CHAPTER - 3

REVIEW OF LITERATURE

3.1 REVIEW OF LITERATURE

In literature, several analytical methods are reported for the determination of sofosbuvir and daclatasvir, ombitasvir, paritaprevir, ritonavir and abacavir, dolutegravir, lamivudine and bictegravir, emtricitabine, tenofovir alafenamide from its bulk, pharmaceutical formulation and biological fluids by using RP-HPLC-UV, LC-MS/MS, UV-VIS spectroscopy, HPTLC detection analytical techniques.

3.2 DACLATASVIR AND SOFOSBUVIR

A simple and precise method was developed for the assay of sofosbuvir from tablet formulation. The several spectrophotometric and HPLC methods have been reported for determination of sofosbuvir individually or in combination with other drugs in pharmaceutical dosage forms. Hence, in the present study, a new, sensitive, suitable and cost effective reversed-phase high performance liquid chromatographic method was developed and validated for the determination of sofosbuvir in bulk and pharmaceutical formulation. In RP-HPLC method, the analyte were resolved by using isocratic program, methanol and phosphate buffer (45:55 v/v) pH-3 was used as mobile phase, at a flow rate of 0.9 ml/min, on HPLC system containing UV-visible detector with Workstation Software and Greece C_{18} column (4.6 × 250 mm; 5 µm). The detection was carried out at 261 nm. The method gave the good resolution and suitable retention time. The results of analysis in the method were validated in terms of accuracy, precision, specificity, linearity, limit of detection, limit of quantification and robustness. The method does not require use of expensive reagent and also less time consuming, it can be performed routinely in industry for routine analysis of marketed product of sofosbuvir [24].

A stability indicating RP-HPLC method was developed and validated for the determination of sofosbuvir using C_{18} column (250 mm x 4.6ID, 5 µm) with mobile phase of methanol: water (70:30 v/v) with a flow rate of 0.8 ml/min. The detection was done at 261 nm. The retention time of sofosbuvir was 4.819 min. Sofosbuvir was subjected to stress conditions including acidic, alkaline, oxidation, photolysis and thermal degradation. The degraded products were well resolved from the pure drug with significantly different retention time values. Linearity was found to be 20-100 µg/ml with significantly high value of correlation coefficient. The method was validated for linearity, accuracy, precision, robustness and recovery. The limits of detection and quantification were 0.44 µg/ml and 1.33µg/ml respectively [25].

A simple, sensitive, precise, and accurate isocratic reverse phase high pressure liquid chromatographic method has been developed and validated for the estimation of sofosbuvir in bulk and tablet dosage form. To optimize, a column Phenomenex prodigy ODS-3V (150 mm x 4.6 mm, 5 µm), mobile phase mixture of methanol and (0.1%) tri-fluro acetic acid as buffer having pH of 3.2 in the ratio of (30:70 v/v) found to be an efficient system for elution of drug with good peak shape as well as retention time 2.990 min, flow rate 1.0 ml/min at UV wavelength of 260nm. Quantitative linearity was obeyed in the concentration range of 100 to 600 µg/ml, the regression equations of concentration over their peak areas were found to be $Y = 18864x + 58306$ $R^2 = 0.996$, where Y is the peak area and X is the concentration of drug. The number of theoretical plates obtained was 2604.352 which indicate the efficient performance of the column. The limit of detection was 0.01 µg/ml and limit of quantification was 0.03 µg/ml, which indicates the sensitivity of the method the high percentage recovery indicates that the proposed method is highly accurate. No interfering peaks were found in the chromatogram indicating that excipients

used in tablet formulation did not interfere with the estimation of the drug by the proposed RP-HPLC method [26].

A rapid and precise reverse phase high performance liquid chromatographic method has been developed for the validation of sofosbuvir, in its pure form as well as in tablet dosage form. Chromatography was carried out on a Hypersil C_{18} (4.6Ã—150mm, 5Âµ) column using a mixture of Methanol (100% v/v) as the mobile phase at a flow rate of 1.0 mL/min, the detection was carried out at 265 nm. The retention time of the sofosbuvir was 3.515 Â±0.02min. The method produce linear responses in the concentration range of 20-100Âµg/mL of sofosbuvir. The method precision for the determination of assay was below 2.0% RSD. The method is useful in the quality control of bulk and pharmaceutical formulations [27].

The present work is concerned with application of simple, precise, accurate, reproducible and specific RP-HPLC method for estimation of sofosbuvir in bulk. Separation of SFS was successfully achieved on a Hisil C_{18} (4.6 x 250mm, 5 µm) waters or equivalent in an isocratic mode utilizing phosphate buffer (4.0 pH): methanol (50:50%v/v) at a flow rate of 0.8 mL /min and eluate was monitored at 262 nm, with a retention time of 1.01 minutes. The method was validated and the response was found to be linear in the drug concentration range of 5 µg/mL to 30 µg/mL. The values of the slope, intercept and the correlation coefficient were found to be 0.07, -0.4 and 1.000 respectively. The RSD values for system precision and method precision were found to be 0.19% (Intra-day), 0.21% (Inter-day) and 0.20% (Intra-day), 0.23% (Inter-day) respectively [28].

Specific, accurate, simple, selective and stability-indicating RP-HPLC method is developed and validated for simultaneous determination of sofosbuvir and ledipasvir in tablet dosage form for assay and dissolution methods. RP-HPLC method was performed

on the Eclipse XDB C_{18} column (250 mm X 4.6 mm, 5 µm particle size, using buffer solution of pH 3.0 containing 0.02 M potassium dihydrogen phosphate and 5.7 mM hexane sulfonate: acetonitrile (50:50 v/v) as the mobile phase at a flow rate of 1.5 ml/min, injection volume 10 µL and UV detection at 254 nm. This method is validated according to BP, USP and ICH requirements for new methods, which include accuracy, precision, selectivity, robustness, ruggedness, LOD, LOQ, linearity and range. Linear relationships were obtained in the ranges of 40-500 µg/mL and 30-300 µg/ mL with correlation coefficients of 0.9998, 0.9996, 0.9996 and 0.9993 at RT value of 2.429 min and 4.529 min for sofosbuvir and ledipasvir respectively for assay content and dissolution rate. The forced degradation studies as acidity, alkalinity, oxidation, heat, and thermal, humidity and photo degradation were performed according to ICH guidelines. The accurate determination of both drugs is very important for Forensic and Criminal Investigations from the point of view of Forensic pharmacy [29].

A reversed phase high-performance liquid chromatographic (RP-HPLC) method and a direct ultra-violet spectrophotometric method, were adopted and validated for the quantification of sofosbuvir, which is a new antiviral agent used for treatment of patients with hepatitis C virus (HCV). Validation parameters such as linearity, accuracy, precision, specificity, limits of detection and quantification were determined according to the guidelines of International Conference on Harmonization (ICH)-Q2B. The RP-HPLC method was applied on Hypersil TM ODS C_{18} column (150 × 4.6 mm, 5 µm) as a stationary phase. The mobile phase was optimized according to the polarity of the studied drug. It was methanol: acetonitrile (90:10, v/v), pumped using an isocratic mode with flow rate of 1 mL/min and UV detection at 260 nm. The UV spectrophotometric method was performed for the studied drug at 260 nm. The calibration curves were linear in the ranges of 2-60 µg/mL and 5-40 µg/mL for the RP-HPLC and UV spectrophotometric methods,

respectively. The proposed methods are accurate, sensitive and precise, so they can be successfully adopted for the reliable determination of sofosbuvir content in its tablet form[30].

In this work, a stability indicating and validated UPLC method has been developed for estimation of sofosbuvir (API) in bulk and its formulations (Sovaldi®). The chromatographic separation was achieved on a Waters BEH C_{18} column (2.1 × 100 mm, 1.7 µm) in an isocratic elution mode with flow rate 0.4 mL/min, the mobile phase of acetonitrile and water (30:70) in 0.1% formic acid (pH ~2-3). The optimized method is linear over the concentration range of 20-120 ppm; the Limit of Quantification (LOQ) and Limit of detection are 0.063 and 0.03 µg/mL respectively [31].

In this work, a rapid and sensitive ultra-performance liquid chromatography tandem mass spectrometry (UPLC-MS/MS) method for the determination of ledipasvir, sofosbuvir and its metabolite GS-331007 in rat plasma was developed. The analytes and the internal standard (diazepam) were separated on an Acquity UPLC BEH C_{18} chromatography column (2.1mm × 50mm, 1.7µm) using gradient elution with a mobile phase of acetonitrile and 0.1% formic acid in water at a flow rate of 0.4 mL/min. The detection was performed on a triple quadrupole tandem mass spectrometer by multiple reaction monitoring (MRM) mode to monitor the precursor-to-product ion transitions of m/z 889.8→130.1 for ledipasvir, m/z 530.3→243.1 for sofosbuvir, m/z 261.5→113.1 for GS-331007 and m/z 285.2→193.1 for diazepam (IS) using a positive electrospray ionization interface. The method was validated over a concentration range of 2-500 ng/mL for ledipasvir, 10-2000 ng/mL for sofosbuvir and 10-2000 ng/mL for GS-331007. Total time for each chromatography was 3.0min. The intra- and inter-day precision and accuracy of the quality control samples at low, medium, and high concentration levels exhibited relative standard

deviations (RSD)<10.2% and the accuracy values ranged from -9.8% to 11.2%. The method was successfully applied to a pharmacokinetic study of ledipasvir, sofosbuvir and GS-331007 in rats [32].

A novel and sensitive LC–MS/MS method was developed and validated for determination of sofosbuvir (SF) using eplerenone as an internal standard. The Xevo TQD LC–MS/MS was operated under the multiple-reaction monitoring mode using electrospray ionization. Extraction with tert-butyl methyl ether was used in sample preparation. The prepared samples were chromatographed on Acquity UPLC BEH C_{18} (50 × 2.1 mm, 1.7 μm) column by pumping 0.1% formic acid and acetonitrile in an isocratic mode at a flow rate of 0.35 mL/min. Method validation was performed as per the US Food and Drug Administration guidelines and the standard curves were found to be linear in the range of 0.25–3500 ng/mL for sofosbuvir. The intra- and inter-day precision and accuracy results were within the acceptable limits. A very short run time of 1 min made it possible to analyze more than 500 human plasma samples per day. A very low quantification limit of sofosbuvir allowed the applicability of the developed method for determination of sofosbuvir in a bioequivalence study in human volunteers[33].

A rapid and sensitive ultra-performance liquid chromatography tandem mass spectrometry (UPLC-MS/MS) method for the determination of ribavirin, sofosbuvir and its metabolite GS-331007 in rat plasma was established. The analytes and the internal standard (midazolam) were separated on an Acquity UPLC BEH C_{18} chromatography column (2.1mm×50mm, 1.7μm) using gradient elution with a mobile phase of acetonitrile and 0.1% formic acid in water at a flow rate of 0.4mL/min. The detection was performed on a triple quadrupole tandem mass spectrometer by multiple reaction monitoring (MRM) mode to monitor the precursor-to-product ion transitions of m/z 245.1→113.1 for ribavirin,

m/z 530.3→243.1 for sofosbuvir, m/z 261.5→113.1 for GS-331007 and m/z 326.2→291.1 for midazolam (IS) using a positive electrospray ionization interface. The method was validated over a concentration range of 5-1000 ng/mL for ribavirin, 10-2000ng/mL for sofosbuvir and 10-2000 ng/mL for GS-331007. Total time for each chromatograph was 3.0min. The intra- and inter-day precision and accuracy of the quality control samples at low, medium, and high concentration levels exhibited relative standard deviations (RSD) <10.0% and the accuracy values ranged from -10.6% to 11.6%. The method was successfully applied to a pharmacokinetic study of ribavirin, sofosbuvir and GS-331007 in rats [34].

A sensitive and rapid method for quantitation of sofosbuvir in human plasma has been established using ultra performance liquid chromatography-electrospray ionization tandem mass spectrometry (UPLC-ESI-MS/MS). Sofosbuvir d3 was used as an internal standard. sofosbuvir and internal standard in plasma sample were extracted using ethyl acetate (liquid liquid extraction). A centrifuged upper layer was then evaporated and reconstituted with the mobile phase of 0.5% formic acid: methanol (30:70, v/v). The reconstituted samples were injected into a Gemini C_{18} (50 × 4.6mm, 5µm) column. Using MS/MS in the multiple reaction monitoring mode, sofosbuvir and sofosbuvir d3 were detected without severe interferences from human plasma matrix. Sofosbuvir produced a protonated precursor ion ($[M+H]^+$) at m/z 428.35 and a corresponding product ion at m/z 279.26. The internal standard produced a protonated precursor ion ($[M+H]^+$) at m/z 431.38 and a corresponding product ion at m/z 282.37. The calibration curves for the analyte was linear ($R2 \geq 0.9956$, n=4) over the concentration range of 4.063-8000.010 ng/mL. Stability studies revealed that sofosbuvir was stable in plasma during bench top (7h at room temperature), in injector (20h), at the end of five successive freeze and thaw cycles and long term at -70°C±15°C for 15 days. The developed method was validated as per the guidelines of

USFDA and the obtained results were found to be within the limits and could be successfully employed for the determination of sofosbuvir in human plasma for regular and pharmacokinetic studies [35].

Sofosbuvir is a novel direct acting antiviral agent against hepatitis C virus. In the present work, a rapid, specific and reproducible isocratic reversed phase high performance liquid chromatography (RP-HPLC) method has been developed and validated for the determination of sofosbuvir in the presence of its stressed degradation products. Sobosbuvir was subjected to hydrolysis (acidic, alkaline and neutral), oxidation, photolysis and thermal stress, as per international conference on harmonization (ICH) conditions. The drug showed degradation under oxidative, photolysis, acid and base hydrolysis stress conditions. However, it was stable under thermal and neutral hydrolysis stress conditions. Chromatographic separation of the drug from its degradation products was performed on Inertsil ODS-3 C_{18} (250 mm × 4.6 mm i.d., 5 µm) column using a green mobile phase of methanol: water 70:30 (v/v). The degradation products were characterized by LC–MS-MS and the fragmentation pathways were proposed. The developed method was validated as per ICH guidelines. No previous method was reported regarding the degradation behaviour of sofosbuvir [36].

A new validated bioanalytical method based on LC tandem MS has been developed for the simultaneous extraction and determination of sofosbuvir and ledipasvir in human plasma using antiviral daclatasvir as an internal standard (IS). Liquid-liquid extraction of samples was used for the purification and preconcentration of the analytes from a human plasma matrix. Good and consistent recoveries were obtained, with average extraction recoveries of 91.61 and 88.93% for sofosbuvir and ledipasvir, respectively. The chromatographic separation of the three analytes was achieved within only 2.8 min by an isocratic mobile

phase consisting of 10 mM ammonium acetate, which was then adjusted to pH 4.0 by acetic acid-acetonitrile-0.1% methanolic formic acid (12 + 25 + 63, v/v/v) flowing through a C_{18} Zorbax eclipse plus column (5 µm, 100×4.6 mm; Agilent). Multiple reaction monitoring transitions were measured in positive ion mode for sofosbuvir, ledipasvir, and daclatasvir (IS). A detailed validation of the method was performed and the standard curves were found to be linear in the range of 0.5 to 2500 and 5 to 2100 ng/mL for sofosbuvir and ledipasvir, respectively, applying weighted (1/X(2)) linear regression. The developed method was applied to the analysis of the two drugs after a single oral administration of Harvoni 400/90 mg film-coated tablets containing 400 mg sofosbuvir and 90 mg ledipasvir to four healthy volunteers [37].

A Novel simple, precise and economical reverse phase high performance liquid chromatographic method has been developed and validated for the estimation of daclatasvir in single dosage form HPLC – WATERS Model NO.2690 series compact system consisting of Inertsil-C_{18} ODS column with a mobile phase constituting of acetonitrile and methanol (70:30) Flow rate 1ml/min and detection was carried out at 230nm. The selected chromatographic conditions were found to effectively separate daclatasvir (Rt: 2.658 min). The developed method was validated for linearity, accuracy, precision, LOD, LOQ, robustness, ruggedness and for system suitability parameters as per ICH guidelines. Linearity for daclatasvir was found in the range of 20-80 µg/ml, respectively. The method was found to be robust. The proposed method could be used for routine analysis of daclatasvir in single dosage forms. The bulk drug was subjected to forced degradation studies like acid, alkali, oxidative, thermal conditions [38].

A sensitive, simple, selective and accurate HPLC method was developed and validated for analysis of antiviral drug daclatasvir (BMS-790052, DCV) in pure form and in tablet

dosage form in the presence of its degradation products. The chromatographic separation achieved by isocratic elution on Hypersil BDS C_{18}, 4.6×150 mm, 5µm column at 25ºC. The mobile phase was a mixture of 0.05M potassium dihydrogen phosphate (pH-4.5) and acetonitrile in ratio of 50:50 (v/v). The injection volume was 10 µl. The flow rate was 1ml/ minute. The detection wavelength was 320 nm. The developed method was validated as per ICH guidelines; it was precise, accurate and robust. The calibration curve of daclatasvir was linear in range 0.5- 100µg/ml with a correlation coefficient ≥ 0.999. Also the validated method was helpful for rapid routine analysis as the run time was less than 3 minute; the retention time for daclatasvir was about 2.33 minute. The method was successfully applied to analysis of daclatasvir in tablet form and the recovery was from 99.71% to 100.86% [39].

A simple and selective reversed-phase stability-indicating liquid chromatographic method has been developed and validated for the determination of daclatasvir in drug substance and drug product. Daclatasvir was subjected to acidic, alkaline, oxidative, thermal and photo-degradation study. The LC method was based on isocratic elution of daclatasvir and its degradation products on a reversed-phase C_{18} Hypersil column using a mobile phase consisting of phosphate buffer (10 mM, 1 mL), triethylamine: acetonitrile (60:40 v/v) at a flow rate of 2 mL min^{-1}. Quantitation was achieved with UV detection at 312 nm. Linearity, accuracy, and precision were found to be acceptable over the concentration range of 0.75–120 µg mL^{-1}, with regression coefficient value of 0.9999, and with limit of detection and quantitation of 0.148 and 0.447 µg mL^{-1}, respectively. Peak purity was checked for principle drug and its alkali induced degradation product, and the pathway of alkaline hydrolysis of daclatasvir was suggested by LC/MS [40].

A selective and specific high-performance liquid chromatography method for the determination of daclatasvir enantiomers has been developed and validated. Various immobilized polysaccharide-based chiral stationary phases were used to define a

separation strategy utilizing normal-phase and polar organic chromatography modes. Excellent resolution between daclatasvir and its enantiomer was achieved on amylose tris (3-chlorophenylcarbamate) stationary phase, namely CHIRALPAK ID-3, using binary gradient containing acetonitrile: diethylamine and methanol: diethylamine as the mobile phase. The flow rate of the mobile phases was maintained at 1.0 mL min−1 while the column oven temperature was maintained at 40 °C. The column effluent was monitored by UV detection at 315 nm. In comparison with isocratic method, the binary gradient method offered excellent peak shape and improved resolution between daclatasvir and its enantiomer while maintaining the specificity with dia-stereomers. The method was found to be precise, accurate, and linear (R2 > 0.999). Limit of detection and limit of quantitation of the enantiomer were found to be 0.083 µg mL−1 as and 0.25 µg mL−1, respectively. Recovery of the enantiomer was found to be in the range of 90 to 112 % [41].

Daclatasvir is an inhibitor of hepatitis C virus NS5A protein that is used for the therapy of chronic hepatitis. So far, published methods for analysis of daclatasvir in plasma are exclusively based on mass spectrometry, which is not always available in standard clinical laboratories. Thus, we wished to develop and validate a simple, but still reliable and sensitive high-performance liquid chromatography (HPLC) assay with UV detection for the quantification of daclatasvir, feasible for a wide-spread clinical routine use. The method consisted of solid-phase extraction of daclatasvir using Waters Oasis HLB 1cc cartridges, reversed-phase liquid chromatography with a Waters XTerra RP_{18} (150mm×4.6mm, 3.5µm) column and a mobile phase of ammonium acetate buffer (pH 5.0, 10mM) and acetonitrile (56:44, v/v), and UV detection at 318nm. This assay proved to be sensitive (lower limit of quantification of 0.05µg/mL), linear (correlation coefficients ≥0.997), specific (no interference with various potentially co-administrated drugs), reproducible (both intra-day and inter-day coefficients of variation ≤8.9%), and accurate

(deviations ranged from -2.2 to 8.0% and from -6.5 to 9.2 % for intra-day and inter-day assays, respectively). The method was applied to therapeutic monitoring of patients undergoing daclatasvir therapy for hepatitis C and showed to be reliable and robust. Thus, this method provides a simple, sensitive, precise, and reproducible assay for dosing daclatasvir that can be readily adaptable to routine use by clinical laboratories with standard equipment. In addition, the stability of daclatasvir in plasma was evaluated under various conditions, including after the heating procedure required for inactivation of infectious viruses and in different light exposure conditions. These studies evidenced photo-instability of the compound under sunlight exposure over time. Thus, blood sampling and the whole handling procedure have to be performed quickly and with minimal light exposure[42].

Daclatasvir dihydrochloride (DCH) is a new drug gained its FDA approval on July 24, 2015 for treatment of hepatitis C. As there are no reported UV spectrophotometric methods for estimation of daclatasvir dihydrochloride, the present work was aimed at development of accurate and precise spectrophotometric method for its estimation by absorbance maxima method. The working standard solution of 10 µg/ml was scanned in the wavelength range of 400-200 nm. Absorption maximum, lambda max was found at 214 nm. Calibration curve was obtained with good correlation coefficient value of 0.986. Linearity was observed in concentration range of 2-12 µg/ml. Method accuracy was revealed by recovery studies obtained in between 99.95 and 100.09[43].

A comprehensive stability indicating HPLC with diode array detection method was developed for the determination of the recently approved antiviral drug daclatasvir dihydrochloride (DCV) which is used for the treatment of chronic Hepatitis C Virus (HCV) genotype 3 infections. Effective chromatographic separation was achieved using Waters C8 column (4.6 × 250 mm, 5 µm particle size) with isocratic elution

of the mobile phase composed of mixed phosphate buffer pH 2.5 and acetonitrile in the ratio of 75:25 (by volume). The mobile phase was pumped at a flow rate of 1.2 mL/min, and quantification of DCV was based on measuring its peak areas at 306 nm. Daclatasvir eluted at retention time 5.4 min. Analytical performance of the proposed HPLC procedure was thoroughly validated with respect to system suitability, linearity, range, precision, accuracy, specificity, robustness, detection and quantification limits. The linearity range was 0.6–60 μg/mL with correlation coefficient > 0.99999. The drug was subjected to forced degradation conditions of neutral, acidic and alkaline hydrolysis, oxidation and thermal degradation. The proposed method proved to be stability-indicating by resolution of the drug from its forced-degradation products. The validated HPLC method was successfully applied to analysis of the cited drug in its tablets [44].

The objective of the present work is to develop a simple, efficient, and reproducible spectrophotometric method for the quantitative estimation of hepatitis-C drugs - Daclatasvir and Sofosbuvir in its active pharmaceutical ingredient (API) form. The developed ultraviolet spectrophotometric method for the quantitative estimation of hepatitis-C drugs - Daclatasvir and Sofosbuvir is based on measurement of absorption at a wavelength maximum (lambda max) of 317 and 261 nm using methanol as solvent. The method was validated in terms of specificity, precision, linearity, accuracy, and robustness as per the ICH guidelines. The method was found to be linear in the range of 50-150% for daclatasvir and in the range of 43-143% for sofosbuvir. The percentage recovery values were in the range of 99.4-100.6% for daclatasvir and in the range of 99.7-100.6% for sofosbuvir at different concentration levels. Relative standard deviation for precision and intermediate precision results were found to be <2%. The correlation coefficient value observed for daclatasvir and sofosbuvir drug substances was not <0.99, 0.99, respectively. Results obtained from the validation experiments prove that the developed method is

quantified for the estimation of daclatasvir and sofosbuvir drug substances. The developed method can be successfully applied for routine analysis, quality control analysis, and also suitable for stability analysis of daclatasvir and sofosbuvir in API form as per the regulatory requirements[45].

Specific, accurate, simple, selective and stability-indicating RP-HPLC method is developed and validated for simultaneous determination of sofosbuvir and ledipasvir in tablet dosage form for assay and dissolution methods. RP-HPLC method was performed on the Eclipse XDB C_{18} column (250 mm X 4.6 mm, 5 µm particle size, using buffer solution of pH 3.0 containing 0.02 M potassium dihydrogen phosphate and 5.7 mM hexane sulfonate: acetonitrile (50:50 v/v) as the mobile phase at a flow rate of 1.5 ml/min, injection volume 10 µL and UV detection at 254 nm. This method is validated according to BP, USP and ICH requirements for new methods, which include accuracy, precision, selectivity, robustness, ruggedness, LOD, LOQ, linearity and range. Linear relationships were obtained in the ranges of 40-500 µg/mL and 30-300 µg/mL with correlation coefficients of 0.9998, 0.9996, 0.9996 and 0.9993 at RT value of 2.429 min and 4.529 min for sofosbuvir and ledipasvir respectively for assay content and dissolution rate. The forced degradation studies as acidity, alkalinity, oxidation, heat, and thermal, humidity and photo degradation were performed according to ICH guidelines. The accurate determination of both drugs is very important for Forensic and Criminal Investigations from the point of view of Forensic pharmacy [46].

A reversed-phase high-performance liquid chromatographic method was developed for the simultaneous determination of sofosbuvir and ledipasvir in tablet dosage form. The analysis was performed on Luna analytical column 250 × 4.6 mm, 5 µm, octyl silica packing (Si–$[CH_2]_7$–CH_3) C8, using ammonium acetate buffer solution pH 7.0 and acetonitrile 35:65 % v/v as mobile phase at flow rate of 0.7 mL min^{-1} for isocratic elution.

Detection of sofosbuvir and ledipasvir was performed on a UV detector at 245 nm. The retention times of sofosbuvir and ledipasvir were 4.468 ± 0.013 min and 8.242 ± 0.012 min, respectively, and the total run time was 20 min. The method was validated according to the requirements of the United States Pharmacopeia (category I). The overall recovery of both analytes was 100 ± 1 %; the relative standard deviation for precision and intraday precision was less than 2.0 %. The method was linear with correlation coefficient (r) >0.9999, limits of detection 0.485 and 0.175 µg mL^{-1}, and limits of quantification were 1.619 and 0.586 µg mL^{-1} for sofosbuvir and ledipasvir, respectively. The method was successfully applied to the assay and in vitro dissolution studies of sofosbuvir and ledipasvir in tablet dosage form [47].

A new RP-HPLC method was developed for the simultaneous assay of sofosbuvir and ledipasvir in combined dosage form, using Inertsil ODS column (Make: 150 mmx4.6 mm I.D; particle size 5µm and a mobile phase composed of TFA- Buffer(pH -2.0), acetonitrile and methanol (30:50:20% v/v/v) at a flow rate of 1.0mL/min. The retention times of sofosbuvir and ledipasvir were found to be 3.205 and 3.774 min, respectively. Linearity was established for sofosbuvir and ledipasvir in the concentration ranges of 40-120ìg/ml and 10-30ìg/ml, respectively. Regression analysis showed a correlation coefficient of greater than 0.999 for sofosbuvir and ledipasvir. The percentage recoveries of sofosbuvir and ledipasvir were found to be in the range of 99.2 to 100.9% and 98.40 to 100.9% respectively. This proposed RP-HPLC method can be successfully employed for simultaneous quantitative analysis of sofosbuvir and ledipasvir in various combined formulations available in the local pharmacies[48].

A novel reversed phase high performance liquid chromatography (RP-HPLC) method has been developed for the estimation of sofosbuvir and velpatasvir drug product by liquid

chromatography. The chromatographic separation was achieved on C18 column (XTerra RP18 150*4.6, 5um) at ambient temperature .The separation achieved employing a mobile phase consists of 0.1%v/v trifluoro acetic acid in water: methanol (42:58). The flow rate was 1.0 ml/ minute and ultra violet detector at 269nm. The average retention time for sofosbuvir and velpatasvir found to be 3.44 and 4.68 min. The proposed method was validated for selectivity, precision, linearity and accuracy. All validation parameters were within the acceptable range. The assay methods were found to be linear from 80-240 µg/ml for sofosbuvir and 20-60µg/ml for velpatasvir[49].

A rapid and sensitive stability indicating RP-HPLC method was developed for simultaneous estimation of velpatasvir and sofosbuvir in combined tablet formulations. Chromatography was carried out on a Discovery® C_{18} HPLC Column (250 x 4.6 mm; 5µ particle size) by eluting with a mobile phase consisting of a 60:40 v/v mixture of 0.1% orthophosphoric acid in water and acetonitrile (ACN) at a flow rate of 1.0 mL/ minute. The detection wavelength was set at 240 nm. Accuracy was assessed by using standard addition method. The developed HPLC method was validated with respect to precision, specificity, accuracy, linearity and robustness. Forced degradation studies on the formulation were conducted by adopting the proposed method to assess the stability of the analytes under acid, base, peroxide, thermal and photolytic conditions and suitability of the method to resolve the degradation products [50].

A new sensitive high performance liquid chromatographic method for the estimation of ambroxol and azithromycin in combined dosage form has been developed. Chromatography was carried out on a Hypersil C_{18} column (250×4.6 mm, 5µ) with a flow rate of 1.0 mL/min of mobile phase and UV detection at a wavelength of 240nm and ambient column temperature with a mobile phase of phosphate buffer(pH-3.5) and

acetonitrile in the ratio of 45:55%v/v as the mobile phase. Acyclovir was used as an internal standard for this study. The retention times for ambroxol and azithromycin were found to be 3.326min and .4.472 min respectively. The proposed method was found to be linear in the concentration range of 10-30µg/ml for ambroxol and 50-150µg/ml for azithromycin respectively. The method was validated as per ICH guidelines and was found to be suitable for bioequivalence and pharmacokinetic studies [51].

3.3 OMBITASVIR, PARITAPREVIR AND RITONAVIR

Determination of paritaprevir and ritonavir in rat liver tissue samples. We successfully validated a UPLC-MS/MS method to measure paritaprevir and ritonavir in rat liver using deuterated internal standards (d8-paritapervir and d6-ritonavir). The method is linear from 20 to 20,000 and 5 to 10,000 pg on the column for paritaprevir and ritonavir, respectively, and is normalized per milligram tissue. Interday and intraday variability ranged from 0.591 to 5.33% and accuracy ranged from -6.68 to 10.1% for quality control samples. The method was then applied to the measurement of paritaprevir and ritonavir in rat liver tissue samples from a pilot study. The validated method is suitable for the measurement of paritaprevir and ritonavir within rat liver tissue samples for PK studies [52].

A simple, Accurate, precise method was developed for the simultaneous estimation of the ritonavir, ombitasvir and paritaprevir in tablet dosage form. Chromatogram was run through Ascentis C_{18} 150 x 4.6 mm, 5µ. Mobile phase containing acetonitrile and water in the proportion of 60:40 was pumped through column at a flow rate of 0.8ml/min. Temperature was maintained at 30°C. Optimized wavelength for ritonavir, ombitasvir and paritaprevir was 265nm. Retention time of ritonavir, ombitasvir and paritaprevir were found to be 2.147 min; 2.732 min and 3.790 min. %RSD of system precision for ritonavir, ombitasvir and paritaprevir were and found to be 0.4, 1.0 and 1.0 respectively. %RSD of

method precision for ritonavir, ombitasvir and paritaprevir were and found to be 0.5, 0.4 and 0.8 respectively. % recovery was obtained as 100.30%, 100.19% and 100.15% for ritonavir, ombitasvir and paritaprevir respectively. LOD, LOQ values are obtained from regression equations of ritonavir, ombitasvir and paritaprevir were 0.14ppm, 0.44ppm, 0.06ppm, 0.19ppm and 0.42ppm, 1.28ppm respectively. Regression equation of ritonavir was y = 28427x + 10360, ombitasvir was y = 8648x + 422.2 and of paritaprevir was y = 39168x + 17464. Retention times are not as much as different techniques so the method developed were basic and conservative that can be received in standard Quality control test in Industries. Retention times are decreased so the method developed basic and conservative embraced in general Quality control test in Industries [53].

The objective of the present study was to develop and validate a novel reverse phase high performance liquid chromatographic (RP-HPLC) method, for simultaneous determination of ritonavir (RIT), ombitasvir (OMB) and paritaprevir (PAR) in bulk mixtures, and in tablets.

Determination of the drugs ritonavir (RIT), ombitasvir (OMB), and paritaprevir (PAR), was carried out applying Hypersil BDS C_{18} column (250 mm X 4.6 mm i.e., 5 µm particle size), with photodiode array detector at λmax of 254 nm. The mobile phase applied for the current study composed of two solvents, i.e. A (0.01N % w/v potassium di-hydrogen orthophosphate buffer, pH 3.0 adjusted with dilute orthophosphoric acid) and B (acetonitrile). The mobile phase was pumped at a flow rate of 1.0 ml/min in the isocratic mode. The validation study with respect to specificity, linearity, precision, accuracy, and robustness, limit of detection (LOD) and limit of quantification (LOQ) was carried out employing the ICH guidelines. Ritonavir, ombitasvir, and paritaprevir showed linearity of response between 12.5-75 µg/ml for ritonavir, 3.125-18.75 µg/ml for ombitasvir and

18.75–112.5 µg/ml for paritaprevir, with a correlation coefficient (R2) 0.999, 0.999,0.999 for RIT, OMB, and PAR respectively. The % recovery obtained was 99.82±0.14 % RIT, OMB 100.03±0.96 % and for 99.96±0.26 % PAR. The LOD and LOQ values for RIT, OMB, PAR were obtained to be 0.02, 0.019and0.02, µg/ml and 0.07, 0.06 and 0.07 µg/ml, respectively. The method also exhibits good robustness for different chromatographic conditions like wavelength, flow rate, mobile phase, and injection volume. The method was successfully employed, for the quantification of RIT, OMB, and PAR, in the quality control of in-house developed tablets, and can be applied for industrial use[54].

A sensitive, simple, selective, and accurate HPLC method was developed and validated for the Simultaneous analysis of antiviral drugs, ombitasvir, paritaprevir, ritonavir, and ribavirin used for chronic hepatitis C virus genotype 4 infection in Egyptian patients with or without compensated cirrhosis. The chromatographic separation was achieved by isocratic elution on a reversed-phase analytical column [Magellen® C_{18} (10µm, 150 x 4.6 mm) column] at ambient temperature. The mobile phase was a mixture of 0.1M phosphate buffer (ph 7) and acetonitrile in ratio of 25:75 (v/v), injection volume was 20 µl, flow rate was 1ml/ minute and the detection wavelength was 243nm. The developed method was validated as per ICH guidelines; it was precise, accurate and robust. The calibration curves of the four drugs were linear in range:5-150µg/ml for ribavirin, 1.8-60 µg/ml for paritaprevir, and 2.5–50 µg/ml for ritonavir,2.25–36µg/ml for ombitasvir with a correlation coefficient ≥0.999. The validated method was helpful for rapid routine analysis as the run time was less than 6 minutes; the retention time was 1.298, 2.82,4.115 and 5.786 minute and LOD was found to be 1.2, 0.8, 0.7 and 0.06 µg/ml and LOQ 3.6, 2.4, 2.1 and 0.21 µg/ml for ribavirin, paritaprevir, ritonavir and ombitasvir respectively. The method was successfully applied to analysis of these drugs in their tablet dosage for MS with accepted % recovery for each one [55].

Stability indicating RP-HPLC method was developed for the simultaneous quantitation of sofosbuvir and velpatasvir in its pharmaceutical dosage form and validated. The drugs were separated on Discovery C_{18} (150mm x 4.6mm, 5µ) column using 0.01N potassium dihydrogen phosphate buffer and acetonitrile (50:50%v/v) as mobile phase on isocratic mode. The mobile phase is pump into the column at flow rate of 1.0ml/min and column oven temperature is maintained at 30ºC. The drugs were detected at a wavelength 240nm. The retention time for sofosbuvir and velpatasvir were found to be 2.32min and 3.34min respectively. The developed method is validated in accordance with ICH guidelines. The method was found to be accurate, precise, specific and robust. The method obeys Beer's law at a concentration range of 100µg/ml – 600µg/ml of sofosbuvir and 25µg/ml – 150µg/ml of velpatasvir, with correlation coefficient of 0.999 for both the drugs. The drugs were found to be stable and less prone to degradation when they are subjected to various stress conditions [56].

The present study describes the development and validation of a simple, rapid, selective and economical reverse phase high performance liquid chromatography-diode array detection (HPLC-DAD) method for the simultaneous determination of paritaprevir (PAR), ombitasvir(OMB), dasabuvir (DAS) and ritonavir (RIT) in bulk and pharmaceutical preparations. The proposed method was carried out using an RPC18 column (150 × 4.5 mm, 3.5 µ), with a mobile phase consisting of 10 mM phosphate buffer (pH 7)and acetonitrile (35:65, v/v) at a flow rate of 1 ml/min and a detection wavelength of 254 nm. Sorafenib (SOR) was selected as the internal standard to ensure that the quantitative performance was high. The method was validated based on its specificity, linearity, limit of detection, limit of quantitation, accuracy, precision, robustness and stability. The calibration curves for PAR, DAS, RIT and OMB were linear at 2.5–60, 1.25–30, 1.7–40 and 0.42–10 µg/ml, respectively, and all of the correlation coefficients

were >0.999. The proposed method was successfully applied for the determination of ombitasvir/paritaprevir/ritonavir/dasabuvirin tablets, without interference from the excipient peaks. Hence, the method can be applied for the routine quality control analysis of the studied drugs, either in bulk or dosed forms [57].

A simple, precise, specific and accurate reverse phase HPLC method has been developed for the determination of ritonavir in bulk and pharmaceutical dosage forms. The chromatographic separation was achieved on Symmetry C_{18} (4.6 x 100mm, 3.5 µm) column using a mixture of buffer: acetonitrile (50:50) as the mobile phase at a flow rate 1.0 ml/min. Linearity was observed in concentration range of 50-150 µg/ml. The retention time of ritonavir was 5.1 min. The analyte was monitored using UV detector at 239 nm. Results of analysis were validated statistically and by recovery studies. The method was validated according to the ICH guidelines with respect to specificity, linearity, accuracy, precision and robustness[58].

A simple, robust and selective and sensitive spectrophotometric method has been developed for the determination of ritonavir in pharmaceutical formulations. The method was based on the scanning of methanolic solution of the drug and methanolic solution of formulation. The method showed high sensitivity with linearity range from 10 to 20 µg/mL. The lower limit of detection (LOD) was found to be 1.1 µg/mL and the limit of quantization (LOQ) was determined as the lowest concentration was found to be 3.3 µg/mL. The variables that affected the reaction were carefully studied and optimized. The proposed method was applied successfully for the determination of ritonavir in pharmaceutical formulations. The percentage recovery was found to be 99.426 ± 0.59 (n = 9) for pharmaceutical formulation[59].

A simple, precise, accurate and repeatable method for bulk and tablet dosage form of ritonavir have been developed using spectrophotometric method. Differential spectrophotometric method was used. The developed method was validated according to ICH (Q2R1) guidelines and was found to be accurate, precise and specific. Amplitude difference was taken at absorbance maxima at 246 nm and absorbance minima at 266 nm. Linearity range was found to be within the concentration range of 10-30 µg/mL. Limit of detection and quantification was found to be 2.62 and 7.96 µg/mL respectively. The proposed method was found accurate in the range of 95.5 to 105.5%. It can be successfully applied for the estimation of ritonavir in bulk and pharmaceutical dosage forms. The results of the analysis were validated statistically and by recovery studies[60].

3.4 ABACAVIR, DOLUTEGRAVIR AND LAMIVUDINE

A simple, accurate and reproducible RP-HPLC method has been developed for the simultaneous determination of lamivudine, zidovudine and abacavir in tablet dosage forms. Chromatography was carried out on a HiQ Sil C 18 V column using a mobile phase consisting of 0.01 M potassium dihydrogen ortho-phosphate (pH 3.0) and methanol (55:45 v/v) at a flow rate of 0.8 mL/min. The detection was made at 272 nm and stavudine was used as the internal standard for this study. The retention times for lamivudine, abacavir and zidovudine were found to be 3.8, 6.3, 8.1 min. respectively. The calibration curves were linear over the range 5-250 µg/mL for both zidovudine and abacavir and 5-140 µg/mL for lamivudine. The proposed method was validated as per ICH and USP guidelines and it was found suitable for the routine quality control analysis of the drugs in tablet dosage forms [61].

A simple, rapid, specific, stability-indicating method was developed and validated for the simultaneous estimation of Abacavir sulfate, Lamivudine, and Dolutegravir sodium in

pharmaceutical dosage form using RP-HPLC. The chromatographic separation was done using BDS column of dimensions 250mm x 4.6mm, 5µ particle size with a mobile phase consisting of potassium dihydrogen phosphate buffer and acetonitrile in the ratio 45:55%v/v run on an isocratic mode of flow rate 1.0ml/min. The column oven temperature was maintained at 30ºC. The detection was done at a wavelength of 240nm. The developed method was validated in accordance with ICH guidelines, evaluating accuracy, precision, ruggedness, robustness, LOD, LOQ, stability parameters and found to be within the limits. The method obeys Beer's law in the concentration range of 150µg/ml-900µg/ml for Abacavir, 75µg/ml-450µg/ml for Lamivudine and 12.5µg/ml-75µg/ml for Dolutegravir with correlation coefficients of 0.9999, 0.9996 and 0.9999 for the three drugs respectively. Forced degradation studies were conducted by exposing the standard drug solution to the various stressed conditions such as acidic, basic, oxidative, thermal, neutral and photolytic conditions. The net degradation for the drugs was found to be within the limits [62].

A fresh selective, rapid, accurate, precise and RP-HPLC stability-indicating method was developed and validated for the quantitative simultaneous determination of dolutegravir and lamivudine in the bulk as well as pharmaceutical dosage form. A chromatographic separation was done by using Inertsil ODS 3V (250 × 4.6 mm, 5 µm) column and mobile phase composed of phosphate buffer, pH-3.0: acetonitrile : methanol (50:20:30% v/v/v) with flow rate of 1.0 mL/min, and the detection of eluents was carried out at a wavelength of 257 nm utilizing a PDA detector. The drugs, dolutegravir and lamivudine, were subjected to varied conditions like base hydrolysis, acid hydrolysis, oxidation, thermal, photochemical and UV. The suggested method was analysed statistically and validated to fulfil requirements of International Conference on Harmonisation (ICH) and the validation

covered accuracy, precision, linearity, limit of detection (LOD), limit of quantification (LOQ), robustness, ruggedness and specificity [63].

A Simple, accurate, specific and rugged reverse phase liquid chromatographic method was developed for the simultaneous estimation of Lamivudine, Tenofovir, and Dolutegravir in bulk and tablet dosage form. A reverse phase gradient program has been developed to separate the all four active ingredients. The ingredients present in different concentrations and chromatographic behaviour 0.05 M Phosphate buffer pH 6.2 ± 0.05 adjusted with dilute potassium hydroxide solution, Acetonitrile was used as mobile phase. A gradient programing has been done, on a reverse phase C18 column (250 × 4.6 mm, 5 micron) with a flow rate 1 mL/min, monitored at 260 nm. The mean retention times of Lamivudine, Tenofovir, and Dolutegravir were found to be 2.8, 5.2 and 11.5 min respectively. Linearity of Lamivudine, Tenofovir, and Dolutegravir was found to be 27–162 µg/mL, 27–162 µg/mL and 4.5–28 µg/mL respectively. The proposed method was validated in terms of Linearity, Range, Accuracy, Precision, Specificity, Robustness and stability studies and the method is successfully applied to the estimation of Lamivudine, Tenofovir, and Dolutegravir in combined tablet dosage form[64].

A reverse phase liquid chromatographic method for the simultaneous determination of lamivudine and abacavir in pure and tablet formulation was developed and validated. The method was found tosimple, precise and accurate. The separation was carried out using Phenomenex C18(250 x 4.6 mm, 5 µm particle size) column, with amobile phase consisting of phosphate buffer (pH 7.8) and methanolin the ratio of 50:50 % v/v. The flow rate was set at 1.0 mL/min anddetection was monitored at 216 nm. The retention times of lamivudine and abacavir were found to be 3.147 and 6.367 min, respectively. The linearity was found in the concentration range of and 80-280µg/mL and 75-450µg/mL for lamivudine and abacavir, respectively. The liquid chromatography method was extensively

validated for linearity, accuracy, precision, and robustness. All these analytical validation parameters were found satisfactory and the %RSD was determined which indicates the usefulness of method for determination of lamivudine and abacavir in bulk drug and tablet formulation[65].

A method has been developed and validated for the estimation of abacavir, lamivudine and zidovudine by high performance liquid chromatography (HPLC) on a C18 column with UV detection at 270 nm. The mobile phase composition that provides an optimal resolution of components in an acceptable elution time in water: methanol (70: 30 v/v) with 0.1 % potassium dihydrogen phosphate pH 3.2 (adjusted with ortho phosphoric acid). The powdered tablet were extracted with methanol: water (50:50 v/v) mixture and after addition of stavudine, an internal standard subjected to HPLC analysis and assayed by comparison of analyte to internal standard peak areas to concentration ratios. The method was successfully applied to pharmaceutical formulation because no chromatographic interferences from the tablet excipients were found. The method retained its accuracy and precision when the standard addition technique was applied [66].

The mechanism of the RP-HPLC is the retention, by the interaction of non-polar hydrocarbon chain of stationary phase with non-polar parts of the sample molecules. This method had been developed for simultaneous determination of antiretroviral drugs which are widely used such as, abacavir sulphate and lamivudine in tablet dosage form and was carried on column Inertsil ODS (150×4.6, 5µm) with UV detection at 254 nm using a mobile phase composition of mixed phosphate buffer (pH 4.0) and acetonitrile at a flow rate of 1 ml/min. The proposed method was validated in terms of linearity, accuracy, precision, robustness, ruggedness, specificity, limit of detection and limit of quantification as per ICH and USP guidelines and it was found suitable for the routine quality control

analysis of the drugs in tablet dosage forms. Linearity of abacavir and lamivudine were found in the range of 20-120µg/ml and 10-60 µg/ml respectively. The limit of detection was found to be 0.0049 and 0.0268 for abacavir and lamivudine respectively. Limit of quantification was found to be 0.0184 µg/ml and 0.0150 µg/ml for abacavir and lamivudine respectively. Hence, it was concluded, chromatographic method developed for abacavir sulphate and lamivudine said to be rapid, simple, specific, sensitive, precise, accurate and reliable that can be effectively applied for routine analysis in research institutions, quality control department in industries, approved testing laboratories, bio-pharmaceutics and bio-equivalence studies and in clinical pharmacokinetic studies[67].

The method was established using Agilent C18 (250 × 4.6 mm, i.d., 5 µm) column, a mobile phase consisting of 0.05M phosphate buffer pH 6.2 (solvent A) and acetonitrile (solvent B) 60:40 v/v at a flow rate of 1 mL/min with isocratic elution, injecting 10 µL sample into the chromatographic system. The eluted compounds were detected by using PDA Detector at a detection wavelength of 260 nm and the temperature was maintained at 30°C. Result: Retention times for the three compounds were found to be 3.09 min, 6.19 min and 9.61min for lamivudine, tenofovir alafenamide, and dolutegravir respectively. The linearity range was 10-80 µg/ml for three drugs with values of LOD found to be 0.56, 0.39µg, 1.35µg and LOQ were found to be 1.50µg, 0.99µg and 3.61 µg for lamivudine, tenofovir alafenamide and dolutegravir respectively which were linear enough showing correlation coefficient 0.999 in all the cases. Conclusion: The proposed method is therefore, suitable for the purpose in quality-control laboratories for quantitative analysis of the drugs individually and in the combined dosage form. The method was found to be as it is simple and rapid with tremendous precision and accuracy. The method can be used as a routine quality control method for triple combined dosage forms [68].

A simple and rapid high performance liquid chromatographic method was developed and validated for simultaneous estimation of abacavir, lamivudine and dolutegravir in their tablet dosage form. The method was established using non polar column-Kromasil 250 mm × 4.5 mm, 5 µm, mobile phase as buffer: acetonitrile (65:35) at a flow rate of 1 mL/min with isocratic elution, injecting 10 µL sample into the chromatographic system. The eluted compounds were detected by using PDA Detector at detection wavelength of 257 nm and temperature was maintained at 30 °C. Retention times for the three compounds were found to be 2.250 min, 2.734 min and 9.633 min for lamivudine, abacavir and dolutegravir, respectively. The linearity range was 15 to 90 ppm, 30 to 180 ppm and 2.5 to 15 ppm with values of LOD found to be 0.08 µg, 0.06 µg, 0.03 µg and LOQ were found to be 0.2 µg, 0.19 µg and 0.10 µg for lamivudine abacavir and dolutegravir, respectively which were linear enough showing correlation coefficient 0.999 in all the cases. The present method was specific, sensitive, reproducible, precise, rapid and simple [69].

A method employing high performance liquid chromatography (HPLC) with tandem mass spectrometry (MS) has been developed and validated for the simultaneous determination of clinically relevant levels of zidovudine (AZT) and lamivudine (3TC) in human serum. The method incorporates a fully automated ultrafiltration sample preparation step that replaces the solid-phase extraction step typically used for HPLC with UV detection. The calibration range of the dual-analyte LC-MS/MS method is 2.5-2,500 and 2.5-5,000 ng ml-1 for AZT and 3TC, respectively, using 0.25 ml of human serum. The lower limit of quantification was 2.5 ng ml^{-1} for each analyte, with a chromatographic run time of approximately 6 min. Overall accuracy, expressed as bias, and inter- and intra-assay precision are < +/- 7 and < 10% for AZT, and < +/- 5 and < 12.1% for lamivudine over the full concentration ranges. A cross-validation study demonstrated that the LC-MS/MS method afforded equivalent results to established methods consisting of a radioimmuno-

assay for AZT and an HPLC-UV method for lamivudine. Moreover, the LC-MS/MS was more sensitive, allowed markedly higher-throughput, and required smaller sample volumes (for 3TC only). The validated method has been used to support post-marketing clinical studies for combivir a combination tablet containing AZT and lamivudine [70].

A HPLC-MS-MS method was developed and validated to measure lamivudine and zidovudine simultaneously in small volumes of human seminal plasma. Sample preparation was simple and rapid, requiring 25 microliter of sample, the use of isotopically labelled lamivudine and zidovudine as internal standards and ultrafiltration through a molecular mass cut-off membrane. Lamivudine and its internal standard were separated from zidovudine and its internal standard with isocratic HPLC. Detection was carried out using tandem mass spectrometry. This validated method was used to analyze seminal samples obtained from six HIV-positive patients prescribed lamivudine and zidovudine[71].

A sensitive liquid chromatography tandem mass spectrometry (LC-MS/MS) assay was developed and validated to facilitate the assessment of clinical pharmacokinetics of dolutegravir (DTG) in plasma samples. This work describes an assay system requiring only a 20μL aliquot of human plasma that is subjected to a simple acetonitrile protein precipitation containing a stably labelled isotope of DTG used as an internal standard. Chromatography was performed on an XT Bridge C_{18}, 2.1mm×50mm, reversed phase analytical column, using a 60:40 acetonitrile/water mobile phase containing 0.1% formic acid. Detection of the analyte and internal standard was achieved by ESI positive ionization tandem mass spectrometry. The precursor/product transitions (m/z) monitored was 420.1/136.0 and 428.1/283.1 for DTG and DTG-IS, respectively. The dynamic range of this assay extends from 5 to 10,000ng/mL, with a mean coefficient of determination (r, mean±SD) of 0.9996±0.0003. The mean precision values for calibration standards ranged from 0.7 to 4.1%, while accuracy values were 98.3 to 102.0%. Validation results

demonstrated high accuracy (≤6.5% deviation) and high precision (≤9.1% CV) for the quality control samples. This assay system provides an accurate, precise, and sensitive method for DTG quantitation and was successfully applied to clinical research samples as part of a phase I/II paediatric clinical trial [72].

A combined bio-analytical assay for abacavir, a reversed transcriptase inhibitor, and mycophenolic acid (MPA), based on reversed-phase liquid chromatography and both ultraviolet (UV) absorption and fluorescence detection, is reported. Both analytes are extracted from plasma with acetonitrile. After centrifugation, evaporation of the supernatant and reconstitution in water, the sample is injected into the chromatograph. Abacavir is detected using UV detection at 285 nm and MPA spectrofluorometrically at 345 and 430 nm for excitation and emission, respectively. The method has been validated in the 80-2000 ng/ml range for abacavir and in the 10-10,000 ng/ml range for MPA for 200-microl plasma samples. The lower limits of quantification are 80 and 10 ng/ml for abacavir and mycophenolic acid, respectively. Precisions and accuracies are < or = 8% in the valid concentration ranges of both analytes[73].

Lamivudine has been widely used in the treatment of HIV disease. A reliable, sensitive reversed phase high performance liquid chromatography (RP-HPLC) method was developed and validated for lamivudine in rabbit plasma. The method was developed on Hypersil BDS C_{18} column (250 mm×4.6 mm, 5 μm) using a mobile phase of 0.25% triethylamine buffer (pH 3.0): acetonitrile (70:30, v/v). The efficient was monitored by UV detector at 256 nm. The total run time was 15 min with a flow rate of 1.0 mL/min. Calibration curve was linear over the concentration range of 25-2000 ng/mL. The retention times of lamivudine and internal standard (Nelfinavir) were 8.78 min and 10.86 min, respectively. The developed RP-HPLC method can be successfully applied for the quantitative pharmacokinetic parameter's determination of lamivudine in rabbit model[74].

A simple, precise, accurate and rapid high performance thin layer chromatographic method has been developed and validated for the simultaneous estimation of Lamivudine and Abacavir sulphate in combined dosage forms. The stationary phase was pre-coated silica gel 60F254. The mobile phase used was a mixture of (Acetone: chloroform: methanol 4: 4: 2 v/v/v). The detection of spot was carried out at 265nm. The method was validated in terms of linearity, accuracy, precision and specificity. The calibration curve was found to be linear between 500 to 3000 ng with regression coefficient of 0.9998. The proposed method can be successfully used to determine the drug content of marketed formulation[75].

Simple, sensitive, precise, and specific high-performance liquid chromatographic (HPLC) and high-performance thin-layer chromatographic (HPTLC) methods for the determination of dolutegravir sodium in bulk drug and pharmaceutical dosage form were developed and validated. In the HPLC method, analysis of the drug was carried out on the ODS C18 column (150 × 4.6 mm, 5 μm particle size) using a mixture of acetonitrile: water (pH 7.5) in the ratio of 80:20 v/v as the mobile phase at the flow rate 1 mL/min at 260 nm. This method was found to be linear in the concentration range of 5-35 μg/mL. The peak for dolutegravir sodium was observed at 3.0 ± 0.1 minutes. In the HPTLC method, analysis was performed on aluminium-backed plates pre-coated with silica gel G60 F254 using methanol: chloroform: formic acid in the proportion of 8:2:0.5 v/v/v as the mobile phase. This solvent system was found to give compact spots for dolutegravir sodium with the Rf value 0.77 ± 0.01. Densitometric analysis of dolutegravir sodium was carried out in the absorbance mode at 265 nm. Linear regression analysis showed good linearity with respect to peak area in the concentration range of 200-900 ng/spot. The methods were validated for precision, limit of detection (LOD), limit of quantitation (LOQ), accuracy, and specificity. Statistical analysis showed that both of the methods are repeatable and specific

for the estimation of the said drug. The methods can be used for routine quality control analysis of dolutegravir sodium[76].

A rapid, simple, accurate, and economical spectrophotometric method has been developed and validated for the assay of the anti-retroviral agent lamivudine in active pharmaceutical ingredients (API) and in its tablet formulation. The analysis is based on the UV absorbance maxima at about 270nm wavelength of lamivudine, using methanol as solvent. A sample of API was dissolved in methanol to produce a solution containing 10 µg/mL of lamivudine. Similarly, a sample of ground tablets were extracted with methanol, centrifuged, and diluted with the same solvent. The absorbance of the sample preparation was measured at 270 nm against the solvent blank, and the assay was determined by comparing with the absorbance of a similarly prepared 10 µg/mL standard solution of lamivudine. The calibration graph was rectilinear from 5 µg/mL to 15 µg/mL for lamivudine with the correlation coefficient being more than 0.999. The relative standard deviation of the replicate determination was about 0.5%. The percent recovery was within the range of 98%-102%, indicating insignificant interference from the other ingredients in the formulation. The method can be applied for the routine QC quantitation of lamivudine in API and tablet formulation[77].

A simple, rapid, precise and accurate spectrophotometric method has been developed for quantitative analysis of Dolutegravir sodium in tablet formulations. The initial stock solution of Dolutegravir sodium was prepared in methanol solvent and subsequent dilution was done in water. The standard solution of Dolutegravir sodium in water showed maximum absorption at wavelength 259.80 nm. The drug obeyed Beer-Lamberts law in the concentration range of 5-40 µg/ mL with coefficient of correlation (R"2) was 0.9992. The method was validated as per the ICH guidelines. The developed method can be

adopted in routine analysis of Dolutegravir sodium in bulk or tablet dosage form and it involves relatively low cost solvents and no complex extraction techniques [78].

A spectrophotometric method for the assay of lamivudine in pure form and in dosage form was developed in this study. The method was based on charge-transfer complex formation between the drug, which acted as n-donor while chloranilic acid and 2,3-dichloro-5,6-dicyano-1,4-benzoquinone (DDQ) acted as a p-acceptor in a non-aqueous solvent in each case. Chloranilic acid was found to form a charge-transfer complex in a 1:1 stoichiometry with lamivudine (lamivudine-chloranilic acid) with a maximum absorption band at 521 nm. Also, DDQ was found to form a charge-transfer complex in a 1:1 stoichiometry with lamivudine (lamivudine-DDQ) with a maximum absorption band at 530 nm. The pH was obeyed at acid range. The complexes obeyed Beer's law at a concentration range of 0.04 - 0.28 mg/ml. The thermodynamic parameters calculated at different temperatures included the molar absorptivity, association constant, free energy change, enthalpy and entropy. The proposed method has been conveniently applied in the analysis of commercially available lamivudine tablet with good accuracy and precision[79].

The present work was undertaken to develop and validate a rapid and consistent UPLC method in which the peaks will appear in a short period as per ICH Guidelines. The UPLC separation was achieved on a Symmetry C18 (2.1 × 100mm, 1.7mm, Make: BEH) or equivalent in an Isocratic Mode. The mobile phase was composed of Phosphate Buffer (60%) [pH 3.0] & Methanol (40%) [UPLC Grade] The flow rate was monitored at 0.25 ml per min. The wavelength was selected for the detection was 280 nm. The run time was 3 min. The retention time found for the drugs lamivudine, abacavir, and zidovudine was 1.019 min, 1.271 min & 1.617 min respectively. The % recovery was found to be 98.0%-99.0% for the drug abacavir. The % recovery was found to be 98.0% – 99.6% for the drug lamivudine. The % recovery was found to be 98.2% – 98.6% for the drug zidovudine. The

linearity was established in the range of 20 to 60 ppm for the drug abacavir & 10 to 30 ppm for the drug lamivudine & 20 to 60 ppm for the drug zidovudine. The LOD for the drugs abacavir, lamivudine, and zidovudine were found to be 0.002 µg/ml, 0.003 µg/ml, & 0.005 µg/ml, respectively. The LOQ for the drugs abacavir, lamivudine, and zidovudine were found to be 0.008 µg/ml, 0.01 µg/ml & 0.02 µg/ml respectively. Overall, the proposed method was found to be suitable, sensitive, reproducible, specific and accurate for the quantitative determination of the drug in tablet dosage form[80].

3.5 BICTEGRAVIR, EMTRICITABINE AND TENOFOVIR ALAFENAMIDE

Shirkhedkar et.al, were reported two simple, rapid, accurate and economical 'Zero order UV-spectrophotometry' and 'first order derivative' methods have been developed for estimation of tenofovir in bulk and tablets [81].

Nevase et.al, were reported spectrophotometric method for estimation of tenofovir disoproxil fumarate tablet dosage form [82].

Soumya et.al were reported simultaneous determination of tenofovir disoproxil fumarate and lamivudine by UV-Spectrophotometric Method. The absorption maxima of both drugs were found at 260nm and 280nm and obeyed Beer's law in the range of 5-45µg/ml (y = 0.021x + 0.002; r2 = 0.999) and 2-16µg/ml (y = 0.061x + 0.004; r2 = 0.998) respectively for TDF and LAM in acetonitrile : 0.1N HCl (20:80) solvent system[83].

Choudhari has reported that spectrophotometric simultaneous determination of tenofovir disoproxilfumarate and emtricitabine in combined tablet dosage form by ratio derivative, first order derivative and absorbance corrected methods and its application to dissolution study [84].

Bhavsar et.al, has reported RP-HPLC method for simultaneous estimation of tenofovir disoproxilfumarate, lamivudine, and efavirenzin combined tablet dosage form. The

separation was based on the use of a Kromasil C_{18} analytical column (150 × 4.6mm, I.D., 5 μm). The mobile phase consisted of a mixture of 70 volumes of methanol and 30 volumes of 10 mM phosphate buffer (pH5.0). This parathion was carried out at 40°C temperature with a flow rate of 1 ml/min[85].

Komaroju et. al, has reported a simple, precise, accurate and rapid RP-HPLC method with PDA detector has been developed and subsequently validated for the simultaneous estimation of emtricitabine and tenofovir disoproxil fumarate in pure and tablet dosage form[86].

Gangrade et.al, has reported reversed phase high performance liquid chromatographic determination of impurities of tenofovir disoproxil fumarate in which a mobile phase consisting of 0.05M sodium dihydrogen phosphate containing 1 ml of triethyl amine, pH adjusted to 2.3 with ortho phosphoric acid and acetonitrile in volume ratio of 60:40 at a flow rate of 1.0 ml/min a Grace Vydac C_{18}, 25cm, 5mcm column was used as stationary phase. Quantitation was performed using UV – VIS variable wavelength detector at 260 nm[87].

Barkil et.al, reported that the relevance of a combined UV and single mass spectrometry detection for the determination of tenofovir in human plasma by HPLC in therapeutic drug monitoring. Anatlantis-d C_{-18} analytical column is 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 [88].

Delahunty et.al, reported Sensitive assay for determining plasma tenofovir concentrations by LC/MS/MS. After addition of a defovirasan internal standard, trifluoroacetic acid was used to produce a protein-free extract. Chromatographic separation was achieved with a

polar-RP Synergi, 2.0 mm x 150 mm, reversed-phase analytical column. The mobile phase was 3% acetonitrile /1% aceticacid [89]

Bezy et.al, reported Simultaneous analysis of several antiretroviral nucleosides in rat-plasma by high-performance liquid chromatography with UV using acetic acid / hydroxylamine buffer Test of this new volatile medium-pH for HPLC-ESI-MS/MS [90].

Takahashi M et.al, reported the determination of plasma tenofovir concentrations using a conventional LC-MS method. A rapid and conventional LC-MS method, validated by estimating the precision and accuracy for inter- and intra-day analysis in the concentration range of 0.019-1.567mg/ml [91].

Bennetto-Hood et. al. reported the development of a sensitive and specific liquid chromatography/ mass spectrometry method for the determination of tenofovir in human plasma. Plasma samples were prepared by solid-phase extraction performed on Waters Oasis cation-exchange cartridges (30mg). Chromatographic separation was performed iso-critically on a reversed-phase Waters Atlantis dC18 column (2.0 x 100 mm, 3 μm). The mobile phase consisted of a hydroxylamine/acetic acid buffer (pH 6.75) and methanol (93:7, v/v) [92].

Sentenac et.al, has reported Sensitive determination of tenofovir in human plasma samples using reversed-phase liquid chromatography. A solid-liquid extraction procedure was coupled with a reversed-phase HPLC system. The system requires a mobile phase containing Na(2)HPO(4) buffer, tetrabutyl ammonium hydrogen sulphate and acetonitrile for different elution through a C_{18} column with UV detection [93].

Chandra et.al, reported that an application of high-performance thin-layer chromatographic method for the simultaneous determination of amivudine and tenofovir disoproxilf umarate in pharmaceutical dosage form[94].

Havele et.al, reported stress studies of tenofovir disoproxil fumarate by HPTLC in bulk drug and pharmaceutical formulation [95].

Joshi et.al, reported HPTLC method for the simultaneous estimation of emtricitabine and tenofovirin tablet dosage form. The mobile phase used was a mixture of chloroform: methanol (9:1v/v). The detection of spots was carried out at 265 nm. The method was validated in terms of linearity, accuracy, precision, and specificity. The calibration curve was found to be linear between 200 to 1000 ng with regression coefficient of 0.9995[96].

Vishnu et.al, reported that spectrophotometric simultaneous determination of tenofovir disoproxil fumarate and emtricitabine in combined tablet dosage form by ratio derivative, first order derivative and absorbance corrected methods and its application to dissolution study [97].

Nimje et.al, reported application of UV- spectrophotometric method for estimation of emtricitabine in bulk and capsule [98].

Bhaskar et.al, reported a simple UV-spectrometric determination of emtricitabine in pure form and in pharmaceutical formulation[99].

Peepliwal et.al, reported the determination of emtricitabine in human plasma by RP-HPLC with UV-detection. The protein precipitation method results in high extraction efficiency for FTC (>85%) with of drug free plasma. A Phenomenex Luna C18, 150 x 4.6 mm, 5 micron column with methanol/10m.mol phosphate buffer, pH-3.2 (15:85,v/v) were

used to provide sharper peaks for FTC and lamivudine (internal standard, IS) at 280 nm[100].

Ghorpade et.al, reported simultaneous determination of emtricitabine and tenofovir by area under curve and dual wave length spectrophotometric method. For the AUC method, the wave length ranges between 242-248 nm and 269-275 nm were selected with reference to the absorbance curves plotted between the wave lengths of 200-400nm. In the second method, dual methods in which two wave lengths were selected for each drug in a way so that the difference in absorbance is zero for another drug. Emtricitabine shows equal absorbance at 230.696 nm and 250 nm, where the differences in absorbance were measured for the determination of Tenofovir [101].

Prashant et.al, reported a validated stability-indicating RP-HPLC method for the simultan determination of tenofovir, emtricitabine, and efavirenz and statistical approach to determine the effect of variables. The drugs individually, and in combination, were subjected to forced degradation (thermal, photolytic, hydrolytic, and oxidative stress conditions) and accelerated stability studies ($40 \pm 1°C/75 \pm 3\%$ RH for three months). Successful separation of combined drugs from degradation products was achieved by gradient elutionon are verse-phase C_{18} column, using a mobile phase containing phosphate buffer (pH-3.5) : acetonitrile at 1.5 mL min^{-1} flow rate, detection wavelength 256 nm[102].

Seshachalam et.al, reported the development and validation of a stability-indicating liquid chromatographic method or determination of emtricitabine and related impurities in drug substance [103].

Raju et.al, reported that simultaneous RP-HPLC method for the estimation of the emtricitabine, tenofovir disoproxil fumerate and efavirenzin tablet dosage forms [104].

Soni et.al, reported simultaneous estimation of tenofovir and emtricitabine in human plasma using HPLC after protein precipitation extraction. In the present study, reverse phase high performance liquid chromatographic method was developed and validated for the simultaneous estimation of TNF and FTC in human plasma using stavudine as the internal standard. Protein precipitation extraction procedure utilizing perchloric acid was employed to extract the drugs from human plasma. Similarly various RP-HPLC methods for determination of emtricitabine and tenofovir are reported [105].

Hamarapurkar et.al, reported the HPLC method for the determination of emtricitabine and related degradation substances [106].

Kumar et.al, reported validated HPTLC method for the determination of emtricitabine as bulk drug and in capsule dosage form [107].

Nikalje et.al, reported HPTLC method development, validation for simultaneous determination of efavirenz, emtricitabine and tenofovirin combined tablet formulation and forced degradation studies [108].