CHAPTER - 1

INTRODUCTION
1.1 LIQUID CHROMATOGRAPHY

Liquid chromatography (LC) defined in its simplest terms can be regarded as the separation of components of a mixture based upon the rates at which they elute from a stationary phase typically over a mobile phase gradient. Differing affinities of the mixtures components for the stationary and mobile phase’s leads to their separation, since certain components will be more attracted to the mobile phase and will elute quickly whilst others will be retained by the stationary phase for longer and therefore will elute more slowly, i.e. have a later retention time (RT). The simplest forms of LC are based upon the separation of a sample components either on paper with a solvent, known as paper chromatography or on a thin layer of stationary phase coated onto a slide which is placed in solvent, thin-layer chromatography (TLC).

Column chromatography based LC, a much more powerful technique than the planar paper chromatography and TLC methods and with a greater sample capacity, was first credited to Mikhail S. Tswett, a Russian born botanist who in the early 1900s successfully separated plant pigments from a complex extract using a basic calcium carbonate and alumina packed glass column stationary phase and liquid solvent mobile phase. For column chromatography, flow of the mobile phase solvent is achieved in several ways. Gravity or a vacuum may be used where columns are not designed to withstand high pressures, as with the open glass columns employed by Tswett. Such columns employ large diameter solid phase particles (>50 μm), so that there is less resistance to flow and less chance of building up pressure [1]. By employing smaller solid-phase particle sizes (<10 μm), improved chromatographic separation can be achieved due to the overall greater solid-phase surface area. However, due to smaller particles having a greater flow resistance, there is a need for greater pressures to drive the liquid mobile phase solvent through the column. Thus high pressure pumps and columns packed within metal casing are employed to create and withstand the high pressures necessary for the technique known as high performance liquid chromatography (HPLC).

The technique known as HPLC was first coined by C. Horváth in 1970, who by combining high pressure pumps (~500 psi) and strengthened packed columns, created the high pressures and strengths of column necessary for the technique then
known as high pressure LC. Tremendous leaps in technology were seen over the following decade with new instruments being produced that could develop ~6000 psi of pressure and with directly incorporated injector systems, detectors and columns. By combining improved instruments with improved column chemistries (smaller particles and higher pressures), improved separations were achieved, therefore the technique was re-coined as high performance liquid chromatography, thus preserving the original HPLC acronym. HPLC is now one of the most widely used tools in analytical chemistry, having been applied to the analysis of a huge range of samples from across science and industry [2]. Almost any compound that dissolves in liquid can be analyzed, even in trace concentrations as low as parts per trillion. Traditional detectors for liquid chromatography include refractive index, electrochemical, fluorescence, and ultraviolet-visible (UV-Vis) detectors. Some of these generate two dimensional data; that is, data representing signal strength as a function of time. Others, including fluorescence and photo diode array (PDA) UV-Vis detectors generate three dimensional data [3]. These latter methods can be extremely useful for detecting certain classes of compound that either absorbs UV or fluoresce (e.g. kaempferols, quercetins and spermidines); indeed PDA detectors have frequently been linked online to HPLC or UHPLC prior to MS in bioanalytical studies to provide a further dimension to aid in the characterization of the detected analyte species [4-7]. However, to acquire the volume of information from an analyte required for full structural elucidation, it is a necessity to employ MS and or NMR spectroscopy detectors

1.2 MASS SPECTROMETRY

A mass spectrometer in simple terms functions to detect the mass-to-charge ratio ($m/z$) and abundance of the various analytes generated during ionization of a sample extract or chromatographic fraction [8]. Ionization is a key step since ions are far more easily manipulated than neutral molecules. Unfortunately differing analytes vary in their ionization efficiencies, i.e. the proportion of metabolite in solution converted to ions in the gas phase. Once ionized the mass analyzer detects the ion abundance and $m/z$, which can be related back to the analytes absolute molecular weight. A data system (run on a personal computer) is responsible for the storage of the paired $m/z$ and abundance values as well as their processing and display in a mass spectral
format. The three principal components found in all varieties of MS are an ionization source, a mass analyzer and a detector; all three components are maintained under vacuum to optimize the transmission of ions to the analyzer and detector [9].

**Advantages of LC/MS detection**

Liquid chromatography coupled with mass spectrometry detection (LC/MS) is one of the most powerful analytical tools for organic compound analysis. The key advantages of using LC/MS methods over HPLC methods include:

*Selectivity* – Co-eluting peaks can be isolated by mass selectivity and are not constrained by chromatographic resolution

*Peak assignment* – A unique chemical fingerprint for the compound of interest is generated, ensuring correct peak assignment in the presence of complex matrices.

*Molecular weight information* – Confirmation and identification of known and unknown compounds.

*Structural information* – Controlled fragmentation enables structural elucidation.

*Rapid method development* – Provides easy identification of eluted analytes without retention time validation.

*Sample matrix adaptability* – Decreases sample preparation time.

*Quantitation* – Quantitative and qualitative data can be obtained easily with limited instrument optimization.

**Instrumentation**

Mass spectrometers work by ionizing molecules and then sorting and identifying the ions according to their mass-to-charge ($m/z$) ratios. An LC/MS detector consists of three major components: an ion source that generates ions at atmospheric pressure, a mass analyzer which filters ions, and a detector that detects ions. As chromatographic peaks elute from the LC column and transfer to the ion source, two main processes occur. First, the ion source produces charged molecules or ions, and second the mobile phase is removed. Once the ions are created, they are extracted from the ion source and transferred to the mass analyzer. Sample ions are then filtered by the quadrupole mass analyzer according to their mass-to-charge ratio ($m/z$) prior to detection. The following sections will discuss the ionization methods, mass analyzers.
and detectors commonly applied in current LC-MS based pharmaceutical and bioanalysis applications.

**Interfacing the LC and MS**
There has been a major focus on improving the interface between the LC and the MS. The Liquid chromatography uses high pressure to separate a liquid phase and produces a high gas load. Mass spectrometry requires a vacuum and a limited gas load. For example, common flow from an LC is 1 mL/min. of liquid which, when converted to the gas phase, is 1 Liter/min. However, a typical mass spectrometer can accept only about 1 mL/min. of gas. Furthermore, an LC operates at near ambient temperature whereas an MS requires an elevated temperature. There is no mass range limitation for samples analyzed by the LC but there are limitations for an MS analyzer. Finally, LC can use inorganic buffers and MS prefers volatile buffers. There are a number of possible interfaces with LC and the main purpose is to evaporate the mobile phase and transfer the analytes to a gaseous phase suitable for ionization and to switch from the high or atmospheric pressures at which chromatographic separation was achieved to the lower pressures required for mass analysis. The main interfaces are described below.

*Atmospheric pressure electrospray ionization*
API electrospray (API-ES) is useful in analyzing samples that become multiply charged such as proteins, peptides, and oligonucleotides, as well as in analyzing samples that are singly charged, such as benzodiazepines and sulfated conjugates. API-ES can be used to measure the molecular weights of most polymers, peptides, proteins, and oligonucleotides up to 150,000 Daltons quickly and with high mass accuracy. In biopharmaceutical applications, chemists use API-ES to speed protein characterization, to accurately identify and characterize post-translational modifications, and to quickly confirm the molecular weight of synthetic peptides. Electrospray relies in part on chemistry to generate analyte ions in solution before the analyte reaches the mass spectrometer. The LC eluent is sprayed (nebulized) into a chamber at atmospheric pressure in the presence of a strong electrostatic field and heated drying gas. The electrostatic field causes further dissociation of the analyte molecules.
The electrostatic field causes further dissociation of the analyte molecules. The heated drying gas causes the solvent in the droplets to evaporate. As the droplets shrink, the charge concentration in the droplets increases. Eventually, the repulsive force between ions with like charges exceeds the cohesive forces and ions are ejected (desorbed) into the gas phase. These ions are attracted to and pass through a capillary sampling orifice into the mass analyzer.

**Atmospheric pressure chemical ionization**

In APCI, the LC eluent is sprayed through a heated (typically 250°C – 400°C) vaporizer at atmospheric pressure. The heat vaporizes the liquid. The resulting gas-phase solvent molecules are ionized by electrons discharged from a corona needle. The solvent ions then transfer charge to the analyte molecules through chemical reactions (chemical ionization). The analyte ions pass through a capillary sampling orifice into the mass analyzer. APCI is applicable to a wide range of polar and non-polar molecules. It rarely results in multiple charging so it is typically used for molecules less than 1,500 u. Due to this, and because it involves high temperatures, APCI is less well-suited than electrospray for analysis of large biomolecules that may be thermally unstable. APCI is used with normal-phase chromatography more often than electrospray is because the analytes are usually non-polar.
Atmospheric pressure photo-ionization (APPI) for LC/MS is a relatively new technique. As in APCI, a vaporizer converts the LC eluent to the gas phase. A discharge lamp generates photons in a narrow range of ionization energies. The range of energies is carefully chosen to ionize as many analyte molecules as possible while minimizing the ionization of solvent molecules. The resulting ions pass through a capillary sampling orifice into the mass analyzer. APPI is applicable to many of the same compounds that are typically analyzed by APCI. It shows particular promise in two applications, highly nonpolar compounds and low flow rates (<100 μl/min), where APCI sensitivity is sometimes reduced. In all cases, the nature of the analyte(s) and the separation conditions has a strong influence on which ionization technique: electrospray, APCI, or APPI, will generate the best results. The most effective technique is not always easy to predict.
Compared to previous LC/MS interfaces, the API-ES and APCI interfaces are relatively rugged. In many cases, existing methods may be used with little or no adaptation. Some instruments allow flow rates of 1 to 2 ml/min without splitting. One of the most critical factors in adapting LC methods is the choice of buffer. Nonvolatile buffers interfere with good MS performance. For the best long-term performance, it is highly recommended that the method be modified to use a volatile buffer.

**Mass analyzers**

All mass analyzers determine the mass of an ion and mass to charge ratio and measure gas-phase ions. There are four main types of mass analyzers:

**Sector mass analyzer**

This is the traditional magnetic sector mass spectrometer in which ions created in the ion source are accelerated with high voltages into the analyzer magnetic field. The radius of curvature in a given magnetic field of the sector is a function of m/z (mass to charge ratio). Ions of differing masses can be separated and detected by a single detector by varying the magnetic field or the source voltage to scan the mass range.
Sector MS is capable of separating all ions all the time but can only detect one mass at a time.

**Quadrupoles**

A quadrupole MS consists of four parallel rods (quads) equally spaced around a central axis. Ions are introduced along the axis of the poles. The ions are accelerated at low voltages, and by applying different voltages to the different quadrupoles, conditions can be established in which only ions with a particular m/z ratio can pass through to the ion detector. Effectively only one ion is monitored; this is known as single ion monitoring (SIM) and is the most sensitive method for a single quad.

**Ion trap MS**

This mass analyzer works by trapping ions and then detecting them based on their m/z ratios. The ion trap is a variation of the quad mass filter and uses the same principles to trap the ions. After trapping, the ions are detected by placing them in unstable orbits causing them to leave the trap. The ion trap is used in drug metabolism but are not as suitable for high-throughput bio-analysis as quads in a tandem MS.

**Time of flight (TOF) analyzers**

These analyzers are based on the fact that ion velocity is mass dependant. They consist of an ion source, a ‘flight’ tube and a detector. Each mass enters the flight tube at different velocities, small mass ions having a higher velocity. The mass ions separate as they pass down the tube and arrive at the detector. TOF has ideal characteristics for structural analysis and are often combined in modern MS with quads.

**Detectors**

Detection of ions is based upon their charge or momentum. For large signals a faraday cup is used to collect ions and measure the current. Older instruments used photographic plates to measure the ion abundance at each mass to charge ratio. Most detectors currently used amplify the ion signal using a collector similar to a photomultiplier tube. These amplifying detectors include: electron multipliers, channeltrons and multichannel plates. The gain is controlled by changing the high
voltage applied to the detector. A detector is selected for its speed, dynamic range, gain, and geometry. Some detectors are sensitive enough to detect single ions.

**Tandem mass spectrometry**

Tandem mass spectrometry also called mass spectrometry–mass spectrometry (MS–MS) because the instrument contains two mass spectral analyzers in tandem; between the two analyzers is a collision gas cell. Generally soft ionization techniques do not cause fragmentation of the ionized particles. The basic approach of MS–MS is the measurement of mass to charge ratios of ions before and after fragmentation of the selected ion by collision with a high-pressure gas (normally helium). This collision process is called collision induced dissociation (CID). There are two main methods for ion monitoring i.e. single ion monitoring (SIM) and selective reaction monitoring (SRM) or multiple reactions monitoring (MRM).

### 1.3 BIOANALYTICAL METHOD VALIDATION

Validation is a process required by law and the concept is described by regulatory agencies in guidance documents [10-14] into acceptable practices. Method validation is not a onetime process that can be ignored once completed, but a constant, evolving process. A well executed and documented validation serve as evidence to regulatory agencies that the method in question is complaint and performs as intended. To be successful, applicants should remain up to date with the current thinking of regulatory agencies and anticipate the changes that will occur in regulations.

The main objective of bioanalytical method validation is to demonstrate the reliability of a method for the determination of an analyte in a specific biological matrix, such as blood, serum, plasma, urine, or saliva. Moreover, if an anticoagulant is used validation should be performed using the similar anticoagulant as used in study samples. Generally a full validation should be performed for each species and matrix concerned. The main characteristics of a bioanalytical method that are essential to ensure the acceptability of the performance and the reliability of analytical results are; selectivity, lower limit of quantification, the response function and calibration range (calibration curve performance), accuracy, precision, matrix effects, stability of the analyte (s) in the biological matrix and stability of the analyte (s) and of the internal
standard in the stock and working solutions and in extracts under the entire period of storage and processing conditions. During method validation and analysis of study samples, a blank biological matrix will be spiked with the analyte(s) of interest using solutions of reference standard(s) to prepare calibration standards, quality control samples and stability samples. During validation it is important that the quality of the reference standard and ISID is ensured, as its purity may affect the outcome of the analysis, and therefore the outcome of the study data. Therefore the reference standards used during the validation and study sample analysis should be obtained from an authentic and traceable source.

Selectivity
The analytical method should be able to differentiate the analyte(s) of interest and ISTD from endogenous components in the matrix or other components in the sample. Selectivity should be proved using at least 6 individual sources of the appropriate blank matrix, which are individually analyzed and evaluated for interference. Selectivity is acceptable where the response in blank matrix is less than 20% of the lower limit of quantification for the analyte and 5% for the ISTD. It may also be necessary to investigate the extent of any interference caused by metabolites of the drug(s), interference from degradation products formed during sample preparation, and interference from possible co-administered medications. Co-medications normally used in the subject population studied which may potentially interfere should be taken into account at the stage of method validation or on a study specific and compound specific base.

Carry-over
Carry-over should be addressed and minimised during method development. During validation carry-over should be assessed by injecting blank samples after a high concentration sample or calibration standard at the upper limit of quantification. Carry over in the blank sample following the high concentration standard should not be greater than 20% of the lower limit of quantification and 5% for the ISTD.
**Lower limit of quantification**

The lower limit of quantification (LLOQ) is the lowest concentration of analyte in a sample which can be quantified reliably, with an acceptable accuracy and precision. The LLOQ is considered to be the lowest calibration standard. In addition, the analyte signal of the LLOQ sample should be at least 5 times the signal of a blank sample. The LLOQ should be adapted to expected concentrations and to the aim of the study.

**Calibration curve**

The response of the instrument with regard to the concentration of analyte should be known, and should be evaluated over a specified concentration range. The calibration standards should be prepared in the same matrix as the matrix of the intended study samples by spiking the blank matrix with known concentrations of the analyte. Ideally, before carrying out the validation of the analytical method it should be known what concentration range is expected. This range should be covered by the calibration curve range, defined by the LLOQ being the lowest calibration standard and the upper limit of quantification (ULOQ), being the highest calibration standard. The range should be established to allow adequate description of the pharmacokinetics of the analyte of interest. A minimum of six calibration concentration levels should be used, in addition to the blank sample (processed matrix sample without analyte and without IS) and a zero sample (processed matrix with IS). The back calculated concentrations of the calibration standards should be within ±15% of the nominal value, except for the LLOQ for which it should be within ±20%. At least 75% of the calibration standards, with a minimum of six calibration standard levels, must meet this criterion. In case a calibration standard does not comply with these criteria, this calibration standard sample should be rejected, and the calibration curve without this calibration standard should be re-evaluated, including regression analysis.

**Accuracy**

The accuracy of an analytical method describes the closeness of the determined value obtained by the method to the nominal concentration of the analyte (expressed in percentage). Accuracy should be assessed on samples spiked with known amounts of the analyte, the quality control samples (QC samples). The QC samples should be
spiked independently from the calibration standards, using separately prepared stock solutions, unless the nominal concentration(s) of the stock solutions have been established. The QC samples are analyzed against the calibration curve, and the obtained concentrations are compared with the nominal value. The accuracy should be reported as percent of the nominal value. Accuracy should be evaluated for the values of the QC samples obtained within a single run (the within run accuracy) and in different runs (the between-run accuracy).

**Within-run accuracy**

Within-run accuracy should be determined by analysing in a single run a minimum of 5 samples per level at a minimum of 4 concentration levels which are covering the calibration curve range: the LLOQ, within three times the LLOQ (low QC), around 50% of the calibration curve range (medium QC), and at least at 75% of the upper calibration curve range (high QC). The mean concentration should be within 15% of the nominal values for the QC samples, except for the LLOQ which should be within 20% of the nominal value.

**Between -run accuracy**

For the validation of the between-run accuracy, LLOQ, low, medium and high QC samples from at least three runs analysed on at least two different days should be evaluated. The mean concentration should be within 15% of the nominal values for the QC samples, except for the LLOQ which should be within 20% of the nominal value.

**Precision**

The precision of the analytical method describes the closeness of repeated individual measures of analyte. Precision is expressed as the coefficient of variation (CV). Precision should be demonstrated for the LLOQ, low, medium and high QC samples, within a single run and between different runs, i.e. using the same runs and data as for the demonstration of accuracy.

**Within-run precision**

For the validation of the within-run precision, there should be a minimum of five samples per concentration level at LLOQ, low, medium and high QC samples in a
single run. The within-run CV value should not exceed 15% for the QC samples, except for the LLOQ which should not exceed 20%.

**Between –run precision**
For the validation of the between-run precision, LLOQ, low, medium and high QC samples from at least three runs analysed on at least two different days should be evaluated. The between-run CV value should not exceed 15% for the QC samples, except for the LLOQ which should not exceed 20%.

**Dilution integrity**
Dilution of samples should not affect the accuracy and precision. If applicable, dilution integrity should be demonstrated by spiking the matrix with an analyte concentration above the ULOQ and diluting this sample with blank matrix (at least five determinations per dilution factor). Accuracy and precision should be within the set criteria, i.e. within ±15%. Dilution integrity should cover the dilution applied to the study samples.

**Matrix effect**
Matrix effects should be investigated when using mass spectrometric methods, using at least 6 lots of blank matrix from individual donors. Pooled matrix should not be used. For each analyte and the IS, the matrix factor (MF) should be calculated for each lot of matrix, by calculating the ratio of the peak area in the presence of matrix (measured by analyzing blank matrix spiked after extraction with analyte), to the peak area in absence of matrix (pure solution of the analyte). The IS normalised MF should also be calculated by dividing the MF of the analyte by the MF of the IS. The CV of the IS-normalised MF calculated from the 6 lots of matrix should not be greater than 15 %. This determination should be done at a low and at a high level of concentration (maximum of 3 times the LLOQ and close to the ULOQ).

**Stability**
Evaluation of stability should be carried out to ensure that every step taken during sample preparation and sample analysis, as well as the storage conditions used do not affect the concentration of the analyte. Stability of the analyte in the studied matrix is evaluated using low and high QC samples (blank matrix spiked with analyte at a
concentration of a maximum of 3 times the LLOQ and close to the ULOQ) which are analyzed immediately after preparation and after the applied storage conditions that are to be evaluated. The QC samples are analyzed against a calibration curve, obtained from freshly spiked calibration standards, and the obtained concentrations are compared to the nominal concentrations. The mean concentration at each level should be within ±15% of the nominal concentration. Stability of the stock and working solutions should be tested with an appropriate dilution, taking into consideration the linearity and measuring range of the detector. Stability studies should investigate the different storage conditions over time periods that equal or exceed those applied to the actual study samples. At minimum the following stability tests should be evaluated:

a) stability of the stock solution and working solutions of the analyte and internal standard
b) freeze and thaw stability of the analyte in the matrix from freezer storage conditions to room temperature or sample processing temperature
c) short term stability of the analyte in matrix at room temperature or sample processing temperature
d) long term stability of the analyte in matrix stored in the freezer
e) stability of the processed sample at room temperature or under the storage conditions to be used during the study (dry extract or in the injection phase and autosampler stability)

In the evaluation of freeze-thaw stability, the QC samples are stored and frozen in the freezer at the intended temperature and thereafter thawed at room or processing temperature. After complete thawing, samples are refrozen again applying the same conditions. At each cycle, samples should be frozen for at least 12 hours before they are thawed. The number of cycles in the freeze-thaw stability should equal or exceed that of the freeze / thaw cycles of study samples. During long term stability of the analyte in matrix stored in the freezer, the QC samples should be stored in the freezer under the same storage conditions and at least for the same duration as the study samples. For small molecules it is considered acceptable to apply a bracketing approach, i.e. in case stability has been proved for instance at -70°C and -20°C, it is
not necessary to investigate the stability at temperatures in between. For large molecules (such as peptides and proteins) stability should be studied at each temperature at which study samples will be stored.

While establishing stability of stock and working solutions it is not necessary to study the stability at each concentration level of working solutions and a bracketing approach can be used. It is not required to study the stability of stable-isotope labeled internal standards if it is demonstrated that no isotope exchange reactions occur under the same conditions as the stability of the analyte was demonstrated. At a minimum the stability demonstration shall be performed for both stock solutions and working solutions at the intended storage conditions at high concentration.

1.4 HUMAN IMMUNODEFICIENCY VIRUS

The human immunodeficiency virus (HIV) [15, 16] is a retrovirus that infects cells of the immune system, destroying or impairing their function. As the infection progresses, the immune system becomes weaker and the person becomes more susceptible to infections. The most advanced stage of HIV infection is called as acquired immunodeficiency syndrome (AIDS). It can take 10-15 years for an HIV infected person to develop AIDS [17]. Since the beginning of the epidemic, almost 70 million people have been infected with the HIV virus and about 36 million people have died of AIDS. At the end of 2012, globally 35.3 million people were living with HIV [18]. The burden of the epidemic continues to vary considerably between countries and regions and worldwide an estimated 0.8% of adults aged between 15-49 years are living with HIV. Sub-saharan Africa remains the most severely affected, with nearly 1 in every 20 adults living with HIV and accounting for 69% of the people living with HIV worldwide. HIV infection is usually diagnosed through blood tests detecting the presence or absence of HIV antibodies. Antibodies are produced by an individual’s immune system to fight off foreign pathogens. An HIV test reveals infection status by detecting the presence or absence of antibodies to HIV in the blood. Most people have a window period of usually 3 to 6 weeks during which antibodies to HIV are still being produced and are not yet detectable. This early period of infection represents the time of greatest infectivity, but transmission can occur during all stages of the infection. HIV is transmitted through unprotected sexual
intercourse (anal or vaginal), transfusion of contaminated blood, sharing of contaminated needles and between a mother and her infant during pregnancy, childbirth and breastfeeding [19]. Individuals cannot become infected through ordinary day-to-day contact such as kissing, hugging, shaking hands or sharing personal objects, food or water [20].

Since the beginning of the epidemic, WHO has been leading the global health sector response to HIV. As a cosponsor of the Joint United Nations Programme on AIDS (UNAIDS), WHO leads on the priority areas of HIV treatment, care and HIV / tuberculosis co-infection, and jointly coordinates with UNICEF to work on the elimination of mother-to-child transmission of HIV. United States Food and Drug Administration (USFDA) had also taken initiatives over a variety of HIV / AIDS related issues. The agency primarily serves a review and oversight function in areas related to drugs, biologics and medical devices for the prevention and treatment of HIV / AIDS and AIDS related conditions. Among the regulatory activities related to regulation of therapeutic products, FDA works with sponsors (developers) of new products to assure that clinical trials are well designed, scientifically sound, ethically conducted and appropriately analyzed. It works with the pharmaceutical industry and other researchers in the development of useful immune based therapies, which may contribute to the body's own defense against HIV and improve clinical outcome over drug therapy alone. To support the President's Emergency Program for AIDS Relief (PEPFAR), FDA developed special expedited review procedures that allow FDA to quickly review applications for generic formulations or fixed-dose combinations of approved drugs. FDA's assessment process helps make the drugs available in developing countries.

1.5 ANTIRETROVIRAL THERAPY
HIV / AIDS can be suppressed by combination antiretroviral therapy (ART) consisting of three or more antiretroviral (ARV) drugs. ART does not cure HIV infection but controls viral replication within a person's body and allows an individual's immune system to strengthen and regain the capacity to fight off infections. There are several classes of antiretroviral agents that act on different stages of the HIV life-cycle. The use of multiple drugs that act on different viral targets is
known as highly active antiretroviral therapy (HAART). HAART decreases the patient's total burden of HIV, maintains function of the immune system and prevents opportunistic infections that often lead to death [21]. With ART, people living with HIV can live healthy and productive lives. More than 9.7 million people living with HIV in low and middle income countries were receiving ART at the end of 2012. This is over 30 fold increase in the number of people receiving ART in developing countries between 2003 and 2012 and close to a 20% increase in just one year (i.e between 2011 and 2012).

Anti-retroviral (ARV) drugs can be broadly classified by the phase of the retrovirus life cycle that the drug inhibits. Typical ARV combinations include 2 NRTIs + 1 PI (Protease Inhibitor) or 2 NRTIs + 1 NNRTI (Non-Nucleoside Reverse Transcriptase Inhibitor) [22, 23].

**Entry inhibitors (or fusion inhibitors)**
These drugs interfere with binding, fusion and entry of HIV-1 to the host cell by blocking one of several targets.
Eg: maraviroc and enfuvirtide

**Nucleoside reverse transcriptase inhibitors (NRTI) and nucleotide reverse transcriptase inhibitors (NtRTI)**
These are nucleoside and nucleotide analogs which inhibit reverse transcription. NRTIs are chain terminators such that once incorporated, work by preventing other nucleosides from also being incorporated because of the absence of a 3-hydroxy group. Both nucleoside and nucleotide analogs act as competitive substrate inhibitors. Eg: deoxythymidine, zidovudine, stavudine, didanosine, zalcitabine, abacavir, lamivudine, emtricitabine, and tenofovir.

**Non-nucleoside reverse transcriptase inhibitors (NNRTI)**
NNRTIs inhibit reverse transcriptase by binding to an allosteric site of the enzyme; NNRTIs act as non-competitive inhibitors of reverse transcriptase. NNRTIs affect the handling of substrate (nucleotides) by reverse transcriptase by binding near the active site and causing “molecular arthritis”. NNRTIs can be further classified into 1st generation and 2nd generation NNRTIs. 1st generation NNRTIs are more rigid in
structure and resistance can quickly be developed against them. Because 2nd generation NNRTIs have a more flexible structure, they can adjust more readily and resist mutation more effectively.

Eg: nevirapine, delavirdine, efavirenz, and rilpivirine

**Integrase inhibitors**

The drugs inhibit the enzyme integrase, which is responsible for integration of viral DNA into the DNA of the infected cell. There are several integrase inhibitors currently under clinical trial, and raltegravir became the first to receive FDA approval in October 2007.

Eg: raltegravir, elvitigravir.

**Protease inhibitors**

Protease inhibitors block the viral protease enzyme necessary to produce mature virions upon budding from the host membrane. Particularly, these drugs prevent the cleavage of gag and gag/pol precursor proteins.

Eg: lopinavir, indinavir, nelfinavir, amprenavir and ritonavir.

The life cycle of HIV can be as short as about 1.5 days from viral entry into a cell, through replication, assembly, and release of additional viruses, to infection of other cells [24]. HIV lacks proofreading enzymes to correct errors made when it converts its RNA into DNA via reverse transcription. Its short life-cycle and high error rate cause the virus to mutate very rapidly, resulting in a high genetic variability of HIV. Most of the mutations either are inferior to the parent virus (often lacking the ability to reproduce at all) or convey no advantage, but some of them have a natural selection superiority to their parent and can enable them to slip past defenses such as the human immune system and antiretroviral drugs. The more active copies of the virus, the greater the possibility that one resistant to antiretroviral drugs will be made [25].

When antiretroviral drugs are used improperly, these multi-drug resistant strains can become the dominant genotypes very rapidly. Improper serial use of the reverse transcriptase inhibitors zidovudine, didanosine, zalcitabine, stavudine, and lamivudine can lead to the development of multi-drug resistant mutations [26].
Antiretroviral combination therapy defends against resistance by suppressing HIV replication as much as possible. Combinations of antiretrovirals create multiple obstacles to HIV replication to keep the number of offspring low and reduce the possibility of a superior mutation. If a mutation that conveys resistance to one of the drugs being taken arises, the other drugs continue to suppress reproduction of that mutation. With rare exceptions, no individual antiretroviral drug has been demonstrated to suppress an HIV infection for long; these agents must be taken in combinations in order to have a lasting effect. As a result, the standard of care is to use combinations of antiretroviral drugs. Combinations usually comprise two nucleoside-analogue RTIs and one non-nucleoside-analogue RTI or protease inhibitor [27]. This three drug combination is commonly known as a triple cocktail [28]. Combinations of antiretrovirals are subject to positive and negative synergies, which limit the number of useful combinations. In recent years, drug companies have worked together to combine these complex regimens into simpler formulas, termed fixed-dose combinations. For instance, two pills containing two or three medications each can be taken twice daily. This greatly increases the ease with which they can be taken, which in turn increases adherence, and thus their effectiveness over the long-term. Lack of adherence is a cause of resistance development in medication experienced patients. Patients who maintain proper therapy can stay on one regimen without developing resistance [29]. This greatly increases life expectancy and leaves more drugs available to the individual should the need arise.

1.6 SELECTION OF DRUGS

In the current research work the following antiretroviral drugs; zidovudine (ZDV), lamivudine (3TC), nevirapine (NVP), tenofovir (TFV), emtricitabine (FTC), efavirenz (EFV) and didanosine (DDI) were selected to develop high throughput LC-MS/MS bioanalytical methods for their estimation as individual or in combination in human plasma. The developed methods are useful to monitor the pharmacokinetics and pharmacodynamics to evaluate bioequivalence, therapeutic drug monitoring for these drugs or combinations. These high throughput methods would be highly useful for generic industry to expedite their regulatory filings, which in turn would be useful for the developing countries to have access to the new formulations or combinations in minimum possible time, to effectively control the AIDS epidemic.
**Chapter-2** describes the simultaneous LC-MS/MS method for the estimation of TFV, 3TC and NVP in human plasma. This is a nucleotide and nucleoside analog combination and it is one of the four recommended regimens by WHO for antiretroviral therapy in resource-limited settings. It is supplied as fixed dose combination of TFV and 3TC co-packaged with NVP. The combination is proven to be cost effective in first-line treatment with appropriate therapeutic activity. The current method was the first reported bioanalytical method for this potent formulation and is highly sensitive with an LOQ of 2 ng/mL for TFV and 10 ng/mL for 3TC and ZDV. The method was successfully applied to evaluate the bioequivalence of TFV and 3TC tablets (300 + 300 mg), in healthy human volunteers under fasting condition.

**Chapter-3** describes a high throughput LC-MS/MS method for determination of DDI in human plasma. DDI is a purine nucleoside analog with in-vitro activity against HIV. It is a reverse transcriptase inhibitor used in combination with other drugs as part of highly active antiretroviral therapy (HAART). This was the first reported individual LC-MS/MS method for the determination of DDI using negative electrospray ionization technique. The method is superior over other reported methods with respect to low quantitation limit and high throughput run time. It was successfully applied to a bioequivalence study to compare the pharmacokinetics of 400 mg of DDI delayed release capsules.

**Chapter-4** describes a high throughput LC-MS/MS method for determination of EFV in human plasma. It is a non-nucleoside reverse transcriptase inhibitor (NNRTI) which is used in the treatment of human immunodeficiency virus type 1 (HIV-1). The current method is superior over the reported methods with respect to low quantitation limits, high throughput run time, sample clean-up and low sample processing time. The method was successfully applied to a bio-study to compare the pharmacokinetics of 600 mg of EFV tablets.

**Chapter-5** describes the simultaneous LC-MS/MS method for the estimation of ZDV, 3TC and NVP in human plasma. It is a triple nucleoside reverse transcriptase inhibitor regimen, which is commonly indicated for the treatment of human
immunodeficiency virus type 1 (HIV-1). The combination has low hepatotoxicity and minimal interaction with anti tuberculosis therapy. It is one of the four recommended first line regimens by WHO for the treatment of HIV in adults and children. It is usually supplied in the form of tablets and tablets for oral suspension (TFOS). The current method is superior over the reported methods with respect to sensitive detection limits and high throughput run time. The method was successfully used to compare the pharmacokinetics of tablets for oral suspension (TFOS, test) against the ready to use suspension (reference).

Chapter-6 describes a high throughput LC-MS/MS method for determination of FTC in human plasma. It is a nucleoside reverse transcriptase inhibitor (NRTI) which is used in the treatment of human immunodeficiency virus (HIV) in adults and children. FTC is included in current WHO model List of Essential Medicines (EML) and various international guidelines for the treatment of HIV infection. The developed method is superior over the reported methods with respect to sensitive quantification limits, low sample processing volume with highly selective processing method and high throughput run time. It is successfully applied to a bio-study to compare the pharmacokinetics of 200 mg of FTC capsules.
1.7 REFERENCES


