3.1. LITERATURE REVIEW OF INSTRUMENTAL METHODS OF ANALYSIS OF ATAZANAVIR SULFATE

3.1.1. Schuster et al \[40\] developed a selective, accurate, and reproducible LC-MS-MS assay for the determination of the HIV protease inhibitor atazanavir (BMS-232632) in human plasma samples. The method involved automated solid-phase extraction of atazanavir and a stable isotope analogue internal standard (I.S.). A portion of the reconstituted sample residue was injected onto a C\textsubscript{18}HDO analytical column which was configured with a triple quad mass spectrometer for analyte determination by positive ion electrospray.

3.1.2. Jemal M et al \[41\] developed a selective, accurate, and reproducible LC/MS/MS assay and validated for the determination of the HIV protease inhibitor atazanavir (BMS-232632) in human peripheral blood mononuclear cells (PBMC) samples. In addition to the details of the validated LC/MS/MS method. The method involved automated solid-phase extraction (SPE) of atazanavir and a stable isotope analogue internal standard (I.S.). A portion of the reconstituted sample residue was injected onto an YMC basic analytical column which was connected to a triple quad mass spectrometer for analyte determination by positive-ion electrospray in the selected reaction monitoring (SRM) mode.

3.1.3. Crommentuyn et al \[42\] developed and validated an assay, using liquid chromatography coupled with electrospray tandem mass spectrometry (LC–MS/MS), for the quantification of the novel protease inhibitors (PIs) atazanavir and tipranavir. The sample pre-treatment consisted of protein precipitation with a mixture of methanol and acetonitrile using 100\,\mu l plasma for atazanavir and 50\,\mu l for tipranavir. Chromatographic separation was achieved on an Inertsil ODS3 column (50 mm×2.0 mm i.d., particle size 5 \mu m), with a quick stepwise gradient using an acetate buffer (pH 5) and methanol, at a flow rate of 0.5 ml/min.

3.1.4. Colombo et al \[43\] reported a previously HPLC method for the simultaneous analysis of the new HIV protease inhibitor Atazanavir (ATV) in human plasma, by off-line solid-phase extraction (SPE) followed by HPLC coupled with UV-diode array detection. After viral inactivation by heat (60°C for 60 min), plasma (600 \mu l) with
clozapine (internal standard) is diluted 1:1 with phosphate buffer pH 7 and subjected to a SPE on a C18 cartridge. Matrix components are eliminated with $2 \times 500 \mu l$ of a solution of 0.1% $H_3PO_4$ neutralised with NaOH to pH 7. ATV is eluted with $3 \times 500 \mu l$ MeOH. The resulting eluate is evaporated under nitrogen at room temperature and is reconstituted in 100 $\mu l$ MeOH/H$_2$O 50:50. A 40 $\mu l$ volume is injected onto a Nucleosil 100-5 $\mu m$ C18 AB column. ATV is analysed by UV detection at 201 nm using a gradient elution program with solvents constituted of MeCN and phosphate buffer adjusted to pH 5.14. The mobile phase also contains 0.02% sodium heptanesulfonate, enabling an excellent separation of ATV from the other HIV protease inhibitors (PIs) amprenavir, indinavir, saquinavir, ritonavir, lopinavir, nelfinavir and the non-nucleoside reverse transcriptase inhibitors (NNRTIs) efavirenz and nevirapine.

3.1.5. Dailly et al [44] proposed a HPLC method for determination of atazanavir and of all other protease inhibitors (PI) and non-nucleoside reverse transcriptase inhibitors (NNRTI) in plasma which are currently used to treat HIV patients. All drugs are extracted after a liquid-liquid extraction and separated on a C18 column with a binary gradient elution except lopinavir which is separated without gradient elution. The absorbance is measured at 259 nm except for lopinavir (205 nm) and nevirapine (320 nm).

3.1.6. Colombo et al [45] proposed a sensitive and accurate liquid chromatography–tandem mass spectrometric (LC–MS/MS) method for the intracellular determination of nine antiretroviral drugs in human peripheral blood mononuclear cells (PBMCs). PBMCs are isolated by density gradient centrifugation using Vacutainer™ CPT tubes and cell count is performed with a Coulter® instrument. Single-step extraction of drugs from PBMCs pellets is performed with MeOH 50% (with clozapine added as internal standard, I.S.) and the supernatant is injected onto a 2.1 mm $\times$ 30 mm SymmetryShield™ 3.5 $\mu m$-RP18 column equipped with a 2.1 $\times$ 10 mm guard column. Chromatographic separations are performed using a gradient program with a mixture of 2 mM ammonium acetate containing 0.1% formic acid and acetonitrile with 0.1% formic acid. Analytes quantification is performed by electro-spray ionisation–triple quadrupole mass spectrometry using the selected reaction monitoring (SRM) detection mode. The positive mode is used for the HIV protease inhibitors (PIs) indinavir, amprenavir, saquinavir, ritonavir, nelfinavir, lopinavir, atazanavir and the non-
nucleoside reverse transcriptase inhibitors (NNRTIs) nevirapine, and the negative mode is applied for efavirenz.

3.1.7. Poirier et al \[46\] developed a simultaneous HPLC determination of the new protease inhibitor atazanavir with all the others currently in use (M8 nelfinavir metabolite included) and the 2 widely used non-nucleoside reverse transcriptase inhibitors efavirenz and nevirapine. This simple HPLC method allows the analysis of all these drugs at a single ultraviolet wavelength following a 1-step liquid-liquid extraction procedure. A 500 μl plasma sample was spiked with internal standard and subjected to liquid-liquid extraction using diethyl ether at pH 10. HPLC was performed using a Symmetry Shield RP18 and gradient elution. All the drugs of interest and internal standard were detected with ultraviolet detection at 210 nm.

3.1.8. Tribut et al \[47\] described a user-friendly, validated, and rapid technique for simultaneous quantification of amprenavir, indinavir, lopinavir, nelfinavir (and its M8 metabolite), ritonavir, saquinavir, efavirenz, and nevirapine. Assays were performed after diethyl ether liquid-liquid extraction from 250 μl plasma samples. Chromatographic separation was achieved on an X-TERRA column using a 58% water (with 3 mM pyrrolidine) and 42% acetonitrile mobile phase; 240 nm was used for atazanavir detection.\[47\]

3.1.9. Cateau et al \[48\] proposed a simple high-performance liquid chromatographic method using UV detection and validated for the analysis of atazanavir in human plasma. The sample clean up was carried out using solid-phase extraction with OASIS® MCX cartridge. The chromatographic separation was achieved on a Kromasil® C18 (150 mm × 3 mm, 5 μm) column with a mobile phase consisting of acetonitrile and water (38:62 v/v) delivered isocratically. The effluent of the column was monitored at a wavelength of 210 nm.

3.1.10. Dickinson et al \[49\] reported a precise and accurate method for simultaneous quantification of protease inhibitors (PIs) amprenavir, atazanavir, indinavir, lopinavir, nelfinavir, ritonavir and saquinavir in plasma. An internal standard was added to samples prior to protein precipitation with acetonitrile followed by addition of ammonium formate buffer. Analysis was by HPLC-MS/MS.
3.1.11. Xu et al\textsuperscript{[50]} reported a monolithic-phase based on-line extraction for pharmaceutical component analysis in plasma by LC–MS/MS. In this approach, a short monolithic C18 4.6mm×10mm cartridge is used for high flow extraction at 4 mL/min. Plasma samples were subjected to protein precipitation first with acetonitrile and the supernatant was diluted and loaded onto a monolithic cartridge. Sample elution was accomplished with narrow-bore LC–MS/MS system. A method for determination of Amprenavir (APV) and Atazanavir (AZV) in human plasma was developed with this approach.

3.1.12. Rezk et al\textsuperscript{[51]} reported a validated analytical method that combines atazanavir with 6 HIV-protease inhibitors (indinavir, amprenavir, saquinavir, nelfinavir, ritonavir, and lopinavir) and 2 non-nucleoside reverse transcriptase inhibitors (nevirapine and efavirenz). Using 200 μL of plasma and a simple liquid-liquid extraction method, this analytical method achieved high extraction efficiencies (90.0% to 99.5%). A Zorbax C-18 (150 × 4.6 mm, 3.5 μm) analytical column was used along with a 27-minute linear gradient elution of the mobile phase to provide sharp peaks at 210 nm. This method was validated over a range of 25 to 10,000 ng/ml.

3.1.13. Loregian et al\textsuperscript{[52]} proposed a simple high-performance liquid chromatography method for the determination of the human immunodeficiency virus protease inhibitor atazanavir in human plasma samples and validated. The method involved a rapid and simple solid-phase extraction of atazanavir using Oasis HLB 1 cc cartridges, an isocratic reversed-phase liquid chromatography on an XTerra RP18 (150 mm × 4.6 mm, 3.5 μm) column, and ultraviolet detection at 203 nm. The mobile phase consisted of phosphate buffer (pH 6, 52.5 mM) and acetonitrile (43: 57, v/v).

3.1.14. Verbesselt et al\textsuperscript{[53]} proposed a simple, accurate and fast method for determination of the commonly used HIV protease inhibitors (PIs) amprenavir, indinavir, atazanavir, ritonavir, lopinavir, nelfinavir, M8-nelfinavir metabolite and saquinavir in human plasma. Liquid-liquid extraction was used with hexane/ethylacetate from buffered plasma samples with a borate buffer pH 9.0. Isocratic chromatographic separation of all components was performed on an Allsphere hexyl HPLC column with combined UV and fluorescence detection.
3.1.15. Keil et al \[54\] applied a previously published reversed-phase high-performance liquid chromatography (HPLC) method from their laboratory for quantification of ATV in Therapeutic Drug Monitoring. Assay was done by a PDA detector at 248nm. The assay has met passing requirements for inter-laboratory proficiency testing for 2 years nationally and internationally, with accuracy within ±15% over all test samples. During 2 years, more than 100 batches of analyses have been performed.

3.1.16. Seshachalam et al \[55\] proposed a forced degradation study, successfully applied for the development of a stability-indicating assay method for the determination of atazanavir in presence of its degradation products. The method was developed and optimized by analyzing the forcefully degraded samples. Degradation of the drug was done under acidic, alkaline, oxidative, photolytic and thermal stress conditions. The major impurities are generated in acidic and alkaline conditions. The product was found stable under thermal, photolytic and oxidative conditions.

3.1.17. Weller et al \[56\] proposed an efficient, isocratic high performance liquid chromatography (HPLC) method for determining human immunodeficiency virus (HIV) non-nucleoside reverse transcriptase inhibitors (NNRTIs) and protease inhibitors (PIs) in plasma. The combination of isocratic reversed phase chromatography using an S-3, 3.0 mm × 150 mm column along with low plasma volume (200 μl), rapid liquid-liquid extraction, and detection at a single wavelength (212 nm) over a short run time made this method valuable.

3.1.18. DAvolio et al \[57\] proposed a new method using high performance liquid chromatography coupled with electrospray mass spectrometry (HPLC-MS) and validated, for the quantification of plasma concentration of the new protease inhibitors darunavir (DRV) and other 11 antiretroviral agents (ritonavir, amprenavir, atazanavir, lopinavir, saquinavir, indinavir, nelfinavir and its metabolite M-8, nevirapine, efavirenz and tipranavir). A simple protein precipitation extraction procedure was applied on 50 μl of plasma aliquots and chromatographic separation of drugs and internal standard (quinoxaline) was achieved with a gradient (acetonitrile and water with formic acid 0.05%) on a C-18 reverse phase analytical column with 25 min of analytical run.
3.1.19. ter Heine et al [58] proposed a bioanalytical method for the determination of most commonly prescribed protease inhibitors (atazanavir, darunavir, lopinavir and ritonavir) and non-nucleoside reverse transcriptase inhibitors (efavirenz and nevirapine) was developed and validated according to FDA guidelines. Dried blood spots were punched out of a collection paper with a 0.25 inch diameter punch. The analytes were extracted from the punched-out disc using a mixture of acetonitrile, methanol and 0.2M zinc sulphate in water (1:1:2, v/v/v) containing the internal standards dibenzepine, 13C6-efavirenz and D5-saquinavir.

3.1.20. Cattaneo et al [59] proposed a high-performance liquid chromatographic method for the determination of atazanavir (ATV) in human plasma and validated. The method involves a rapid and simple solid-phase extraction (SPE) of ATV using Bond-elut C18 3 ml cartridge. The separation of ATV from internal standard and endogenous components was achieved using an isocratic elution on a C8 column and an UV detector set at 260 nm. The method was linear from 20 - 10,000 ng/ml.

3.1.21. Dey et al [60] proposed a simple, accurate, precise and cost effective UV-VIS spectrophotometric method for the estimation of atazanavir in bulk and pharmaceutical dosage form. The solvent used was methanol and the \( \lambda_{\text{max}} \) or the absorption maxima of the drug was found to be 250nm. A linear response was observed in the range of 10-50 \( \mu \)g/ml.

3.1.22. Khanage et al [61] proposed a simple, precise and economical spectrophotometric method for the estimation of Atazanavir sulfate in bulk and pharmaceutical formulations. The quantitative determination of the drug was carried out using the first order derivative method. Atazanavir sulfate showed a sharp peak at 254.0 nm in first order derivative spectrum. The drug follows Beer-Lambert’s law in the concentration range of 10-50\( \mu \)g/ml.

3.1.23. Muller et al [62] proposed an efficient HPLC method for the determination of atazanavir in human plasma and validated. A relatively simple mobile phase consisting of acetonitrile–ammonium formate buffer (pH 3; 10mM) (45:55, v/v) was pumped at a low flow rate of 0.3 ml/min through a reverse phase Phenomenex® Luna C18 (5\( \mu \)m, 150mm×2.0mm i.d.) column maintained at 30ºC. Diazepam was used as an internal
standard and the eluent was monitored at 210 nm. The major advantage of this method over previously reported procedures was that the narrow-bore HPLC column used resulted in relatively short retention times for the internal standard (6.8 min) and atazanavir (8.3 min) with excellent peak resolution and associated reduction in solvent usage. Sample preparation involved liquid–liquid extraction using 400µl plasma treated with sodium carbonate (2M) and extracted with a mixed organic solvent consisting of ethyl acetate–n-hexane (50:50, v/v).[62]

3.1.24. Chitturi et al [63] proposed a simple and selective RP-HPLC method for the determination of process impurities and degradation products (degradants) of atazanavir sulfate (ATV) drug substance. Chromatographic separation was achieved on Ascentis® Express C8, (150mm×4.6mm, 2.7µm) column thermostated at 30ºC under gradient elution by a binary mixture of potassium dihydrogen phosphate (pH 3.5, 0.02M) and ACN at a flow rate of 1.0 ml/min. A photodiode array (PDA) detector set at 250nm was used for detection. Stress testing (forced degradation) of ATV was carried out under acidic, alkaline, oxidative, photolytic, thermal and humidity conditions.

3.1.25. Nanda et al [64] proposed two simple, sensitive, rapid spectrophotometric methods developed for simultaneous estimation of Atazanavir Sulfate (ATV) and Ritonavir (RTV) in tablets. First method involves solving simultaneous equations based on measurement of absorbance at two wavelengths 249.5nm and 238.5 nm λmax of ATV and RTV, respectively. Second method is based on area under curve (AUC) and the wavelength ranges selected for analysis were 254.5-244.5 nm for Atazanavir Sulfate and 243.5-233.5 nm for Ritonavir. Beer’s law was obeyed in the concentration range of 10-50 μg/ml and 10-50 μg/ml for ATV and RTV, respectively.

3.2. LITERATURE REVIEW OF INSTRUMENTAL METHODS OF ANALYSIS OF RITONAVIR

3.2.1. Turner et al [65] developed a rapid, sensitive and specific high-performance liquid chromatographic (HPLC) method using UV detection for the determination of nine antiretroviral compounds commonly found in plasma from patients receiving antiretroviral therapy. Analytes include indinavir, saquinavir, ritonavir, amprenavir, lopinavir, delavirdine, efavirenz, nelfinavir and its M8 metabolite. Analytes were
isolated from plasma using tert.-butyl methyl ether and separation achieved via reversed-phase liquid chromatography on a C8 column with a gradient mobile phase. Detection at 210 nm provided adequate sensitivity.

3.2.2. Usami et al [66] developed a simple HPLC method for the simultaneous determination of lopinavir (LPV), ritonavir (RTV) and efavirenz (EFV) to evaluate the efficiency of co-administration of LPV/RTV and EFV in Japanese patients enrolled in a clinical study. The monitoring of LPV plasma concentration is important because co-administration of LPV/RTV with EFV sometimes decreases plasma concentrations of LPV caused by EFV activation of Cytochrome P-450 3A. A solution of acetonitrile, methanol and tetramethylammonium perchlorate (TMAP) in dilute aqueous trifluoroacetic acid (TFA) has been used as the mobile phase in a HPLC method to elute LPV and RTV. We found that a solvent ratio of 45: 5: 50 (v/v/v) of acetonitrile/methanol/0.02M TMAP in 0.2% TFA optimized separation of LPV, RTV and EFV. A column temperature of 30°C was necessary for the reproducibility of the analyses.

3.2.3. Hu et al [67] established an RP-HPLC method for the determination of ritonavir in human plasma and applied its application for the pharmacokinetic study of ritonavir in 9 healthy volunteers. The separation was carried out using Hypersil C18 (4.6 mm x 200 mm, 5 μm) column with mobile phase of 0.05 mol/L phosphate buffer (pH 5.6)-acetonitrile (50:50). The flow rate was 1 ml/min. The detection wavelength for ritonavir was 210 nm. The pharmacokinetic analysis of ritonavir was studied. It was found to be fitted to a one-compartment model. The method is sensitive, accurate and simple, and suitable for the study on pharmacokinetic of ritonavir in body.

3.2.4. E. M. Donato et al [68] described the development and full validation of a stability-indicating HPLC method to quantify ritonavir (RTV) and lopinavir (LPV) in soft gelatin capsules. The method used a LiChrospher_ 100 RP-18 (250 mm x 4.6 mm, 5 μm, Merck) column and isocratic elution. The mobile phase consisted of a mixture of acetonitrile-water-methanol (53:37:10, v/v/v), pumped at a flow-rate of 1.0 ml/min) and UV detection at 210 nm using a photodiode array detector. LPV and RTV were exposed to thermal, photolytic, hydrolytic and oxidative stress conditions, and the stressed samples were analyzed by the proposed method. Degradation studies showed
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that lopinavir is stable in thermal, alkaline and oxidative conditions, while ritonavir
degraded under these conditions. The method was found to be stability-indicating and
can be used for the routine analysis of the association LPV/RTV in soft gelatin
capsules.

3.2.5. Weller et al [69] proposed an efficient, isocratic high performance liquid
chromatography (HPLC) method for determining human immunodeficiency virus
(HIV) non-nucleoside reverse transcriptase inhibitors (NNRTIs) and protease inhibitors
(PIs) in plasma for laboratories participating in clinical trials and therapeutic drug
monitoring (TDM) programs, or conducting small animal research. The combination of
isocratic reversed phase chromatography using an S-3, 3.0mm×150mm column along
with low plasma volume (200µl), rapid liquid–liquid extraction, and detection at a
single wavelength (212 nm) over a short run time makes this method valuable.

3.2.6. Yekkala et al [70] evaluated the recently published gradient LC method for the
determination of related substances in ritonavir (RTV) in the International
Pharmacopoeia. The method used a base-deactivated reversed-phase C18 column kept
at a temperature of 35º C. The mobile phases consist of acetonitrile, phosphate buffer
pH 4.0 and water. UV detection is performed at 240 nm. A system suitability test (SST)
was described to govern the quality of the separation. Since no brand names of columns
were mentioned in pharmacopoeal texts, analysts often have problems to select a
suitable stationary phase which is only described in general terms. So, the separation
towards RTV components was investigated on 18 C18 columns and correlation was
made with the column classification system developed in our laboratory. The method
was further evaluated using a Hypersil BDS C18 column (25cm×4.6mm i.d., 5µm)
which was also used for the development of the method. A central composite design
was applied to examine the robustness of the method. The method showed good
precision, linearity, sensitivity and robustness. Four commercial samples were
examined using this method.

3.2.7. Sulebhavikar et al [71] proposed a rapid and simple high performance thin layer
chromatography (HPTLC) method with densitometry at $\lambda_{\text{max}} = 263$ nm and validated
for simultaneous determination of lopinavir and ritonavir from pharmaceutical
preparation. Separation was performed on aluminum-backed silica gel 60F254 HPTLC
plates as stationary phase and using a mobile phase comprising of toluene, ethyl acetate, methanol and glacial acetic acid, in the volume ratio of 7.0:2.0:0.5:0.5 (v/v) respectively. After development, plates were observed under UV light. The described method had the advantage of being rapid and easy. Hence it can be applied for routine quality control analysis of lopinavir and ritonavir from pharmaceutical preparation and stability studies.

3.2.8. Senthilkumar et al. [72] developed simple, rapid, accurate, precise and validated methods for the simultaneous analysis of Emtricitabine, Tenofovir, Ritonavir and lopinavir by high performance liquid chromatography.

3.2.9. Dias et al. [73] proposed an ultraviolet-derivative spectrophotometric method (UV-D) as an alternative to a previously described liquid chromatographic (LC) method for the quantitative determination of ritonavir in soft gelatin capsules. The spectrophotometric method was based on recording the second-derivative spectra for ritonavir at 222.3nm of its solutions in methanol. The statistical analysis showed that LC and UV-D methods were equivalent to assay ritonavir capsules.

3.2.10. Yadav et al. [74] developed and validated a high throughput and rugged ultra performance liquid chromatography tandem mass spectrometry (UPLC–ESI-MS/MS) method for the selective determination of protease inhibitors – lopinavir (LPV) and ritonavir (RTV) in human plasma. Plasma samples were prepared by solid phase extraction of the analytes and their deuterated analogs as internal standard (IS) using Waters Oasis HLB cartridges. The chromatographic separation was achieved in a run time of 1.2 min on Waters Acquity UPLC BEH C18 column (50mm×2.1mm, 1.7µm) under isocratic conditions. The mobile phase consisted of 10mM ammonium formate, pH 4.0 adjusted with formic acid and methanol (10:90, v/v). The protonated precursor → product ion transitions for lopinavir, ritonavir, d8-lopinavir and d6-ritonavir were monitored on a triple quadrupole mass spectrometer, operating in the multiple reaction monitoring (MRM) and positive ion mode. The method was successfully applied to a bioequivalence study of [200(lopinavir) + 50(ritonavir)] mg tablet formulation in 36 healthy human subjects under fasting conditions.
3.2.11. Rezk et al [75] developed and validated a novel bioanalytical LC–MS method for the simultaneous quantification of the most recently FDA-approved protease inhibitor and non-nucleoside reverse transcriptase inhibitor, etravirine, darunavir, and ritonavir for clinical pharmacology investigations and potential therapeutic drug monitoring. This novel method was developed and validated using a sub-2 μm particle column, and provides excellent chromatographic separation and peak shape for all three analytes and internal standard. Finally, the applicability of the method was investigated with clinical samples and external quality assurance proficiency testing samples.

3.2.12. Phechkrajang et al [76] developed a simple high-performance liquid chromatographic method (HPLC) for the simultaneous determination of lopinavir and ritonavir in syrup. A reversed-phase with isocratic elution was utilized. The mobile phase was a mixture of 10 mM ammonium acetate, pH 7 and acetonitrile (50:50, v/v). The flow rate was operated at 1 ml/min and the presence of two interest compounds was detected by a UV detector at 245 nm. The retention times on the described method were about 12.5 min and 14.7 min for ritonavir and lopinavir, respectively. The developed HPLC method was also validated for important performance characteristics such as linearity, accuracy and precision. The developed method was then applied to determine lopinavir and ritonavir in syrup sample.

3.2.13. Temghare et al [77] developed and validated a rapid and sensitive liquid chromatography-mass spectrometric (LCMS- MS) method for the simultaneous determination of lopinavir and ritonavir in human plasma using abacavir as internal standard. Sample preparation of plasma involved solid phase extraction. The described method had the advantage of being rapid and easy and it could be applied in therapeutic monitoring of these drugs in human plasma.

3.2.14. Carvalho et al [78] developed a micellar electrokinetic chromatography (MEKC) method for the separation of Ritonavir from three available synthetic precursors was developed. The optimized separation was performed in a background electrolyte composed of sodium tetraborate (pH 9.6; 15mM) containing sodium dodecyl sulfate (30mM) and acetonitrile (18%, v/v). Mass spectrometry was used to confirm the identity of the tested substances.
3.2.15. Ponnilavarasan et al \[79\] developed and validated a simple reverse phase high-performance liquid chromatographic (RP-HPLC) method for the quantitative estimation of antiretroviral drugs Lopinavir (LPV) and Ritonavir (RTV). Chromatography was carried out by binary gradient technique on a reversed-phase phenomenex-Luna C18 column using Ambroxol (ABM) as the internal standard. The proposed method was highly sensitive, precise and accurate, which was evident from the LOD value of 30 ng/ml for LPV and 25 ng/ml for RTV hence the present method applied successfully for the quantification of active pharmaceutical ingredient content (API) in the combined formulations of LPV and RTV.

3.2.16. Rao et al \[80\] reported forced degradation of ritonavir (RTV), under the conditions of hydrolysis (acidic, basic and neutral), oxidation, photolysis and thermal stress as prescribed by ICH using LC–MS/MS. Eight degradation products were formed and their separation was accomplished on Waters XTerra® C18 column (250mm×4.6mm i.d., 5μm) using water: methanol: acetonitrile as (40:20:40, v/v/v) mobile phase in an isocratic elution mode by LC. The method was extended to LC–MS/MS for characterization of the degradation products and the pathways of decomposition were proposed.

3.2.17. Suneetha et al \[81\] developed and validated an accurate, sensitive, precise and robust reverse phase high performance liquid chromatographic method for the simultaneous estimation of lopinavir and ritonavir in combined dosage form. Chromatographic separation was conducted on Phenomenex Gemini C18 (250mm x 4.6mm, 5μm) column at room temperature using Potassium hydrogen phosphate buffer (pH adjusted to 6.0 ± 0.1 with dilute potassium hydroxide solution), acetonitrile and methanol in the ration 50:35:15 v/v and at a flow rate of 1.0ml/min, while UV detection was performed at 254nm. The retention time for lopinavir and ritonavir was found to be 6.0 ± 0.2 and 3.7± 0.1min, respectively.

3.2.18. Sudha et al \[82\] developed a validated reverse phase high performance chromatography (HPLC) and high performance thin layer liquid chromatography (HPTLC) methods for the estimation of ritonavir in bulk and in pharmaceutical formulation. The RP-HPLC separation was achieved on Eclipse XBD (C18) RP column (150mmX 4.6mm id, 15μparticle size) using acetonitrile: water (60:40%v/v) as
the mobile phase at a flow rate of 1.4ml/min at an ambient temperature. Quantification was achieved with ultraviolet (SPD-10AV) detection at 209 nm. The HPTLC separation was achieved on the aluminum backed layer of silica gel 60F254 using (Toluene: Ethyl Acetate: Methanol: Glacial Acetic Acid) (7.0:2.0:0.5:0.5, v/v/v/v) as mobile phase. Quantification was achieved with HPTLC detection at 263nm. Both the methods are simple, precise, and sensitive and extended for routine analysis in bulk as well as pharmaceutical formulation.

3.2.19. Chiranjeevi et al [83] developed a simple, precise, specific and accurate reverse phase HPLC method for the determination of ritonavir in bulk and pharmaceutical dosage forms. The chromatographic separation was achieved on Symmetry C18 (4.6 x 100mm, 3.5 μm) column using a mixture of buffer: acetonitrile (50:50, v/v) as the mobile phase at a flow rate 1.0 ml/min. Linearity was observed in concentration range of 50-150μg/ml. The retention time of Ritonavir was 5.1 min. The analyte was monitored using UV detector at 239 nm.

3.3. LITERATURE REVIEW OF INSTRUMENTAL METHODS OF ANALYSIS OF Efavirenz

3.3.1. Montgomery et al [84] developed a stability-indicating high performance liquid chromatographic (HPLC) method for the assay of Efavirenz, a non-nucleoside reverse transcriptase inhibitor used in the treatment of AIDS. The HPLC method, which was used to determine potency in Efavirenz capsules and related substances in Efavirenz drug substance and capsules, was validated per ICH guidelines. This method, which used a cyano column, is capable of separating Efavirenz from its trans-alkene reduction product. This paper discussed the development and validation of a method, which is the first known separation of homologs containing double and triple bonds using reverse-phase HPLC.

3.3.2. Matthews et al [85] reported methods for the quantitative determination of Efavirenz in human plasma and the qualitative assessment of the stereochemical integrity of Efavirenz in post-dose human plasma samples. After the addition of an internal standard, plasma samples were extracted with hexane–methylene chloride (65:35, v/v). The extracts were evaporated to dryness and reconstituted in mobile phase. Upon exposure to UV light, the analyte was found to form fluorescent products;
the major fluorescent product was isolated and identified as a substituted quinoline. Thus, the plasma extracts were analyzed via HPLC with post-column photochemical derivatization and fluorescence detection. Reverse phase chromatography was used for the quantitative assay, whereas chromatography with a column containing a chiral stationary phase (dinitrobenzoyl leucine) was used for the stereochemical assessment. The selective detection method reduces the likelihood of interference by co-administered medications or endogenous species. The stereochemical configuration of Efavirenz was confirmed to remain intact in post-dose human plasma samples. The quantitative method has been successfully utilized to support a study in which a possible drug interaction between co-administered HIV protease inhibitors and Efavirenz was evaluated.

3.3.3. Raju et al \cite{86} established a simultaneous stability indicating RP-HPLC method for the estimation of Emtricitabine, Tenofovir Disoproxil Fumarate and Efavirenz in tablet dosage form. Chromatography was carried on an Inertsil ODS 3V column using gradient composition of 0.02M sodium dihydrogen orthophosphate as mobile phase A and mixture of methanol and water in ratio of 85:15 as mobile phase B at a flow rate of 1.5 ml/min with detection at 265 nm. The retention times of the emtricitabine, tenofovir disoproxil fumarate and efavirenz was about 5.875, 8.800 and 12.020 mins respectively. The method was validated by determining its sensitivity, Linearity, accuracy and precision. The proposed method is simple, fast, sensitive, Linear, accurate, rugged and precise and hence can be applied for routine quality control of emtricitabine, tenofovir disoproxil fumarate and efavirenz in bulk and in tablet dosage form.

3.3.4. Rao et al \cite{87} developed and validated a rapid and accurate isocratic HPLC method for the assay of Efavirenz (EFA) in bulk and pharmaceutical dosage forms. The chromatographic conditions comprise of a Novapak phenyl column. A mixture of phosphate buffer and acetonitrile was used as mobile phase. Quantitation was achieved by UV detection at 247 nm. The proposed method can be used for quality control assay of EFA in bulk and in finished dosage form and for the stability studies as the method separates EFA from its degradation products and excipients.
3.3.5. Mogatle et al [88] reported a pharmacokinetic interaction study between efavirenz (EFV), a non-nucleoside reverse transcriptase inhibitor used in the treatment of HIV-1 infection, and an African traditional medicine, African potato in human subjects was undertaken. This necessitated the development and validation of a quantitative method for the analysis of EFV in plasma. A simple mobile phase consisting of 0.1M formic acid, acetonitrile and methanol (43:52:5, v/v/v) was pumped at a low flow rate of 0.3 ml/min through a reverse phase Phenomenex® Luna C18 (5μm, 50mm × 2.0 mm i.d.) column maintained at 40ºC. Diclofenac sodium was used as an internal standard (IS) and EFV and IS were monitored at 247 nm and 275 nm, respectively. A simple and rapid sample preparation involved the addition of mobile phase to 100μl of plasma to precipitate plasma proteins followed by direct injection of 10μl of supernatant onto the column. The procedures were validated according to international standards with good reproducibility and linear response. Plasma samples were evaluated for short-term (ambient temperature for 6 h) and long-term (−10±2ºC for 60 days) storage conditions and were found to be stable. The method described was cost-effective and has the necessary accuracy and precision for the rapid quantitative determination of EFV in human plasma.

3.3.6. Avery et al [89] developed and validated a combined UPLC–tandem mass spectrometric (UPLC–MS/MS) technique for quantitation of protein free Efavirenz (EFV) as well as total concentrations of EFV in human blood and seminal plasma. The analytical method possesses capabilities for concentration measurements of EFV ranging from 0.5 to 10,000 ng/ml with accuracy. The method employed a racemic fluorinated analogue of EFV (F-EFV) as the internal standard. EFV and F-EFV were eluted from a reverse-phase UPLC column via gradient elution with detection via negative ion multiple reaction monitoring (MRM). EFV and F-EFV, respectively, were detected via the following MRM transitions: \( m/z \ 314.0 > 244.1 \) and \( m/z \ 298.0 > 227.9 \). The time required for the analysis of each sample was 8.0 min. The analytical technique was capable of a reliable detection limit of \( \sim 15–20 \) fmol of EFV injected on column.

3.3.7. Theron et al [90] developed and validated a novel and robust screening method for the determination of the non-nucleoside reverse transcriptase inhibitor, efavirenz (EFV), in human saliva based on high performance liquid chromatography tandem
mass spectrometry (LC–MS/MS). Sample preparation of the saliva involved solid-phase extraction (SPE) on C18 cartridges. The analytes were separated by high performance liquid chromatography (Phenomenex Kinetex C18, 150 mm × 3 mm internal diameter, 2.6 μm particle size) and detected with tandem mass spectrometry in electrospray positive ionization mode with multiple reaction monitoring. Gradient elution with increasing methanol (MeOH) concentration was used to elute the analytes, at a flow-rate of 0.4 mL/min. The total run time was 8.4 min and the retention times for the internal standard (reserpine) was 5.4 min and for EFV was 6.5 min. This novel LC–MS/MS assay is suitable for reliable detection of low EFV concentrations in saliva and can be used as a screening tool for monitoring EFV compliance.

3.3.8. Potale et al \cite{91} developed a sensitive, selective, precise and stability indicating (in accordance with ICH guidelines) High-Performance Thin Layer Chromatographic method of analysis for Efavirenz, to resolve drug response from that of its degradation products. The method employed TLC aluminium plates precoated with silica gel 60 F254 as the stationary phase. The solvent system consisted of dichloromethane: methanol (5: 0.3 v/v). This system was found to give compact spot for efavirenz (Rf value 0.72 ± 0.03). Efavirenz was subjected to stress test conditions like acid, alkali, neutral hydrolysis, oxidation, dry heat and photo degradation. The spot for product of degradation were well resolved from the drug. Densitometric analysis of drug was carried out in the absorbance mode at 247 nm. The linear regression data for the calibration plots showed good linear relationship with r² of 0.998 in the concentration range of 400-2000ng/spot. The result indicated that the drug was susceptible to degradation, to different extent in different conditions.

3.3.9. Foxa et al \cite{92} developed a simple and rapid isocratic, high performance liquid chromatography (HPLC) assay employing solid phase extraction (SPE) for the simultaneous determination of the anti HIV drug, efavirenz, the antituberculosis drug, rifampicin and the desacetyl metabolite of rifampicin in plasma from HIV/tuberculosis infected patients. Using a Zorbax SB-Phenyl reverse-phase analytical column with UV detection, good separation and detection of the drugs was attained within a 10 min run time. This low cost assay can accurately detect efavirenz and rifampicin concentrations within a clinically relevant concentration range using standard chromatography equipment, making it particularly applicable to resource-limited settings.
3.3.10. **D’Avolio et al** [93] developed and validated a sensitive and accurate high performance liquid chromatography–mass spectrometric (HPLC–MS) method for the intracellular determination of 14 antiretroviral drugs in peripheral blood mononuclear cells (PBMCs) for HIV+ patients. PBMCs are isolated by Ficoll density gradient centrifugation and cells count and the relative mean volume is performed with a Coulter® instrument. Extraction of drugs from PBMCs pellets was obtained with methanol: water (70:30, v/v), with quinoxaline added as internal standard, after a sonication step. Supernatant was dried and then dissolved in water: acetonitrile (60:40, v/v), before injection into a 2.1mm × 150mm Atlantis® T3 3µ column. Chromatographic separations were performed using a gradient program with a mixture of water (0.05% formic acid), as mobile phase A and acetonitrile (0.05% formic acid), as mobile phase B. Analytes quantification was performed by electrospray ionisation–single quadrupole mass spectrometry using the selected ion recording (SIR) detection mode. The positive ionization was used for the HIV protease inhibitors (PIs) indinavir, saquinavir, nelfinavir, nelfinavir M8 metabolite, amprenavir, darunavir, atazanavir, ritonavir, lopinavir, tipranavir, the integrase inhibitor (II) raltegravir and the non-nucleoside reverse transcriptase inhibitors (NNRTIs) nevirapine and etravirine, while the negative ionization is applied for efavirenz. Each drug concentration evaluated was expressed in ng/mL and optimized using each patient medium corpuscular volume and cell number. This analytical method is routinely used in our clinical research centre for the assessment of intracellular levels of all PIs, raltegravir and NNRTIs commercially available at present.

3.4. **LITERATURE REVIEW OF INSTRUMENTAL METHODS OF ANALYSIS OF EMTRICITABINE**

3.4.1. **Darque et al** [94] developed an analytical methodology combining solid-phase extraction (SPE) and high-performance liquid chromatography (HPLC) to quantitate the intracellular active 5*-triphosphate (TP) of β-L-2*,3*-dideoxy- 5-fluoro-3*-thiacytidine (emtricitabine) (FTC) in human peripheral blood mononuclear cells (PBMCs). The FTC nucleotides, including 5*-mono-, di-, and triphosphates, were successively resolved on an anion exchange SPE cartridge by applying a gradient of potassium chloride. The FTC-TP was subsequently digested to release the parent nucleoside that was finally analyzed by HPLC with UV detection (HPLC-UV).
Validation of the methodology was performed by using PBMCs from healthy donors exposed to an isotopic solution of [3H] FTC with known specific activity, leading to the formation of intracellular FTC-TP that was quantitated by an anion-exchange HPLC method with radioactive detection. These levels of FTC-TP served as reference values and were used to validate the data obtained by HPLC-UV. This methodology was successfully applied to the determination of FTC-TP in PBMCs of patients infected with human immunodeficiency virus after oral administration of various dosing regimens of FTC monotherapy.

3.4.2. Cass et al [95] reported the separation of FTC enantiomers using an amylose tris[(S)-1-phenylethylcarbamate] coated onto APS-Nucleosil (7 mm particle size, 500 Å pore size, 20% w/w, 15 x 0.46 cm ID) chiral column under polar organic elution mode. Good enantioselectivity (α =1.9) with excellent enantioresolution (Rₛ = 3.3) was achieved by the use of methanol with 0.02% of triethylamine acetate as mobile phase. The method allowed the accurate determination of as low as 0.2% of each enantiomers as an impurity. The validated method proved to be reliable and sensitive for the quantification of both enantiomers as impurity in different batches of Emtricitabine and β-D-(+)-FTC.

3.4.3. Rezk et al [96] developed an accurate, sensitive and simple reverse-phase (RP) high-performance liquid chromatography (HPLC) assay for the simultaneous quantitative determination of Emtricitabine and Tenofovir in human blood plasma is described. Using 200 µL of plasma and BOND ELUT-C18 Varian columns, the solid phase extraction (SPE) method resulted in a clean baseline and high extraction efficiencies (100% for emtricitabine and 98.6% for tenofovir). An Atlantis TM dC-18 analytical column was used along with an 18 min linear gradient elution of phosphate buffer (pH 5.7) and methanol to provide sharp peaks for emtricitabine at 280 nm, tenofovir at 259 nm, and the internal standard 2', 3'-dideoxyuridine (DDU) at 262 nm. This method is suitable for use in clinical pharmacokinetic studies and is nimble enough for therapeutic drug monitoring.

3.4.4. Notari et al [97] developed a HPLC–UV method to quantify simultaneously seven HIV protease inhibitors (amprenavir, atazanavir, indinavir, lopinavir, nelfinavir, ritonavir, and saquinavir; PIs), seven nucleoside reverse transcriptase inhibitors
(abacavir, didanosine, emtricitabine, lamivudine, stavudine, zalcitabine, and zidovudine; NRTIs), and two non-nucleoside reverse transcriptase inhibitors (efavirenz and nevirapine; NNRTIs) in human plasma. The volume of the plasma sample was 600µL. This method involved automated solid-phase extraction with Oasis HLB Cartridge 1 cc (divinylbenzene and N-vinylpyrrolidone) and evaporation in a water bath under nitrogen stream. The extracted samples were reconstituted with 100µL methanol. Twenty microliters of these samples were injected into a HPLC–UV system; the analytes were eluted on an analytical C18 SymmetryTM column (250mm×4.6mm I.D.) with a particle size of 5µm. The mobile phase (0.01M KH₂PO₄ and acetonitrile) was delivered at 1.0 ml/min with linear gradient elution. The total run time for a single analysis was 35 min, the anti-HIV drugs were detected by UV at 240 and 260 nm. The calibration curves were linear up to 10 µg/mL. The absolute recovery ranged between 88 and 120%. The in vitro stability of anti-HIV drugs (0.005–10 µg/ml) in plasma had been studied at 24.0°C. On these bases, a two to four analyte method had been tailored to the individual needs of the HIV-infected patient. The HPLC–UV method here reported has been validated and was applied to monitor PIs, NRTIs, and NNRTIs in plasma of HIV-infected patients. It allowed monitoring the largest number of anti-HIV drugs simultaneously, appearing useful in a routine laboratory, and represents an essential step to elucidate the utility of a formal therapeutic drug monitoring for the optimal follow-up of HIV-infected patients.

3.4.5. Notari et al [98] determined the anti-HIV drugs lamivudine, lopinavir, and ritonavir concentration in the plasma of HIV-infected patients by MALDI-TOF/TOF. The volume of the plasma sample was 600 µL. Plasma samples were extracted by solid-phase (divinylbenzene and N-vinylpyrrolidone) and evaporated in a water bath under a nitrogen stream. The extracted samples were reconstituted with methanol (100 µL), mixed (1 : 1) with a saturated matrix solution (4-hydroxybenzoic acid in 50% acetonitrile-0.1% trifluoracetic acid), and spotted onto the MALDI-TOF/TOF sample target plate. The lamivudine, lopinavir and ritonavir concentration was determined by standard additions analysis. Moreover, emtricitabine (i.e., the fluorinated analog of lamivudine) was used as the internal standard to determine the lamivudine concentration. Values of the lamivudine, lopinavir and ritonavir concentration determined by MALDI-TOF/TOF are in excellent agreement with those obtained by HPLC-UV and HPLC-MS/MS. MALDI-TOF/TOF experiments allowed also the
detection of the ritonavir metabolite R5. Zidovudine was undetectable by MALDI-TOF/TOF analysis because also the minimal laser intensity may induce the anti-HIV drug photolysis. The MALDI-TOF/TOF technique is useful to determine very low concentrations of anti-HIV drugs (0.0025-0.010 pmol/μl).

3.4.6. Li et al\textsuperscript{[99]} established an HPLC method for the determination of the related substances and content of emtricitabine. The HPLC system consisted of mixed-mode C/anion column (250 mm × 4.6 mm, 7 μm) the mobile phase was composed of 0.02 mol/L potassium dihydrogen phosphate (pH 6.0)-acetonitrile (65: 35, v/v) at a flow rate of 0.6 ml/min and the UV detector was set at 279 nm. The column was 25°C and the injection volume was 10 μl. The contents of related substances of sample Emtricitabine was within 1%, the emtricitabine content exceeded 98.5%. This method was simple, sensitive, accurate and suitable for the determination of emtricitabine.

3.4.7. Droste et al\textsuperscript{[100]} reported a sensitive, specific, and simple high performance liquid chromatography assay with fluorometric detection for the determination of the antiretroviral agent emtricitabine in human plasma. Using 500 μL of plasma and Oasis MAX columns, the solid phase extraction (SPE) method results in a clean baseline and high extraction efficiencies (107%). An Atlantis dC18 analytical column was used along with a 15 min linear gradient elution of phosphate buffer and methanol to provide sharp peaks for emtricitabine at excitation and emission wavelengths of 244 and 356 nm, respectively. Participation in an external quality control program resulted in deviations for three different levels of less than 2.4% from the nominal concentrations. This method was suitable for use in clinical trials and therapeutic drug monitoring of HIV-infected patients.

3.4.8. Seshachalam et al\textsuperscript{[101]} developed and validated a novel stability-indicating high-performance liquid chromatographic (HPLC) method for assay and determination of impurities of emtricitabine in drug substance. Emtricitabine was found to be degraded under acidic, alkaline, and oxidative stress conditions and to be more labile under oxidative conditions. The drug proved to be stable to dry heat and photolytic degradation. Resolution of major and minor degradation impurities was achieved on an Intersil ODS-3V column utilizing 10 mM sodium phosphate buffer and methanol (85:15, v/v) as mobile phase. Detection was at 280 nm. Validation studies were
performed as per ICH recommended conditions. The developed method was found to be linear, accurate, specific, selective, precise, and robust.

3.4.9. **Sparidans et al** \[102\] developed and validated a liquid chromatography-tandem mass spectrometric assay for the determination of the antiretroviral nucleoside emtricitabine in human plasma using a simple sample pre-treatment procedure. After addition of 5-deoxy-5-fluorocytidine as the internal standard and protein precipitation with acetonitrile, the supernatant was directly injected in the isocratic chromatographic system using a polar embedded reversed-phase column and formic acid in water-methanol as the eluent. The eluate was completely led into an electrospray interface with positive ionization and the analytes were quantified using triple quadrupole mass spectrometry. The analytes were chemically stable under all relevant conditions and the assay was applied in the analysis of plasma samples of HIV-infected patients treated with the drug.

3.4.10. **Gehrig et al** \[103\] simultaneously determined many antiretrovirals using triple quadrupole mass spectroscopy with electrospray ionisation. For this purpose, spectra and fragmentation patterns of the protease inhibitors amprenavir, atazanavir, indinavir, lopinavir, nelfinavir, ritonavir, and saquinavir, the non-nucleoside reverse transcriptase inhibitors delavirdine, efavirenz, and nevirapine, the nucleoside reverse transcription inhibitors abacavir, didanosine, emtricitabine, lamivudine, stavudine, zalcitabine, and zidovudine, and the nucleotide reverse transcriptase inhibitor tenofovir were evaluated. A bioanalytical method to determine all protease and non-nucleoside reverse transcriptase inhibitors, and zalcitabine and zidovudine concentrations in biological matrices was developed. Samples were prepared by protein precipitation with methanol after addition of three different internal standards. Antiretrovirals were separated by high-performance liquid chromatography on a Nucleosil C18-100 Nautilus column using a gradient of 20 mM ammonium acetate including 0.1% aqueous acetic acid and acetonitrile and detected by electrospray ionisation/tandem mass spectrometry in the negative (efavirenz, stavudine, zidovudine) or positive ionisation mode (all other compounds). The bioanalytical method was successfully validated according to FDA guidelines and applied to plasma and cerebrospinal fluid samples of patients treated for acquired immunodeficiency syndrome (AIDS).
3.4.11. Saux et al [104] developed a simple analytical method in 100 µL of plasma for the simultaneous assay of the 7 nucleoside/nucleotide reverse transcriptase inhibitors (abacavir, didanosine, emtricitabine, lamivudine, stavudine, tenofovir, and zidovudine) currently used for the treatment of HIV-infected patients. After adding the internal standard, 6-beta-hydroxy-theophyline, plasma samples were precipitated with 500µL acetonitrile and the supernatants were evaporated to dryness. The residues were reconstituted with 500 µL of water and 10 µL of the extracts were injected in the chromatographic system. The chromatographic separation was performed with a C-18 column and a gradient mobile phase consisting of a mixture of water and acetonitrile, both containing 0.05% formic acid. Analytes quantification was performed by electrospray ionisation triple quadrupole mass-spectrometry in the positive mode using selected reaction monitoring (SRM). The method has been implemented to assess plasma concentrations of patients infected by HIV and was found suitable for therapeutic drug monitoring.

3.4.12. Robledoa et al [105] provided analytical chemical information on selected new molecular entities (NMEs) which were drugs that had recently been approved by the FDA, the antiretroviral drugs, atazanavir, indinavir and emtricitabine, the antibacterial gemifloxacin, rosuvastatine which is a cholesterol-lowering drug, the anti-cancer drug gefitinib and aprepitant for neurological disorders. Electrospray ionisation-quadrupole ion trap mass spectrometry (ESI-MSn) was employed to generate tandem mass spectrometric (MS2) data of the drugs studied and structural assignments of product ions were supported by quadrupole time-of-flight mass spectrometry (QToF-MS/MS). These fragmentation studies were then utilised in the development and validation of a specific and sensitive liquid chromatographic method (LC–ESI-MS2) to identify and determine these drugs at therapeutic concentration levels in serum after a single protein precipitation procedure with acetonitrile. In addition, this method was compared to the application of gas liquid chromatography-flame ionisation detection (GLC-FID) and differential pulse polarography (DPP) for the analysis of these NMEs in serum.

3.4.13. Appala Raju et al [106] developed a simultaneous stability indicating RP-HPLC method for the estimation of emtricitabine, tenofovir disoproxil fumarate and Efavirenz in tablet dosage form. Chromatography was carried on an Intersil ODS 3V column using gradient composition of 0.02M sodium dihydrogen orthophosphates as mobile
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phase A and mixture of methanol and water in ratio of 85:15 as mobile phase B at a flow rate of 1.5 ml/min with detection at 265 nm. The retention times of the emtricitabine, tenofovir disoproxil fumarate and efavirenz was about 5.875, 8.800 and 12.020 mins respectively. The method was validated by determining its sensitivity, linearity, accuracy and precision. The proposed method was simple, fast, sensitive, linear, accurate, rugged and precise and hence can be applied for routine quality control of emtricitabine, tenofovir disoproxil fumarate and efavirenz in bulk and in tablet dosage form.

3.4.14. D’Avolio et al\textsuperscript{[107]} developed and validated a new solid-phase extraction (SPE) method on a liquid chromatography (LC) coupled with a mass spectrometer for the determination of plasma concentrations of tenofovir (TFN) and emtricitabine (FTC) in HIV infected patients. Chromatographic separation was achieved with a gradient (acetonitrile and water with formic acid 0.05%) on an Atlantis 4.6 mm × 150 mm, reversed phase analytical column. Detection of TFN, FTC, and internal standard (IS) was achieved by electrospray ionization mass spectrometry (ESI-MS) in the positive ion mode. The method did not show any significant interference with antiretrovirals or other concomitant drugs administered to patients, and no significant “matrix effects” were observed. The method was applied for the determination of antiretroviral plasma concentration of HIV-positive patients treated with FTC and/or TFN, in combination with various other antiretrovirals.

3.4.15. Sharma et al\textsuperscript{[108]} developed and validated a simple, rapid reversed-phase high performance liquid chromatographic method for estimation of emtricitabine and tenofovir disoproxil fumarate in tablet dosage form. The estimation was carried out on Luna C18 (25cm x 4.60 mm, particle size 5μm) column with a mixture of acetonitrile: potassium dihydrogen phosphate buffer (pH 3.0 ± 0.05 adjusted with orthophosphoric acid): triethylamine in the ratio of 70:30:0.5(v/v) as mobile phase. UV detection was performed at 260 nm. The method was validated for linearity, accuracy, precision, specificity and sensitivity as per ICH norms. The developed and validated method was successfully used for the quantitative analysis of commercially available dosage form. The retention time was 1.78 and 2.27 min for emtricitabine and tenofovir disoproxil fumarate respectively and total run time was 4 min. at a flow rate of 1.5 ml/min. The high percentage of recovery and low percentage coefficient of variance confirmed the
suitability of the method for the simultaneous estimation of emtricitabine and tenofovir disoproxil fumarate in tablet dosage form.

3.4.16. Joshi et al \[109\] developed and validated a simple, precise, accurate, and rapid high performance thin layer chromatographic method for the estimation of emtricitabine and tenofovir simultaneously in combined dosage form. The stationary phase used was precoated silica gel 60 F 254. The mobile phase used was a mixture of chloroform: methanol (9:1v/v). The detection of spots was carried out at 265nm. The method was validated in terms of linearity, accuracy, precision and specificity. The proposed method can be successfully used to determine the drug content of marketed tablet formulation.

3.4.17. Patel et al \[110\] developed two simple, accurate, economical and reproducible spectrophotometric methods for simultaneous estimation of two component drug mixture of tenofovir disoproxil fumarate and Emtricitabine in bulk and combined tablet dosage form had been developed. The first method employed formation and solving of simultaneous equations using 259nm and 286nm as two analytical wavelengths. The second method was absorbance ratio method, which used 286nm and 247.6nm as two analytical wavelengths. Both methods were statistically validated according to ICH and recovery studies confirmed the accuracy of the proposed method.

3.4.18. Raju et al \[111\] developed a simple, precise, rapid and accurate reverse phase HPLC method in isocratic mode has been developed for the estimation of tenofovir disoproxil fumarate, emtricitabine and efavirenz in tablet dosage form. An Intersil C-18 column with 250 x 4.6 mm, 5 μm particle size, with mobile phase consisting of acetonitrile and buffer containing 1 ml of Triethyl amine in 1000 ml water (pH adjusted to 3.2 with orthophosphoric acid) in the ratio of 60:40 v/v was used. The flow rate was 1.0 ml/min and the effluents were monitored at 265nm. The retention times were 2.513min for tenofovir disoproxil fumarate, 3.576 for emtricitabine and 11.849 min for efavirenz. The method was validated by determining its accuracy, precision and system suitability. The results of the study showed that the proposed RP-HPLC method was simple, rapid, precise and accurate, which was useful for the routine determination of tenofovir disoproxil fumarate, emtricitabine and efavirenz in bulk drug and in its pharmaceutical dosage forms.
3.4.19. Ghorpade et al\(^{[112]}\) developed two methods for the simultaneous determination of emtricitabine and tenofovir by spectroscopy. These two simple, accurate and precise methods include area under the curve (AUC) method and dual wavelength method. From a solvent effect studies and the spectral behaviours of emtricitabine and tenofovir, methanol was selected as solvent. Emtricitabine showed maximum absorbance at 281 nm and tenofovir showed maximum absorbance at 259 nm. For the AUC method, the wavelength ranges between 242-248 nm and 269-275 nm were selected with reference to the absorbance curves plotted between the wavelengths of 200-400 nm. In the second method i.e. Dual method in which two wavelengths were selected for each drug in a way so that the difference in absorbance was zero for another drug. Emtricitabine showed equal absorbance at 230.696 nm and 250 nm, where the differences in absorbance were measured for the determination of tenofovir. Similarly, differences in absorbances at 250 nm and 268.670 nm were measured for determination of emtricitabine. These methods allowed rapid analysis of two drug combination. The results of analysis were validated statistically and by recovery studies. This tablet containing both drugs was assayed using the methods developed, showing a good accuracy and precision.

3.4.20. Sudha et al\(^{[113]}\) developed a simultaneous ultraviolet spectrophotometric method for the analysis of tenofovir and emtricitabine in the combined dosage form (Tenof-Em). Although tenofovir is an old drug but emtricitabine is a novel drug and its combination is also new. The raw materials of the two drugs were solubilised in orthophosphate buffer (pH 3.5). The drugs showed good linearity at 5-30 μg/ml. Both the drugs showed good linearity at 5 – 30 μg/ml in the λ max of tenofovir and emtricitabine was 257nm and 280nm respectively. Quantification studies of the raw materials and the formulation gave good results. The accuracy and the precision of the developed method were confirmed by repeatability studies.

3.5. LITERATURE REVIEW OF INSTRUMENTAL METHODS OF ANALYSIS OF TENOFOVIR DISOPROXIL FUMARATE

3.5.1. Sparidans et al\(^{[114]}\) developed and validated a sensitive and selective reversed-phase liquid chromatographic assay for tenofovir in human plasma. Tenofovir was isolated from a 200 μl plasma sample using protein precipitation with trichloroacetic acid. The fluorescent 1, N\(^6\) - etheno derivative was formed at 98°C in the buffered
extract with chloroacetaldehyde. This derivative was analysed using gradient ion-pair liquid chromatography and fluorescence detection at 254 nm for excitation and 425 nm for emission. The usefulness of the assay was demonstrated for samples obtained from an HIV-infected patient treated with Tenofovir.

3.5.2. **Sentenac et al** [115] developed a new high-performance liquid chromatography assay method for the determination of tenofovir, a nucleotide analogue, in plasma. A solid–liquid extraction procedure was coupled with a reversed-phase HPLC system. The system requires a mobile phase containing Na₂HPO₄ buffer, tetrabutylammonium hydrogen sulfate and acetonitrile for different elution through a C18 column with UV detection. The method proved to be accurate, precise and linear between 10 and 4000 ng/ml. The method was applied to determine trough levels of Tenofovir in 11 HIV-infected patients with virologic failure under multiple antiretroviral therapies. This method was also successfully applied to a pharmacokinetic study in an HIV infected patient with renal failure.

3.5.3. **Rezk et al** [116] reported an accurate, sensitive and simple reverse-phase (RP) high-performance liquid chromatography (HPLC) assay for the simultaneous quantitative determination of emtricitabine and tenofovir in human blood plasma. Using 200 µL of plasma and BOND ELUT-C18 Varian columns, the solid phase extraction (SPE) method results in a clean baseline and high extraction efficiencies (100% for emtricitabine and 98.6% for tenofovir). An Atlantis TM dC-18 analytical column is used along with an 18 min linear gradient elution of phosphate buffer (pH 5.7) and methanol to provide sharp peaks for emtricitabine at 280 nm, tenofovir at 259 nm, and the internal standard 2’,3’ didoxuryridine (DDU) at 262 nm. This method was suitable for use in clinical pharmacokinetic studies and was nimble enough for therapeutic drug monitoring.

3.5.4. **Delahunty et al** [117] developed and validated an LC/MS/MS assay for the determination of tenofovir (TNF) for use with the EDTA anticoagulated human plasma matrix. Heparin-treated plasma and serum matrices were also validated. After addition of adefovir as an internal standard, trifluoroacetic acid was used to produce a protein-free extract. Chromatographic separation was achieved with a Polar-RP Synergi, 2.0mm × 150 mm, reversed phase analytical column. The mobile phase was 3%
acetonitrile/1% acetic acid, aq. Detection of TNF and the internal standard was achieved by ESI MS/MS in the positive ion mode using 288/176 and 274/162 transitions, respectively. The method was linear from 10 to 750ng/ml with a minimum quantifiable limit of 10ng/ml when 250 µl aliquots were analyzed. The usefulness of this LC/MS/MS method to routinely monitor plasma concentrations of TNF was demonstrated along with its ability to assist in the performance of pharmacokinetic studies.

3.5.5. King et al [118] developed and validated an LC–MS–MS methodology for the determination of TFV concentrations, which directly corresponded with the intra-hPBMC (human peripheral blood mononuclear cells), TFV-DP (tenofovir-diphosphate) concentration. To facilitate the evaluation of drug safety, virologic activity, and pharmacokinetics, an anion exchange isolation of tenofovir-diphosphate (TFVDP) from human peripheral blood mononuclear cells (hPBMCs), coupled with dephosphorylation, desaltation, and detection by LC–MS–MS was validated. hPBMCs were harvested from whole blood, lysed, and a suspension of intracellular Tenofovir moieties was produced. TFV-DP was isolated from TFV-monophosphate (TFV-MP) and Tenofovir (TFV), dephosphorylated with acid phosphatase to form TFV and then desalted and concentrated, making it possible for tandem mass spectral detection. This paper outlined the development and validation of the determination of TFV-DP concentrations in femtomoles per million hPBMCs.

3.5.6. Barkil et al [119] developed a sensitive high-performance liquid chromatography method coupled to UV and single mass spectrometry (MS) detection for the determination of tenofovir in human plasma. A solid phase extraction procedure (Bond-Elut® C18 Varian cartridges) provided high extraction efficiency (91% for tenofovir and 68.8% for the internal standard, 3-methylcytidine). An atlantis®-dC-18 analytical column was used with an isocratic mode elution of a mixture (pH 2.5) of ammonium acetate/methanol (98.5:1.5, v/v). Detection was performed at 260 nm and by using the ion at m/z 288. To investigate the potential of the validated method for clinical studies, more than 170 samples from HIV-infected adult patients were then analyzed with this assay. A good correlation was observed between the results obtained with both detectors. However, in several cases discordant results were observed between UV and MS detections. Therefore, Tenofovir can sometimes suffer from interferences using
either UV or single MS detection. We concluded that the double detection allows obtaining a more specific quantification of Tenofovir. The assay was sound and could be used for therapeutic drug monitoring allowing a higher reliability of the results which were transmitted to the medical team.

3.5.7. **Takahashi et al**[^120] developed a rapid and conventional LC-MS method, validated by estimating the precision and accuracy for inter- and intraday analysis. The quantification of tenofovir, a nucleoside reverse transcriptase inhibitor prescribed once daily, in human plasma is important due to a recent increase in its use. HPLC, however, cannot easily detect and quantify Tenofovir because of interfering peaks. This novel method provides a conventional, accurate and precise way to determine Tenofovir in human plasma samples.

3.5.8. **Gomes et al**[^121] developed and validated a rapid and sensitive liquid chromatography–tandem mass spectrometry (LC–MS/MS) method for simultaneous quantification of Tenofovir (TEN) and Emtricitabine (EMT) in human plasma using Chromolith Speed Rod RP18. The mass transition ion-pair has been followed as \( m/z \) 288.10→176.10 for TEN, \( m/z \) 248.20→130.20 for EMT and \( m/z \) 230.10→112.10 for Lamivudine (LAM). The method involves solid phase extraction from plasma, simple isocratic chromatographic conditions and mass spectrometric detection using an API 5000 instrument that enables detection at nanogram levels. Lamivudine was used as the internal standard. Total elution time was as low as 2 min.

3.5.9. **Seshachalam et al**[^122] developed a high-performance liquid chromatographic method for chiral separation of tenofovir enantiomers. The (R) and (S) isomers were separated on Intersil ODS 3V column (150 mm x 4.6 mm i.d., 5 µm) at 258°C. The mobile phase contained the complex of Cu (II) with the optical selector L-phenylalanine (L-PheA). Satisfactory results were achieved with the mobile phase consisting of buffer (3 mM of copper sulfate, 1 mM of L-PheA, and 20 mM of ammonium dihydrogen phosphate, pH adjusted to 4.0) and acetonitrile in the ratio of 95:5. The method was validated for linearity, repeatability, LOD, LOQ, and robustness. The solution stability was studied and found to be stable for the period of 7 days.

3.5.10. **Kandagal et al**[^123] developed and validated a simple reverse-phase high-performance liquid chromatographic method for the determination of Tenofovir
Disoproxil Fumarate (TDF) in pharmaceutical formulations and human plasma samples. Piroxicam (PRX) was used as an internal standard. The assay of the drug was performed on a CLC C18 (5µm, 25 cm x 4.6 mm i.d.) with UV detection at 259 nm. The mobile phase consisted of acetonitrile–water mixture in the ratio of 75:25 (v/v), and a flow rate of 1 ml/min was maintained. Analytic parameters have been evaluated. Within-day and between day precision as expressed by relative standard deviation was found to be less than 2%. The method had been applied successfully for the determination of TDF in spiked human plasma samples and pharmaceutical formulations.

3.5.11. Shirkhedkar et al \cite{124} developed two new, simple and cost effective UV-spectrophotometric and first order derivative methods for estimation of tenofovir disoproxil fumarate in bulk and tablets. Tenofovir disoproxil fumarate was estimated at 260 nm in 0.1N HCl. In first order derivative, it showed amplitude at 273 nm. The proposed methods were successfully applied for the determination of tenofovir disoproxil fumarate in pharmaceutical formulations. The results demonstrated that the procedure was accurate, precise and reproducible (relative standard deviation <2%), while being simple, cheap and less time consuming and can be suitably applied for the estimation of Tenofovir Disoproxil Fumarate in different dosage forms.

3.5.12. Majumder et al \cite{125} developed two new, selective and sensitive visible spectrophotometric methods (method A and B) for the estimation of tenofovir in bulk and in pharmaceutical preparations. Tenofovir was subjected to acid hydrolysis and this acid hydrolyzed drug was used for the estimation. Method A was based on the reaction with 3-methyl-2-benzothiazolinone hydrazone in the presence of ferric chloride, to form a colored species with a $\lambda_{\text{max}}$ at 628.5 nm. Method B was based on the reaction with Folin-Ciocalteu phenol’s reagent under alkaline condition with a $\lambda_{\text{max}}$ at 768 nm. The methods were extended to pharmaceutical formulations and there was no interference from any common pharmaceutical excipients and diluents. The result of analysis had been validated statistically and by recovery studies.

3.5.13. Balaji et al \cite{126} developed a simple, specific, accurate and precise high performance thin layer chromatographic method for determination of tenofovir disoproxil fumarate in bulk and pharmaceutical dosage forms. The method used
aluminium plates coated with silica gel 60F254 as stationary phase and n-butanol: n-hexane (5:5, v/v) as mobile phase. Densitometric evaluation of the separated bands was performed at 260nm. The $R_f$ value of tenofovir disoproxil fumarate was 0.35 $\pm$ 0.02. Results of analysis were validated statistically and by recovery studies. The method was validated according to the ICH guidelines with respect to linearity, accuracy, precision and ruggedness.

3.5.14. Karunakaran et al\cite{127} developed and validated a simple, rapid reverse - phase high performance liquid chromatographic method for the simultaneous estimation of Lamivudine and Tenofovir Disoproxil Fumarate in pure and in tablet dosage form. The estimation was carried out on a Phenomenax Luna C18 (150 mm x 4.6 mm i.d., particle size 5$\mu$m) column with a mixture of acetonitrile: methanol: water in the ratio of 30:50:20 (v/v) as mobile phase. UV detection was performed at 258 nm. The method was validated for linearity, accuracy, precision, specificity and sensitivity as per ICH norms. The developed and validated method was successfully used for the quantitative analysis of commercially available dosage form. The retention time was 3.27 and 4.15 min for lamivudine and tenofovir disoproxil fumarate, respectively. The flow rate was 1.0 ml/ min. The high percentage of recovery and low percentage coefficient of variance confirmed the suitability of the method for the simultaneous estimation of lamivudine and tenofovir disoproxil fumarate in pure and in tablet dosage form.

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