4 (11.8)
EMT	70.1 (11.4)	57.1 (7.6)	64.1 (5.5)
TDF	50.1 (6.6)	53.7 (5.4)	63.2 (6.3)
EFV	56.7 (12.1)	59.0 (10.1)	58.1 (9.5)
AVERAGE	**60.6%**	**60.8%**	**63.9%**

Appendix A6: LLE-PPT recoveries

ANALYTE	LOD (RSD %)	LLOQ (RSD %)	ULOQ (RSD %)
LPV	55.4 (6.8)	60.1 (8.9)	62.1 (7.6)
RTV	56.7 (5.6)	62.9 (4.3)	63.1 (8.5)
EMT	53.7 (9.0)	60.1 (7.9)	65.7 (8.1)
TDF	49.1 (8.3)	50.9 (7.7)	55.2 (9.4)
EFV	46.9 (10.4)	48.7 (11.8)	50.1 (12.1)
AVERAGE	**52.4%**	**56.5%**	**59.2%**

Appendix A7: Q-LLE recoveries

ANALYTE	LOD (RSD %)	LLOQ (RSD %)	ULOQ (RSD %)
LPV	60.7 (5.7)	65.5 (6.1)	72.1(7.1)
RTV	66.9 (8.1)	68.7 (7.6)	73.6 (8.9)
EMT	68.7 (9.7)	60.1 (10.9)	70.1 (11.2)
TDF	53.2 (10.9)	66.9 (8.8)	68.7 (9.1)
EFV	55.2 (13.1)	65.8 (10.2)	67.8 (11.7)
AVERAGE	**60.9%**	**65.4%**	**70.5%**

APPENDIX B: Method detection limits (MDL's) for MRM in ppb

Appendix B1: LLE MDL's

ANALYTE	Regression Equation	R^2	LOD	LLOQ	ULOQ
TDF	y= 5e-05x + 0.00041	0.9556	14.5	29.0	48.3
EFV	y= 1e-05x – 4e-05	0.9771	28.2	56.4	94.1
EMT	y= 2e-05x – 5e-05	0.9867	18.3	36.7	61.2
LPV	y=0.0001x + 0.0051	0.9828	21.8	43.7	72.9
RTV	y=6e-05x + 0.0105	0.9697	17.2	34.4	57.3

Appendix B2: PPT MDL's

ANALYTE	Regression Equation	R^2	LOD	LLOQ	ULOQ
TDF	y= 7e-05x+0.0042	0.9800	26.3	52.0	86.7
EFV	y= 1e-05x+0.005	0.9705	15.3	30.6	51.0
EMT	y=0.0003x+0.0046	0.9556	23.4	46.9	78.1
LPV	y=0.0026x+0.1252	0.9771	18.5	37.1	61.8
RTV	y=0.0091x+0.0707	0.9467	28.6	57.3	95.5

Appendix B3: SPE MDL's

ANALYTE	Regression Equation	R^2	LOD	LLOQ	ULOQ
TDF	y=0.0001x-0.0012	0.9848	15.1	30.3	50.5
EFV	y= 6e-06x-3e-05	0.9936	12.5	25.0	41.7
EMT	y=3e-05x-9e-05	0.9815	8.0	16.1	26.8
LPV	y=0.002x+0.0025	0.9775	16.3	32.7	54.5
RTV	y=0.001x+0.0062	0.9853	19.2	38.5	64.2

Appendix B4: Q-LLE MDL's

ANALYTE	Regression Equation	R^2	LOD	LLOQ	ULOQ
TDF	y=0.0002x+0.0045	0.9609	21.8	43.7	72.9
EFV	y= 7e-05x+8e-05	0.9758	26.3	52.7	87.9
EMT	y=0.0001x-0.0006	0.9654	17.0	34.1	56.8
LPV	y=0.0016x+0.0008	0.9699	19.3	38.6	64.4
RTV	y=0.0421x+0.5059	0.9748	17.9	35.8	59.7

Appendix B5: Q-PPT MDL's

ANALYTE	Regression Equation	R^2	LOD	LLOQ	ULOQ
TDF	y= 0.0021x-0.0218	0.9704	14.8	29.9	49.6
EFV	y= 6e-05x-0.0002	0.9723	16.6	33.3	55.5
EMT	y=0.0002x+0.0029	0.9769	19.6	39.2	65.3
LPV	y=0.0112x-0.1222	0.9813	15.4	30.9	51.5
RTV	y=0.0026x+0.0877	0.9726	18.8	37.6	62.7

Appendix B6: LLE-PPT MDL's

ANALYTE	Regression Equation	R^2	LOD	LLOQ	ULOQ
TDF	y=0.0001x+0.0011	0.9839	20.8	41.7	69.5
EFV	y= 0.0004x+0.0141	0.9748	14.7	29.4	49.0
EMT	y=0.0008x+0.015	0.9625	17.2	34.4	57.4
LPV	y=0.0592x+0.212	0.9744	18.1	36.2	60.3
RTV	y=0.039x+0.0507	0.9819	15.1	30.2	50.4