

**ANALYTICAL METHOD DEVELOPMENT AND
VALIDATION OF SOME ANTI-VIRAL PHARMACEUTICAL
FORMULATIONS**

कुछ एंटी-वायरल फार्मास्युटिकल फॉर्मूलेशन का विश्लेषणात्मक
पद्धति विकास और सत्यापन

A

Thesis

**Submitted for the Award of the Ph.D. degree of
PACIFIC ACADEMY OF HIGHER EDUCATION
AND RESEARCH UNIVERSITY**

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**DEPARTMENT OF PHARMACEUTICAL CHEMISTRY
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RESEARCH UNIVERSITY UDAIPUR**

2023

DECLARATION

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A resolute effort to achieve the target backed by self-confidence is the key to success. A journey is easier when you travel together. Success of any project never depends on individual but it is always teamwork. It is pleasant aspect that I have the opportunity to express my gratitude for all of them. It is said that ‘accomplishments must be credited to those who have put up the foundations of the particular chore’. Here, I pay tribute to **Father - G. Wilson and Mother – G. Ratna Kumari** for lifting me up to this phase of life & being supportive to me, giving me the confidence that ‘We trust you & are always there for you’. I thank them for their love, trust, patience, support & of course, for bearing all kinds of stress, they could, to make me what I am. I owe everything to them. I thanks to my brother **Sri G. Phanikumar**, my loving and caring spouse **Sri Neeraja** & my daughter **G. Sahithi** and my son **G. Charan** for their kind affection, love, trust & support.

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GOLLAPALLI NAGARAJU

ABSTRACT

Pharmaceutical drug products play a vital role in human progress by finding curing for diseases. Today, a majority of the drugs used are of synthetic origin. They are produced in bulk and used for their therapeutic effects in pharmaceutical formulations. There are biologically active chemical substances generally formulated into convenient dosage forms such as tablets, capsules, suspensions, ointments and injectables. These formulations deliver the drug substances in a stable, non-toxic and acceptable form, ensuring its bio-availability and therapeutic activity.

The end user of the drug product has to be assured of the medicine's quality, which makes suitable testing of the materials an area of great concern. Thus, the analytical activities concerning purities in drug products are among the most important issues in modern pharmaceutical analysis.

This subject or topic for this research activity is selected based on the increasing need for the pharmaceutical industry to develop suitable analytical methods. Among various other available techniques, the scope of work was focused on the modern chromatographic techniques such as HPLC and UPLC, which are very powerful and sophisticated techniques and have a wide spectrum of applications in the pharmaceutical industry. The development and validation of such a chromatographic method needs a lot of theoretical study, practical knowledge, skills, capability of application, literature search and visualisation of experimental and extrapolated results and assessment of the right conditions for use and method application and finalization. Some products were reviewed, and it was felt that there is a need to develop new, simple and reliable analytical methods.

Five chromatographic methods were developed for different pharmaceutical drug products. The first method was developed for the estimation of Sofosbuvir and Daclatasvir. The second method was developed for the estimation of Ombitasvir, paritaprevir, ritonavir. The third method was developed for the estimation of

Abacavir, Dolutegravir, and Lamivudine. The fourth method was developed for the estimation of Bictegraviremtricitabine, tenofovirafenamide.

The developed methods were validated according to the ICH (International Conference on Harmonization) guidelines and proved suitable for quality control of the drugs in pharmaceutical preparations.

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CHAPTER - 1

INTRODUCTION



1.1. INTRODUCTION

To know about the composition and structure of matter, Analytical chemistry, a branch of science, is used, by acquiring, practicing and conveying information. It is not confined to definite compounds or reactions and it deals with the study of the natural and artificial materials. Geometrical features like molecular morphologies and species identity are constituted in the properties of analytical chemistry^[1]. The development of its various concepts and theories include safety and quality of food, pharmaceuticals and water, environmental monitoring, biomedical applications and also to support the legal processes (forensics) and diagnose diseases, etc., Analytical chemists play a vital role to support this^[2].

To identify and measure the chemical species in a sample, analytical chemists use different techniques. By comparison of the known substance to a similar substance (whose concentration is known), which is called a standard reference material, and almost every technique will be carried out^[3].

In general, the drugs may be new or partially modified in structure of the existing ones with combinations releasing into the market annually. Frequently, from its introduction into the market to the inclusion of pharmacopoeias, it is being delayed, so there is a lack of analytical methods for these drugs, and for such drugs, this can lead to the development of newer analytical methods.

In the literature, for the drugs, no appropriate analytical methods are available. Excipients cause interference in drug formulation; hence no suitable methods are available for drug analysis. The use of some expensive reagents and solvents leads to the convolution of extraction and separation procedures, which may not be trustworthy^[4].

1.2 CHROMATOGRAPHY- AN OVERVIEW:

To resolve a multi-component mixture into its individual components, chromatography, is a new, well-known and a primary tool of separation and it can be applied both quantitatively and qualitatively. Despite, some other methods like IR spectroscopy, Nuclear Magnetic resonance spectroscopy or Mass spectroscopy etc. are required for the final identification and confirmation.

Tswett. M, in 1806, in Warsaw, invented a new technique, while separating the plant pigments by a column of calcium carbonate, which acted as an adsorbent and the different substances get adsorbed to different extent and this give rise to the different colored bands, at different positions on the column. In Greek, chroma means color and graphos means writing. Hence, he termed the system of colored bands as the chromatogram and the method as chromatography^[5].

To separate coloured as well as colorless substances, recent advances have been made there after. Thus, in general, a sample moves over a stationary phase through the mobile phase in chromatography. Chromatography is one of the best and most likely used analytical techniques, now-a-days and in foreseeable future. It is the cornerstone of molecular analytical chemistry. Recent advancements of chromatography have been introduced by A.P.J. Martin and R.L.M. Synge in 1941, made them noble prize winners^[6].

1.3 HIGH PERFORMANCE LIQUID CHROMATOGRAPHY (HPLC):

HPLC is used to figure out the amount of specific compound in a solution. It supports reliable quantitative range to allow the determination of substances in a single run. This method is considered to be rapid, accurate, precise and specific and offers the ease of

automation. It is because methods using HPLC have more advantages over the conventional methods^[7].

1.4 PRINCIPLE OF HPLC:

A mixture of sample is dissociated into components for its identification, quantification and purification by HPLC, due to the differences in their relative affinities for the mobile phase and stationary phase used. Especially, RP-HPLC, relies on the principle of hydrophobic interactions, as the more non-polar the material is, longer it will be retained. Due to their low affinities and polar nature, most of the drugs elute at a faster rate through the column and so they are separated and detected easily.

The optimization of laboratory resources is ensured by the effective method development, while methods meet the objectives required at each stage of drug development. To approve the drug, at certain stages, method development is required by the regulatory agencies^[8-11].

1.5 METHOD DEVELOPMENT OF DRUGS:

The analyst is enough to know the information of the compound. One can select the most suitable HPLC method development by the physical and chemical characteristics and by a vast literature review. Information regarding the sample can be achieved by molecular weight, structure, functionality, Pka value, UV-spectra and solubility of the compound. By knowing whether the pure compound is organic soluble or water soluble, one can select the best mobile phase and column for HPLC method development. In many laboratories, typical detectors like Mass spectroscopy, UV-Visible detectors are used as they can detect a wide variety of compounds^[12-15].

Analytical methods are intended to establish the identity, purity, physical characteristics and potency of the drugs. To support drug testing against specifications that arise during manufacturing and quality release operators, as well as during long-term stability studies and for safety and characterization studies or evolution of drug performance, methods are developed.

Before the final method optimization, all individual components should be investigated during the preliminary method development. By this, it is easy to evaluate the method performance in each component and streamline the final method optimization. Resolving power, specificity and speed are the major attributes of method development. By combining different factors like composition of solvent, type of stationary phase, mobile phase, P^H and buffers, selectivity can be achieved. For the chromatographic separation, changing solvents and stationary phases, proper range of P^H are the most suitable approaches. Better chromatographic resolution can be achieved by the P^H ranging from 1-12 and the development of the method depends upon column efficiency, selectivity and retention time. For chromatographic separation, mobile phase composition or strength plays a vital role. The most commonly used solvents in RP-HPLC are acetonitrile, methyl alcohol and Tetra hydro furan (having the wavelength of 190, 205 & 212nm). By the selection of right column temperature and changing the mobile phase, the separation of many samples can be enhanced ^[16-19].

1.6 METHOD VALIDATION OF DRUGS:

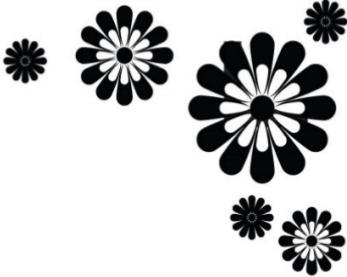
According to ISO definition, validation is defined as “Verification, where the specified requirements are adequate for an intended use.” Method validation can be used for qualitative, semi-quantitative or quantitative methods. The scientific soundness of the measurement or characterization and also to varying extents throughout the regulatory

submission process, the validation of analytical method is required. The method development includes the measurement of the correct substance, in correct amount and in appropriate range. The goal of method validation is to identify the critical parameters and to establish the acceptance criteria of system suitability^[20-21].

The effort done in method development and optimization leads to the effective development of HPLC method and its final performance. For the method development of samples in chromatographic separation, method validation is very important^[22].

1.7 ICH GUIDELINES:

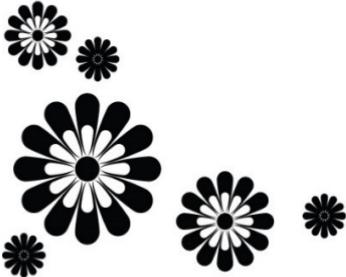
For analytical measures and method authentication, U.S FDA has given some manufacturing directions. The ICH guidance also provides clear text on the method validation of drug analysis. The USP has precise strategies for method validation for compound assessment. USP describes eight steps for endorsement. They are, Linearity, Accuracy, Precision, Selectivity and Specificity, Limit of quantification, Limit of detection, Robustness and Ruggedness^[23].



CHAPTER – 2

OBJECTIVE AND PLAN OF

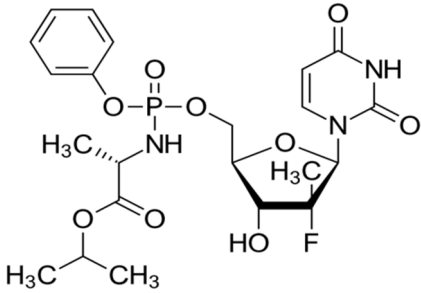
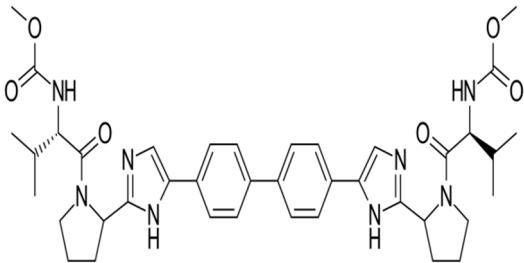
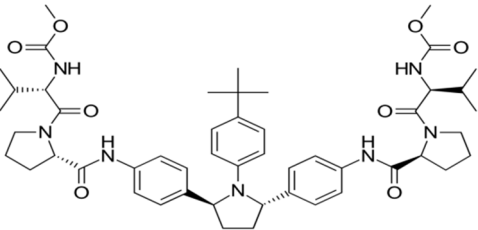
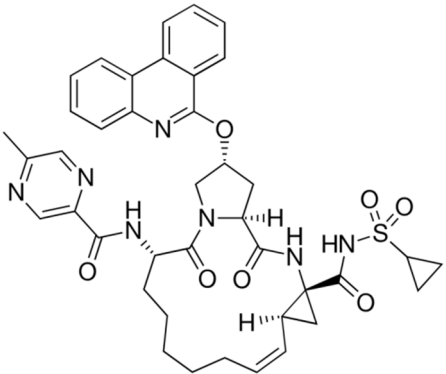
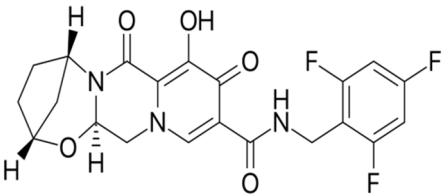
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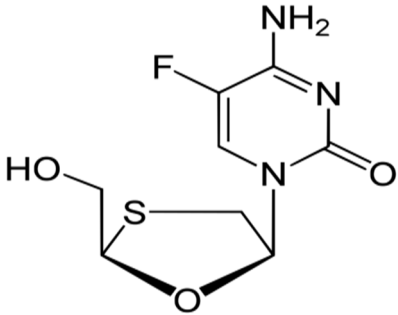
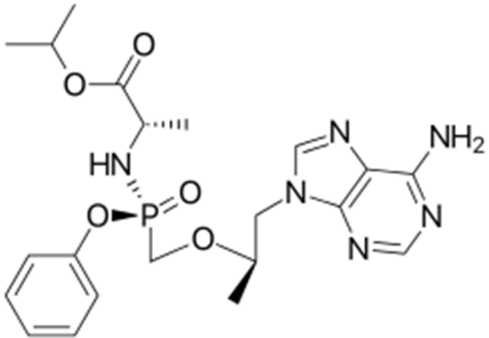


2.1 OBJECTIVES OF WORK

- ✓ To carry out an extensive literature survey before selecting a research problem (selection of drugs).
- ✓ To optimize chromatographic parameters, regression, extraction methods for selected drugs.
- ✓ To develop and validate a analytical method for quantitative determination of selected drugs like Sofosbuvir and Daclatasvir, Ombitasvir, Paritaprevir, Ritonavir and Abacavir, Dolutegravir, Lamivudine and Bictegravir, Emtricitabine, Tenofovir alafenamide in API's by using RP-HPLC, UV detection to explore the applicability of HPLC, the developed and validated methods have to be successfully applied to assess the purity of the marketed pharmaceutical formulation pharmaceutical formulations by using RP-HPLC with UV detection.

Table 2.1: List of drugs selected for research work

S. No.	Name of the Drug	Chemical Structure	Category
1.1	Sofosbuvir		Inhibitor of hepatitis C virus
1.2	Daclatasvir		Inhibitor of hepatitis C virus
2.1	Ombitasvir		Inhibitor of hepatitis C virus
2.2	Paritaprevir		Inhibitor of hepatitis C virus
3.1	Bictegravir		Treatment of HIV-1 and HIV-2 infection

3.2	Emtricitabine		Nucleoside reverse transcriptase inhibitor (NRTI) for the treatment of HIV infection
3.3	Tenofovir alafenamide		Hepatitis B virus (HBV) nucleotide reverse transcriptase inhibitor

2.2 PLAN OF WORK**A) To develop analytical method**

- 1) Selection of solvent for solution preparation.
- 2) Selecting the HPLC separation mode and fixation of parameters like
 - a) Selecting/optimizing the mobile phase and column for analyte and Internal analysis.
 - b) Selecting appropriate gradient/ isocratic mobile phase medium, flow rate, column temperature and pH.
 - c) Selecting the appropriate detector system.
- 3) Tuning of analyte and internal standard for fixation of mass parameters.
- 4) Selecting the extraction procedure for recovery of analyte.

B) To validate different parameters

- 1) System suitability
- 2) Specificity/selectivity
- 3) Linearity and range
- 4) Precision and accuracy
- 5) Accuracy (Recovery)
- 6) Ruggedness
- 7) Robustness
- 8) LOQ and LOD

C) To estimate the drug content in pharmaceutical formulations by RP-HPLC



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 C₁₈ 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 C₁₈ 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-fluoro 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 C₁₈ (4.6Å—150mm, 5Åμ) 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 Å±0.02min. The method produce linear responses in the concentration range of 20-100Åμg/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 C₁₈ (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 C₁₈ 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 C₁₈ 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 C₁₈ 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 C₁₈ 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 C₁₈ (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 C₁₈ 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 C₁₈ (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≥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. Sofosbuvir 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 C₁₈ (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 C₁₈ 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/X²) 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-C₁₈ 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 C₁₈, 4.6×150 mm, 5µm column at 25°C. 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 C₁₈ 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 ($R^2 > 0.999$). 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 RP₁₈ (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 $\leq 8.9\%$), 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 (λ_{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 C₁₈ 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-[CH₂]₈-CH₃) 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 $0.175 \mu\text{g mL}^{-1}$, and limits of quantification were 1.619 and $0.586 \mu\text{g mL}^{-1}$ 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 $5\mu\text{m}$ 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 40-120 $\mu\text{g/ml}$ and 10-30 $\mu\text{g/ml}$, 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, 5µm) 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® C₁₈ 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 C₁₈ 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, PARITAPRE VIR 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-paritaprevir 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 C₁₈ 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 C₁₈ column (250 mm X 4.6 mm i.e., 5 µm particle size), with photodiode array detector at λ_{max} 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 (R^2) 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, 0.019 and 0.02, 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® C₁₈ (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 C₁₈ (150mm x 4.6mm, 5 μ) 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 30°C. 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 100 μ g/ml – 600 μ g/ml of sofosbuvir and 25 μ g/ml – 150 μ g/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 \times 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 μ g/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 C₁₈ (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, 5 μ 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 30°C. 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 150 μ g/ml-900 μ g/ml for Abacavir, 75 μ g/ml-450 μ g/ml for Lamivudine and 12.5 μ g/ml-75 μ g/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 \times 4.6 mm, 5 μ m) 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 $\mu\text{g/mL}$, 27–162 $\mu\text{g/mL}$ and 4.5–28 $\mu\text{g/mL}$ 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 to simple, precise and accurate. The separation was carried out using Phenomenex C18(250×4.6 mm, 5 μm particle size) column, with a mobile phase consisting of phosphate buffer (pH 7.8) and methanol in the ratio of 50:50 % v/v. The flow rate was set at 1.0 mL/min and detection 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 80–280 $\mu\text{g/mL}$ and 75–450 $\mu\text{g/mL}$ 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 20-120 µg/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 9.61 min 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, 0.39 µg, 1.35 µg and LOQ were found to be 1.50 µg, 0.99 µg 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 \times 4.5 mm, 5 μ m, mobile phase as buffer: acetonitrile (65:35) at a flow rate of 1 mL/min with isocratic elution, injecting 10 μ L 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 $^{\circ}$ 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 μ g, 0.06 μ g, 0.03 μ g and LOQ were found to be 0.2 μ g, 0.19 μ g and 0.10 μ g 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 $< \pm 7$ and $< 10\%$ for AZT, and $< \pm 5$ 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 C₁₈, 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 ($\leq 6.5\%$ deviation) and high precision ($\leq 9.1\%$ 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 $\leq 8\%$ 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 C₁₈ column (250 mm \times 4.6 mm, 5 μ m) using a mobile phase of 0.25% triethylamine buffer (pH 3.0): acetonitrile (70:30, v/v). The effluent 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 R_f 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 ($y = 0.021x + 0.002$; $r^2 = 0.999$) and 2-16µg/ml ($y = 0.061x + 0.004$; $r^2 = 0.998$) 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 C₁₈ 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 (pH 5.0). 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 C₁₈, 25cm, 5mm 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. Atlantis-d C₁₈ 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% acetic acid [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 C₁₈ 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 disoproxil fumarate 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 simultaneous 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 ($40 \pm 1^\circ\text{C}/75 \pm 3\% \text{ RH}$ for three months). Successful separation of combined drugs from degradation products was achieved by gradient elution on a reverse-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 for determination of emtricitabine and related impurities in drug substance ^[103].

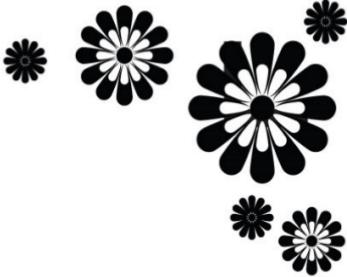
Raju et.al, reported that simultaneous RP-HPLC method for the estimation of the emtricitabine, tenofovir disoproxil fumarate and efavirenz in tablet dosage forms ^[104].

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].

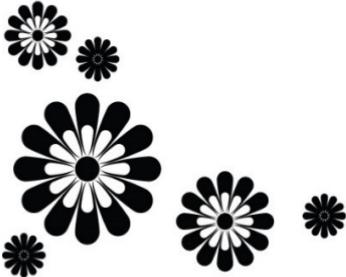
Nikalje et.al, reported HPTLC method development, validation for simultaneous determination of efavirenz, emtricitabine and tenofovirin combined tablet formulation and forced degradation studies ^[108].



CHAPTER - 4

DACLATASVIR AND

SOFOSBUVIR



DRUG PROFILE

SOFOSBUVIR:

Structure:

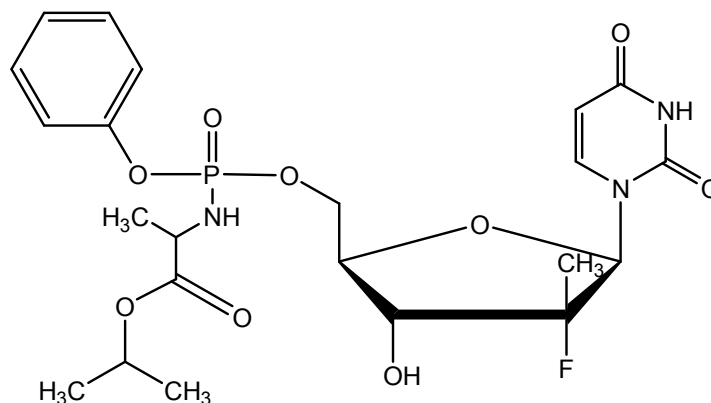
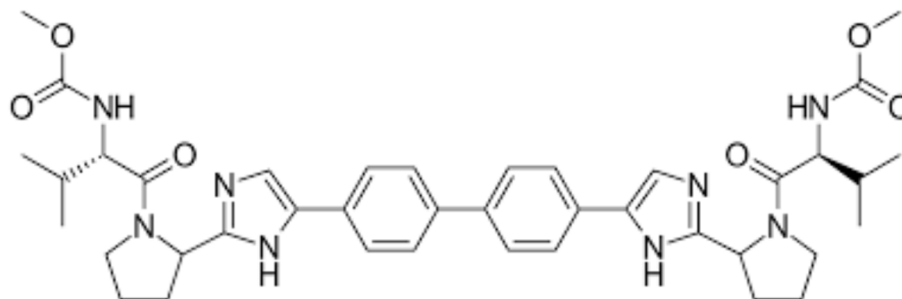


Fig. 4.1: Structure of Sofosbuvir

IUPAC Name	: Isopropyl (2S)-2-[[[(2R,3R,4R,5R)-5-(2,4-dioxypyrimidin-1-yl)-4-fluoro-3-hydroxy-4-methyl-tetrahydrofuran-2-yl]methoxy-phenoxy-phosphoryl]amino] propanoate
Molecular formula	: C ₂₂ H ₂₉ FN ₃ O ₉
Molecular Weight	: 529.453 g/mol.
Solubility	: Soluble in Methanol and water
p_{ka}	: 9.3
Category	: Inhibitor of the hepatitis C NS5B protein. It acts as a high barrier to the development of resistance

DACLATASVIR:**Structure:****Fig. 4.2: Structure of Daclatasvir**

IUPAC Name	: Dimethyl N,N'-([1,1'-biphenyl]-4,4'-diylbis{1H-imidazole-5,2-diyl-[(2S)-pyrrolidine-2,1-diyl][(2S)-3-methyl-1-oxobutane-1,2-diyl]})dicarbamate
Molecular formula	: C ₄₀ H ₅₀ N ₈ O ₆
Molecular Weight	: 738.89 g/mol.
Solubility	: Soluble in Water, Methanol, and Acetonitrile
p_{ka}	: 3.74
Category	: Anti-viral

MATERIALS AND METHODS**Instrumentation**

Chromatographic separation was performed on a Agilent chromatographic system equipped with 1200 series isocratic pump; Rheodyne injector with 20 μ L fixed volume loop, variable wavelength programmable UV detector and the output signal was monitored and integrated by EZICHROME ELITE Chromatographic Software. Double beam UV-Visible spectrophotometer (Labindia-3120) was used to carry out spectral analysis and the data was recorded by UVWIN-5 software. Ultrasonicator (1.5L) was used for degasification of mobile phase and samples. Standard and sample drugs were weighed by using shimadzu electronic analytical balance (AX-220) and pH of the mobile phase was adjusted by using systronics digital pH meter.

Chemicals and solvents

All chemicals and reagents used were of HPLC grade. Pure standards of Daclatasvir and (DCV) Sofosbuvir (SFV) employed in the study were obtained as gift sample from Micro labs, Bangalore. The other reagents used were methanol and acetonitrile from qualigens Ltd. Mumbai, India, triethylamine from Hi-media, Mumbai, India, and HPLC grade water from Merck chemicals, Mumbai, India.

Preparation of standard stock solution

Standard stock solution of Daclatasvir and Sofosbuvir (1mg/mL) were prepared by accurately weighing about 100 mg pure drug and transferring in to 100 mL volumetric flask and dissolved in methanol. The standard stock solution was further diluted with mobile phase to get calibration curve standard concentrations of 5, 10, 15, 20 and 25 μ g/mL of Daclatasvir and 2, 4, 6, 8 and 10 μ g/mL of Sofosbuvir.

Preparation of 0.1% triethylamine buffer (pH - 3.0)

Buffer solution was prepared by taking accurately a quantity of 0.1 mL of triethylamine dissolved in 100 mL HPLC grade water. The pH of the solution was adjusted to 3.0 with ortho- phosphoric acid and degassed for about 30 min in a ultra-bath sonicator.

Preparation of mobile phase

The mobile phase used was acetonitrile, methanol and freshly prepared 0.1% triethylamine buffer solution (pH- 3.0) in the ratio of 25:35:40 (v/v/v) and the mobile phase was filtered through 0.45 μ membrane filter and sonicated before use.

Preparation of sample solution: Accurately weighed twenty tablets were ground to obtain fine powder equivalent to 400 μ g of Sofosbuvir and 60 μ g of Daclatasvir sample were weighed and transferred to 100 ml of volumetric flask and dissolved in diluents. The flask was shaken and volume was made up to mark with diluent to give a primary stock solution. From the above solution 4 ml of solution is pipette out into a 100 ml volumetric flask and volume was made up to mark with diluent to give a solution containing 160 μ g/ml of Sofosbuvir and 24 μ g/ml of Daclatasvir.

METHOD DEVELOPMENT

Method development involves in the evaluation and optimization of the various stages of sample preparation, chromatographic separation, qualification and quantification. Optimization of various parameters was performed in order to develop a selective and sensitive method for analysis on HPLC using UV detection.

Trial -1:

Chromatographic conditions :

Mobile Phase : Water : ACN(50:50%V/V)

Column : Discovery C18 (250mm x 4.6 mm, 5 μ)□.

Detection Wavelength : 245 nm

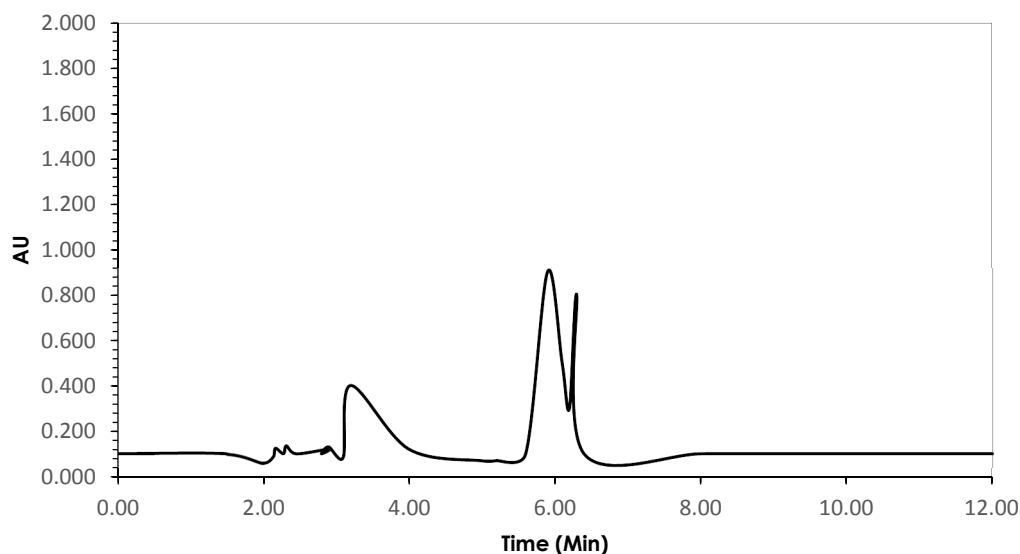


Fig. 4.3: Chromatogram Showing Trial – 1

Conclusion: The baseline and peak shape is not good.

Trail -2:

Chromatographic conditions :

Mobile Phase : Water: ACN(60:40%V/V)

Column Chromolith Speed ROD RP- 18,4.6 X
50mm,5 μ m

Detection Wavelength 250 nm.

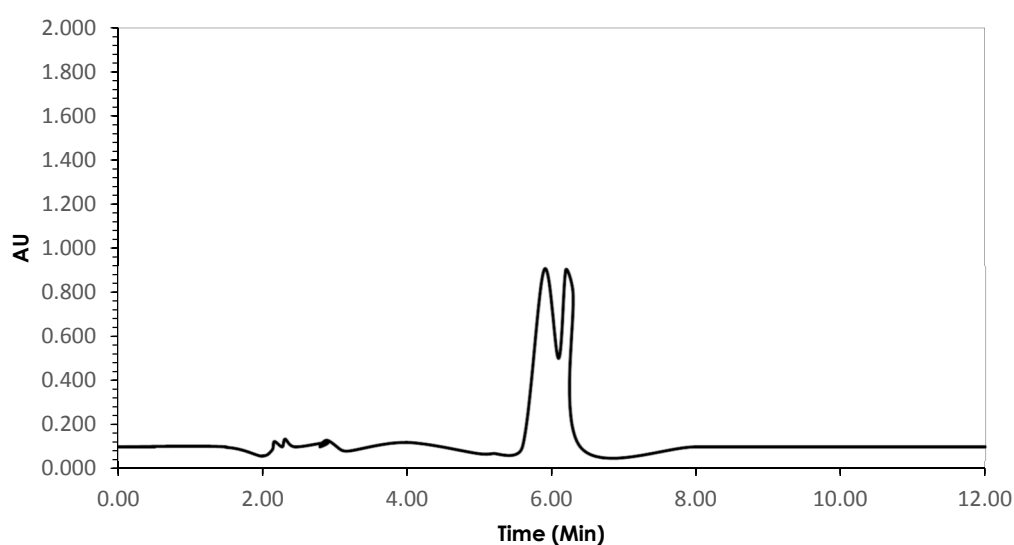


Fig. 4.4: Chromatogram Showing Trial – 2

Selection of Wavelength (λ max): 10 mg of the Sofosbuvir and Daclatasvir standard drug is taken in a 10 ml volumetric flask and dissolved in Acetonitrile and volume made up to the mark, from this solution 0.1ml is pipetted into 10 ml volumetric flask and made up to the mark with the Acetonitrile to give a concentration of 10 μ g/ml. The above prepared solution is scanned in UV between 200-400 nm using Acetonitrile as blank. The λ max was found to be 250nm.

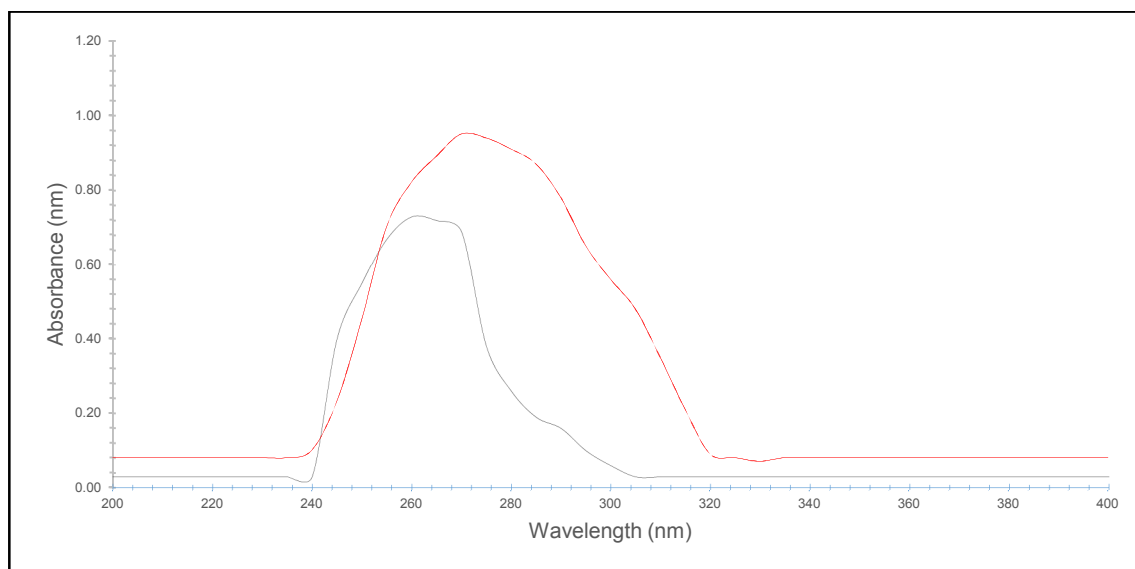


Fig: 4.5: UV spectrum of standard Daclatasvir and Sofosbuvir

Choice of stationary phase: Initially the separation was tried with different columns having different dimensions like diameter and length and pore size. Finally good separation with finest peak shape was achieved with the analytical column Inertsil ODS- C_{18} ; 5 μ m (4.6 X 250mm).

Selection of mobile phase: Several systematic test plans were performed to optimize the mobile phase. Different solvents like methanol, water and acetonitrile in different ratios and different pH values of the mobile phase ratios, by using different buffer solutions in order to get sharp peak and base line separation of the components and without interference of the excipients. Satisfactory peak symmetry, resolved and free from tailing was obtained in mobile phase Acetonitrile: Methanol: 0.1% Triethylamine buffer (pH-3.0) 25:35:40 (v/v/v).

Selection of the mobile phase flow rate: Flow rates of the mobile phase were changed from 0.5-1.0 ml/min for optimum separation. A minimum flow rate as well as minimum run time gives the maximum saving on the usage of solvents. It was found from the experiments that 1.0 mL/min flow rate was ideal for the successful elution of the analyte.

Optimized Chromatographic conditions: After series of trials, the chromatographic conditions was accomplished with following:

Buffer	: 0.1% TFA in water
Mobile Phase	: Acetonitrile: Methanol: 0.1% Triethylamine buffer (pH-3.0) 25:35:40 (v/v/v)
Column	: Inertsil ODS-C ₁₈ column (250 x 4.6 mm, 5 μ)
Flow Rate	: 1.0 ml/min
Temperature	: Ambient
Volume	: 20 μ l
Detector	: 250 nm
Diluent	: Water: Acetonitrile (50:50) column with a mixture of as mobile phase. UV detection was performed at 250 nm

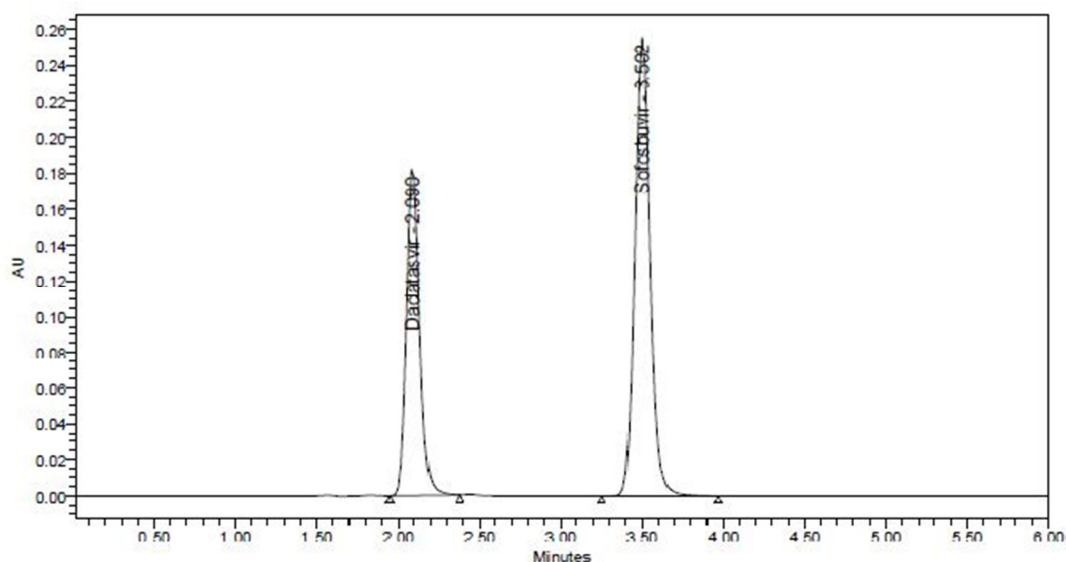


Fig. 4.6: Chromatogram of Daclatasvir and Sofosbuvir

METHOD VALIDATION

Specificity: The chromatogram of standard and sample are identical with nearly same retention time, shown in the Figures 4.25 and 4.26. No interference due to placebo and sample at the retention time of analyte which shows that the method was specific. It was shown in Figures 4.27 and 4.28.

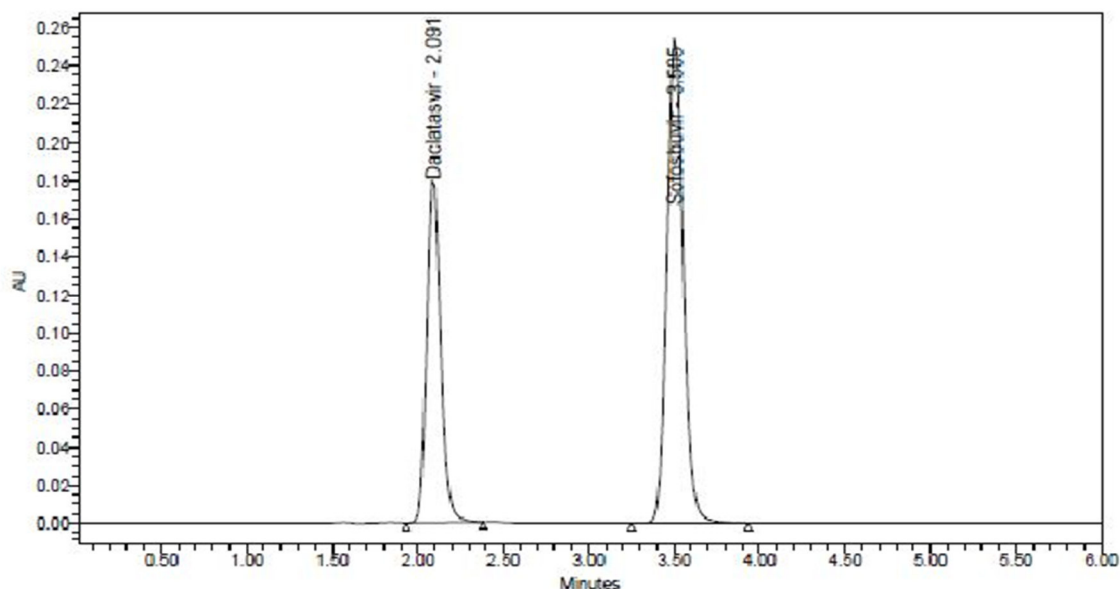


Fig. 4.7: Chromatogram representing specificity of standard

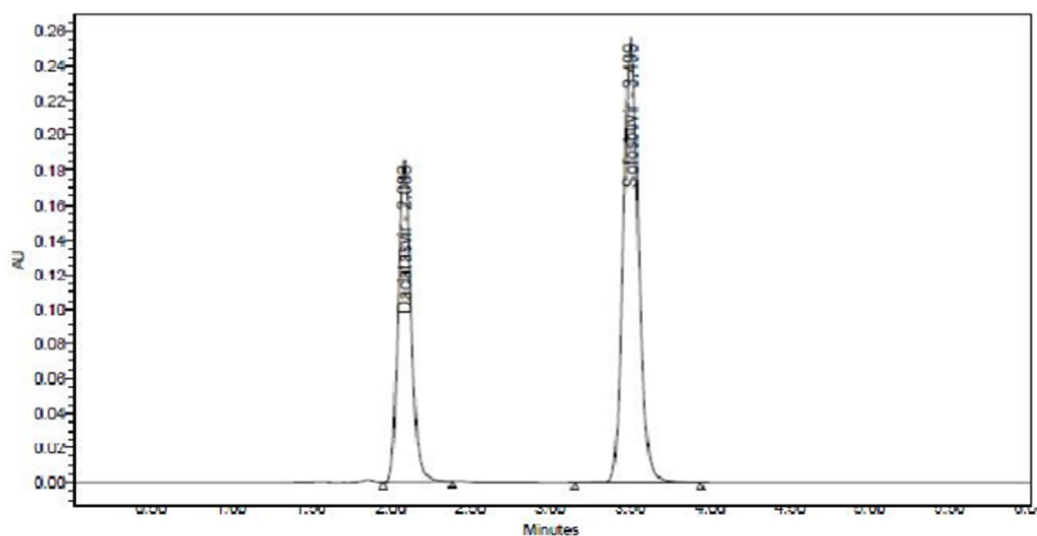


Fig. 4.8: Chromatogram representing specificity of sample

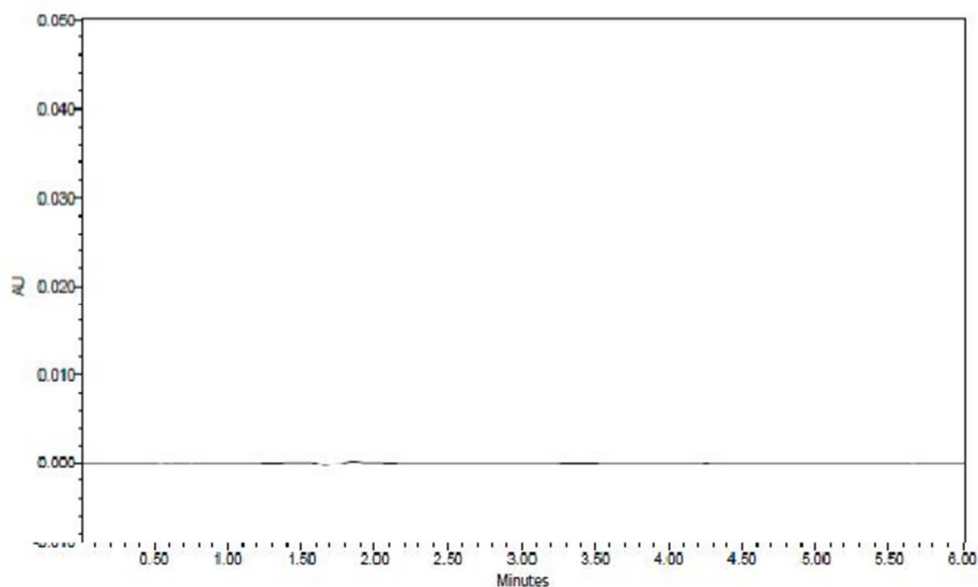


Fig. 4.9: Typical chromatogram of the blank

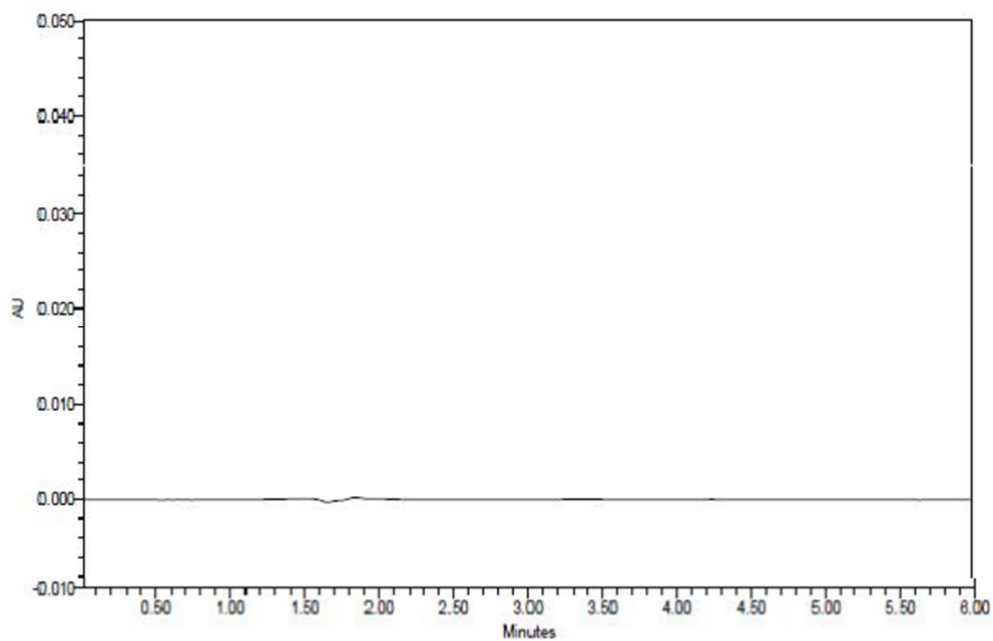


Fig. 4.10: Typical chromatogram of the Placebo

Table 4.1: Specificity data for Sofosbuvir and Daclatasvir

Sr. No.	Sample Name	Rt (min) Sofosbuvir	Rt (Min) Daclatasvir
1	Standard	3.505	2.091
2	Sample	3.499	2.083
3	Blank	-	-
4	Placebo	-	-

Result:

Chromatograms explain that retention time for standard, sample and commercial product of Daclatasvir and Sofosbuvir are same (Table 4.1). This proved that, Excipients have no effect on the analytical method. On the other hand, blank peak did not overlap drug peak. So, the method is highly selective.

System suitability:

The system suitability parameters such as US tailing factor, US theoretical Plates and resolution was achieved by injecting the prepared solution five times individually into the chromatographic system separately (Figures 4.9 to 4.14).

Table 4.2: System suitability data of Sofosbuvir and Daclatasvir

Parameter	Sofosbuvir	Daclatasvir	Acceptance Criteria
Retention time (min)	3.502	2.089	± 10
Theoretical plates	6613	3226	>3000
Tailing factor	1.11	1.15	<2.00
% RSD	0.08	0.27	<2.00

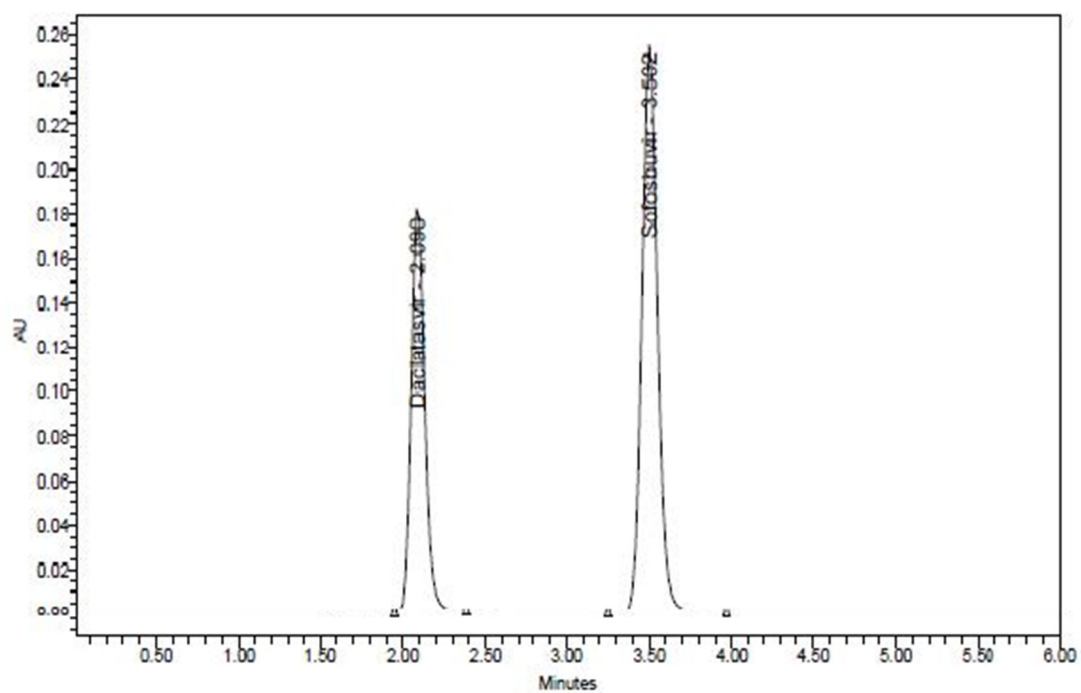


Fig. 4.11: Typical Chromatogram of Standard Injection-1

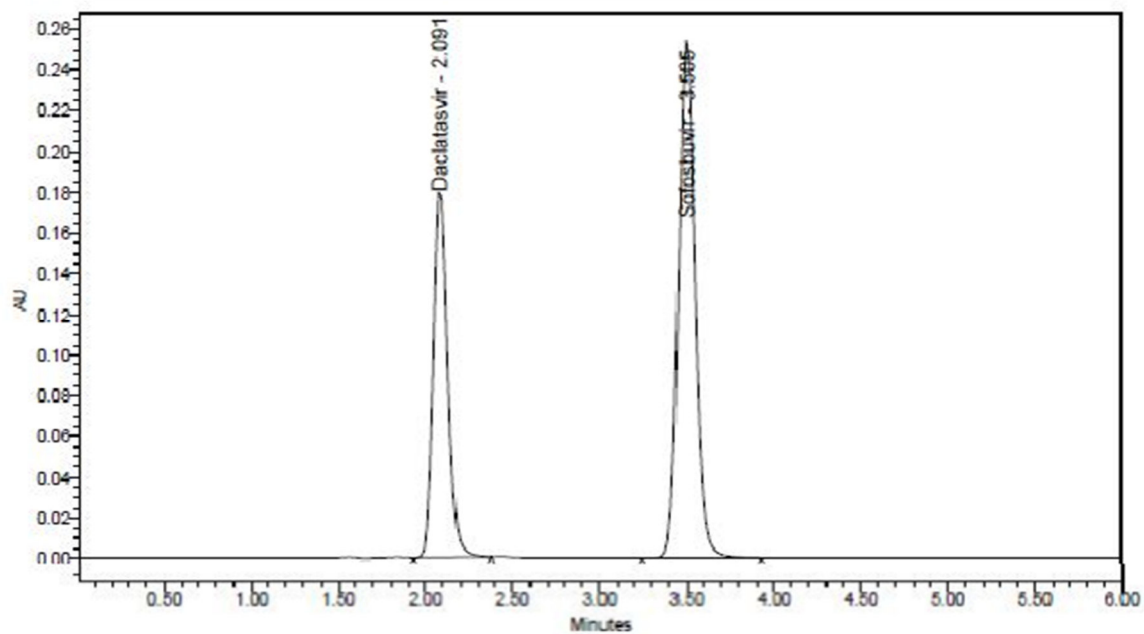


Fig. 4.12: Typical Chromatogram of Standard Injection-2

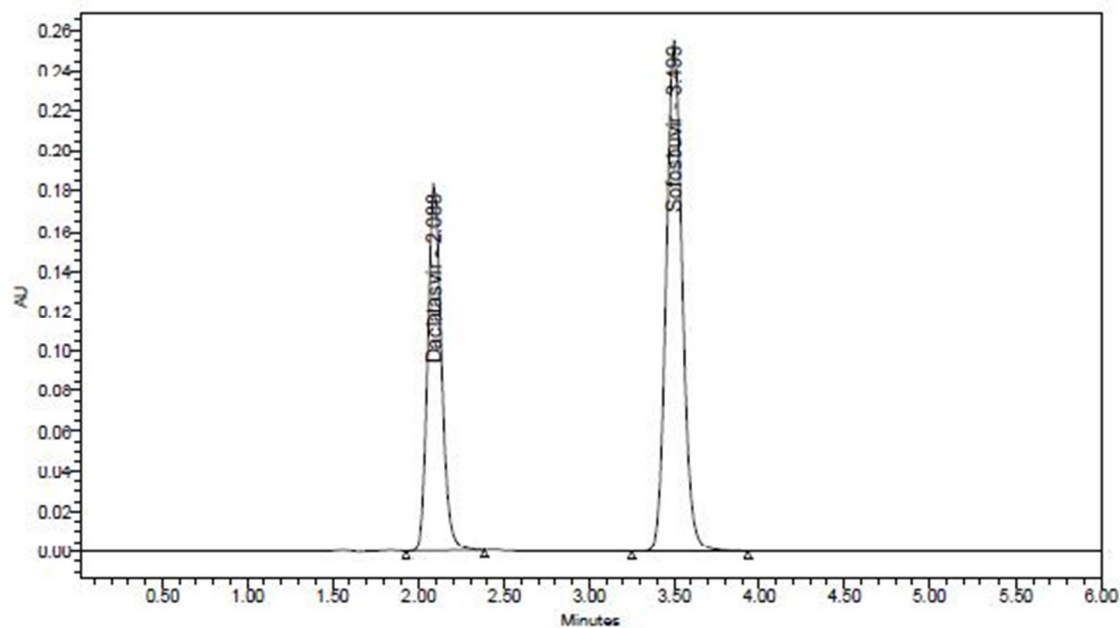


Fig. 4.13: Typical Chromatogram of Standard Injection-3

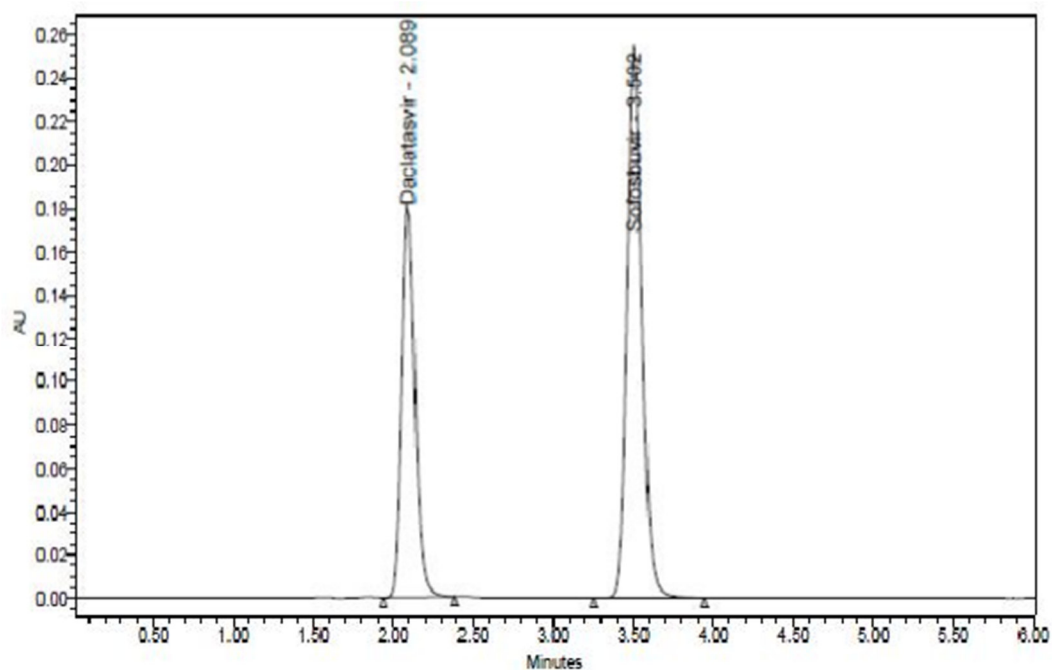


Fig. 4.14: Typical Chromatogram of Standard Injection-4

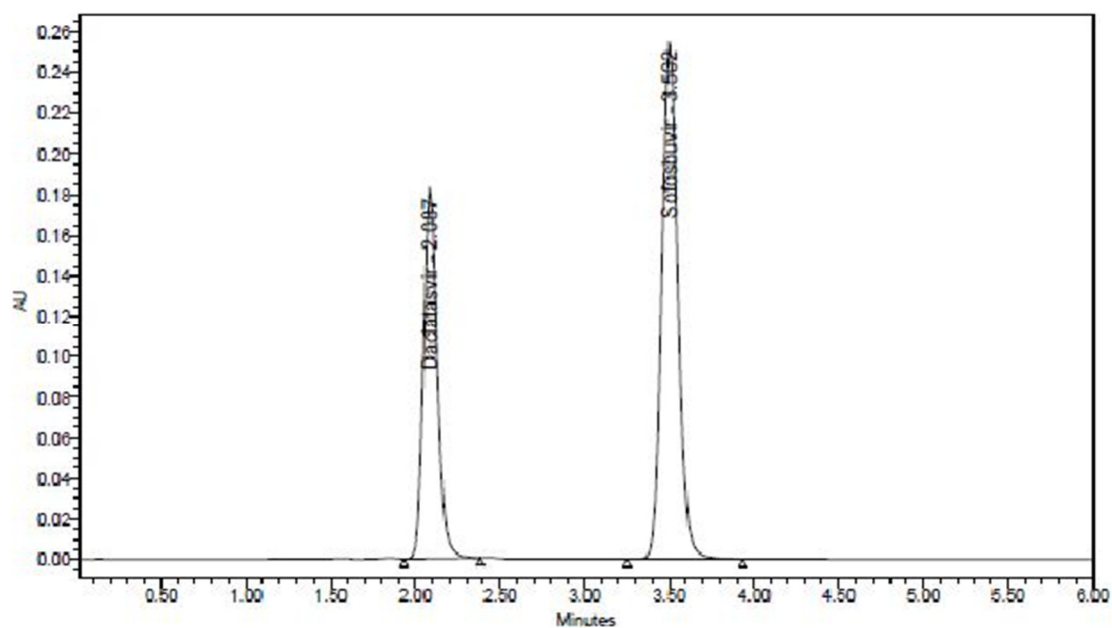


Fig. 4.15: Typical Chromatogram of Standard Injection 5

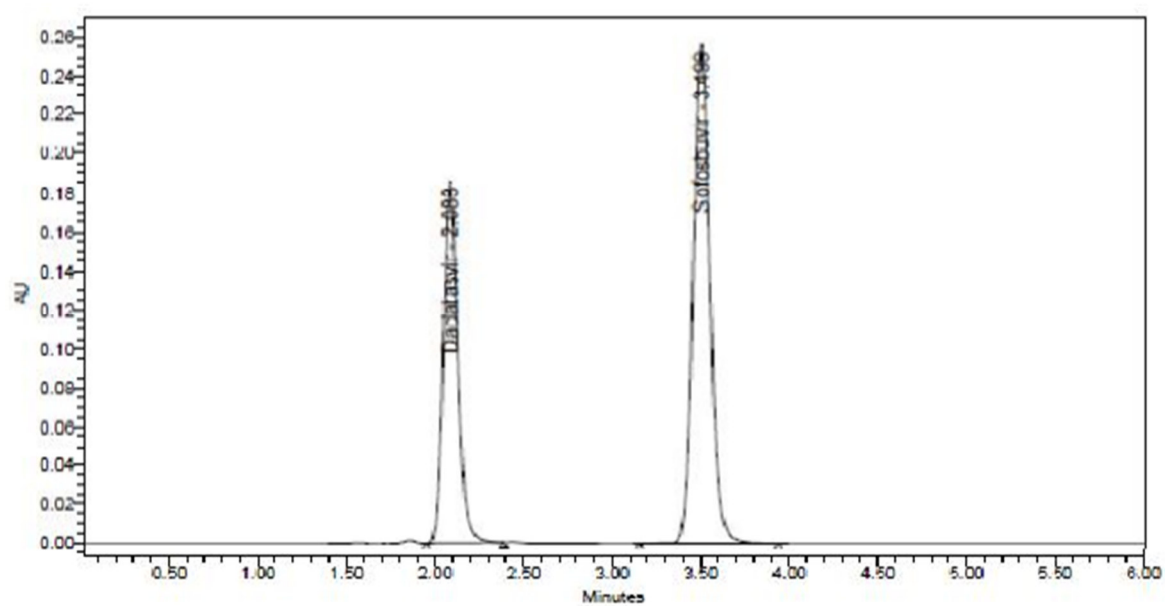


Fig. 4.16: Typical Chromatogram of Sample

Table 4.3: Standard Results of Sofosbuvir and Daclatasvir

S. No.	Sample Name	RT (Min)		Area		USP plate		USP tailing	
		SFB	DCT	SFB	DCT	SFB	DCT	SFB	DCT
1	Injection1	3.502	2.090	1682706	1035661	6647	3257	1.11	1.15
2	Injection 2	3.505	2.091	1682880	1034398	6527	3215	1.11	1.15
3	Injection 3	3.499	2.088	1680431	1030858	6609	3238	1.11	1.15
4	Injection 4	3.502	2.089	1679825	1029640	6642	3237	1.11	1.15
5	Injection 5	3.502	2.087	1680710	1029757	6638	3185	1.11	1.16

Result: Results of system suitability study are summarized in the Table 4.2. Five consecutive injections of the standard solution showed uniform retention time, theoretical plate count, tailing factor and resolution for both the drugs which indicate a good system for analysis, which was shown in Table 4.3.

Linearity: A series of standard concentrations were prepared from 50 % to 150 % of the target concentration of DCV and SFV. Linearity was assessed by performing single measurement at several analyte concentration varying quantities of stock standard solution diluted with the mobile phase to give a concentration of 5, 10, 15, 20 and 25 µg/mL of DCV and 2, 4, 6, 8 and 10 µg/mL of SFV. Injection was made at intervals of 10.0 min. Linearity of DCV was found to be exist between 5-25 µg/mL and for SFV was 2-10 µg/mL. The chromatograms were recorded and linearity graph was plotted by using peak area of drug against respective concentrations to obtain the linearity range. Calibration curve with concentration verses peak areas was plotted by injecting the above prepared

solutions, shown in figures 4.6 and 4.7 and the obtained data were subjected to regression analysis using the least squares method, shown in **Table 4.3**.

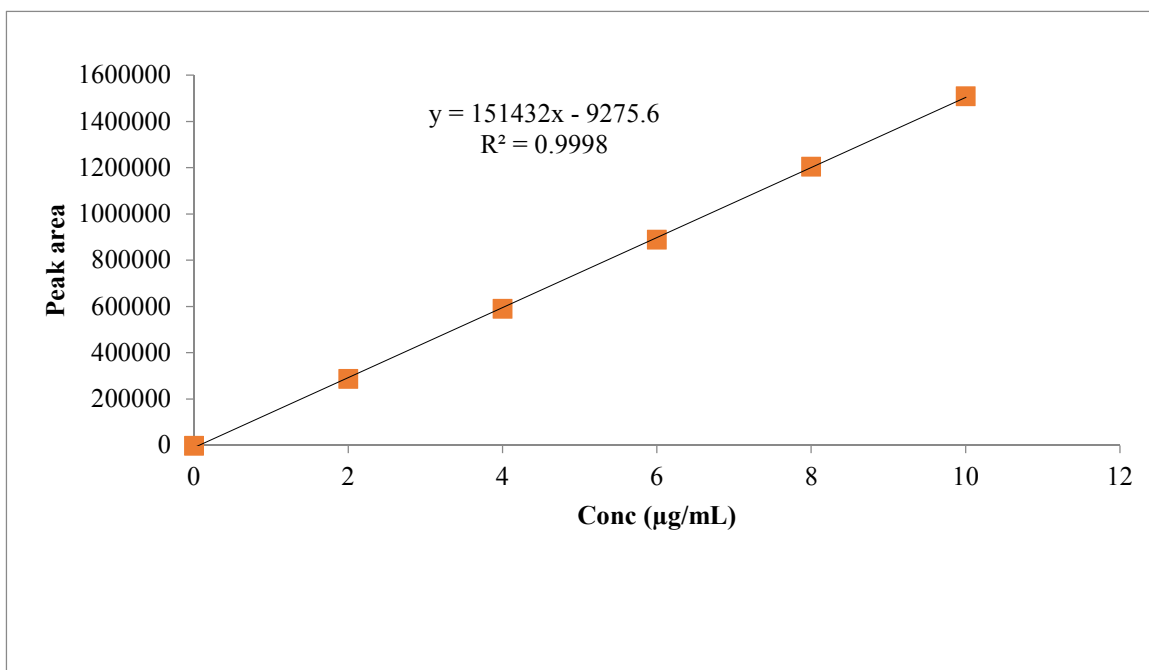


Fig. 4.17: Linearity plot of Sofosbuvir

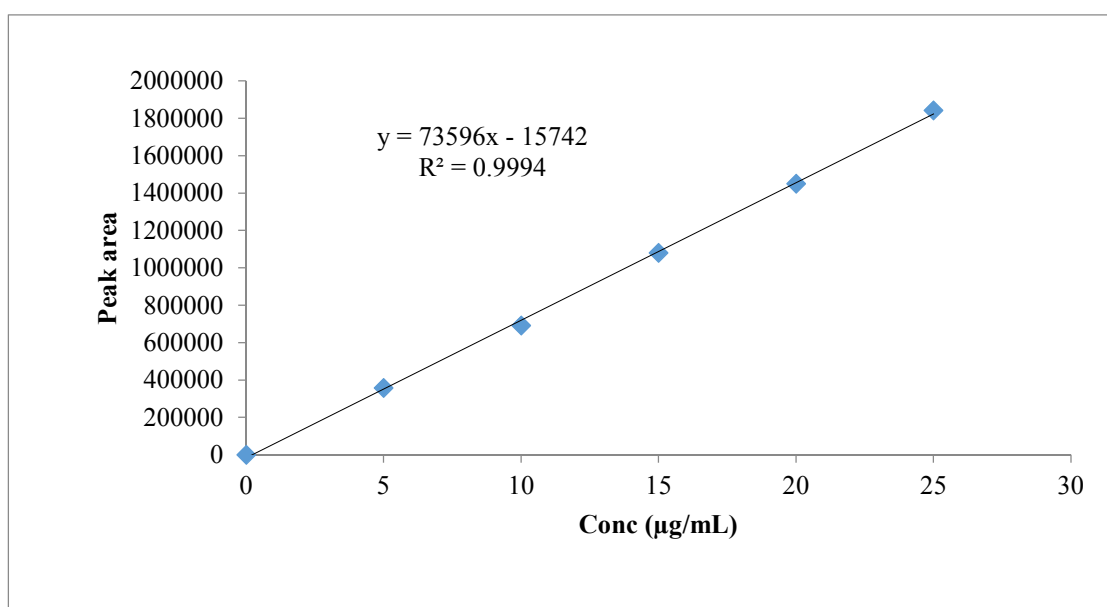


Fig. 4.18: Linearity plot of Daclatasvir

Table 4.4: Linearity data for Sofosbuvir and Daclatasvir

%Level	Concentration µg/mL	Area of Daclatasvirin	Concentration µg/mL	Area of Sofosbuvir
50%	5	358575	2	289124
75%	10	691556	4	591568
100%	15	1081521	6	890541
125%	20	1451468	8	1205846
150%	25	1842135	10	1510214
Concentration range	5-25µg/mL		2-10 µg/mL	
Slope (m)	73596		151432	
Correlation coefficient (r ²)	0.9992		0.9997	

Precision:**Method precision (Repeatability):**

The precision of the instrument was checked by repeated injections and measurement of peak areas and retention times of solutions (n = 6) for 160 µg/ml of Sofosbuvir and 24µg/ml Daclatasvir without changing the parameter of the proposed chromatographic method, shown in Figures 4.17 to 4.20.

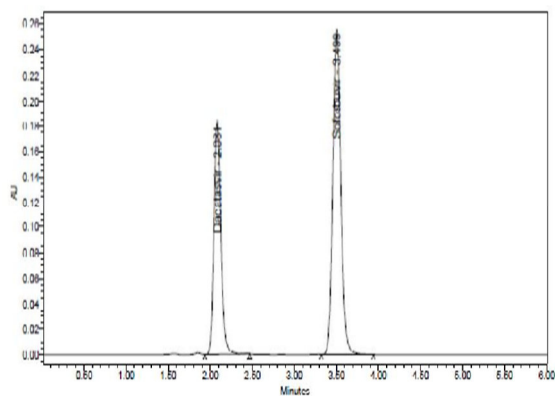


Fig 4.19: Chromatogram for injection 1

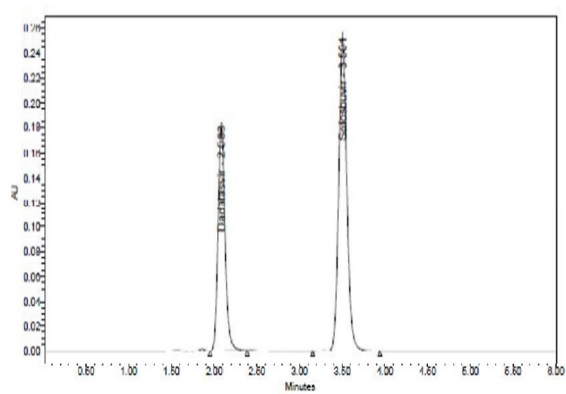


Fig 4.20: Chromatogram for injection 2

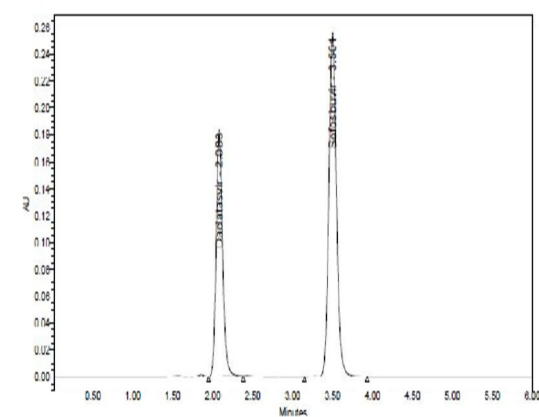


Fig. 4.21: Chromatogram for injection 3

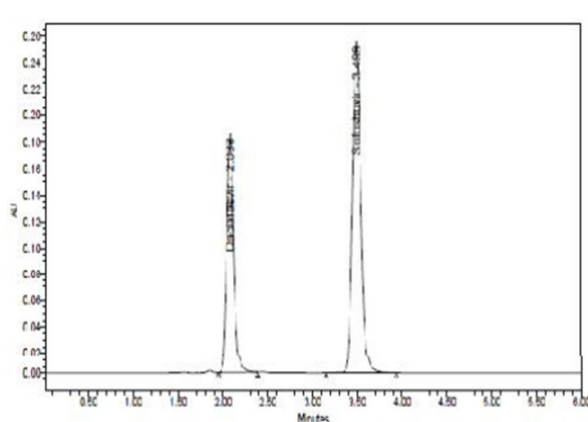


Fig. 4.22: Chromatogram for injection 4

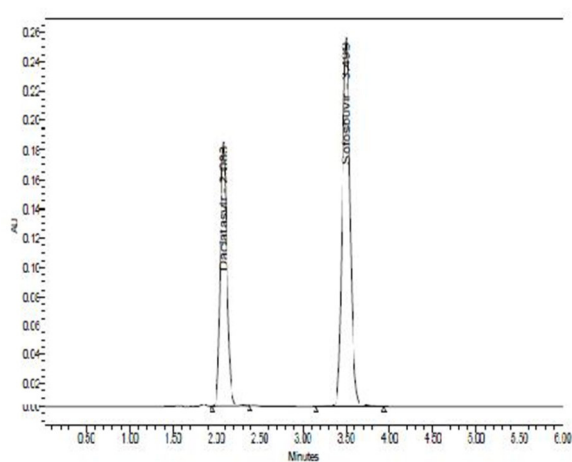


Fig. 4.23: Chromatogram for injection 5

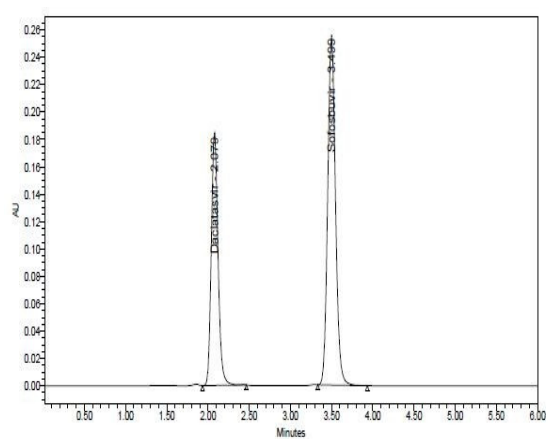


Fig. 4.24: Chromatogram for injection 6

Table 4.5: Summary of peak areas for method precision for Sofosbuvir and daclatasvir

Sample No.	Sofosbuvir			Daclatasvir		
	Retention time (min)	Peak area	% Assay	Retention time (min)	Peak area	% Assay
1	3.499	1686766	99.9	2.083	1035922	99.2
2	3.499	1677924	99.2	2.081	1034144	100.0
3	3.501	1687828	99.2	2.083	1022382	98.9
4	3.501	1679039	99.7	2.082	1038818	99.9
5	3.499	1674492	99.2	2.079	1046773	100.0
6	3.499	1682158	99.3	2.080	1048278	99.6
Mean			99.4			99.6
%RSD			0.31			0.48

Result: Results of variability were summarized in the above table 4.5. Percentage relative standard deviation (%RSD) was found to be less than 2% which proves that method is precise.

Accuracy (Recovery study):

The accuracy of the method was determined by calculating the recoveries of Sofosbuvir and Daclatasvir by analyzing solutions containing approximately 50%, 100% and 150% of the working strength of Sofosbuvir and Daclatasvir (Figures 4.21 to 4.23) and the percentage recovery results obtained are listed in Table 4.6.

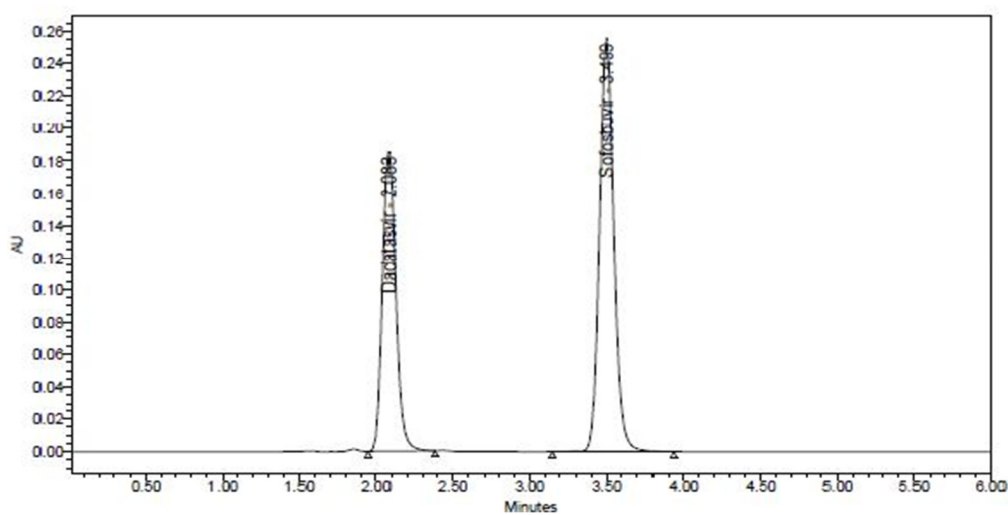


Fig. 4.25: Typical chromatogram for Accuracy 50 %

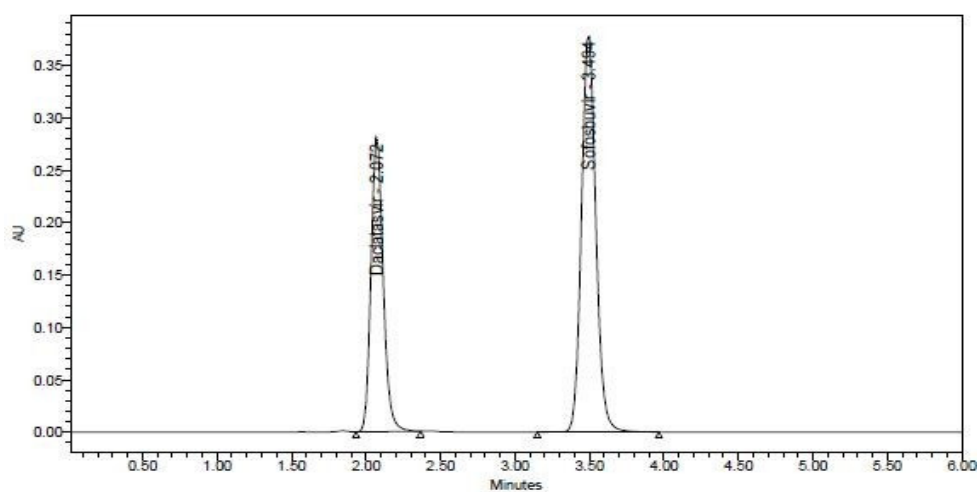


Fig. 4.26: Typical chromatogram for Accuracy 100 %

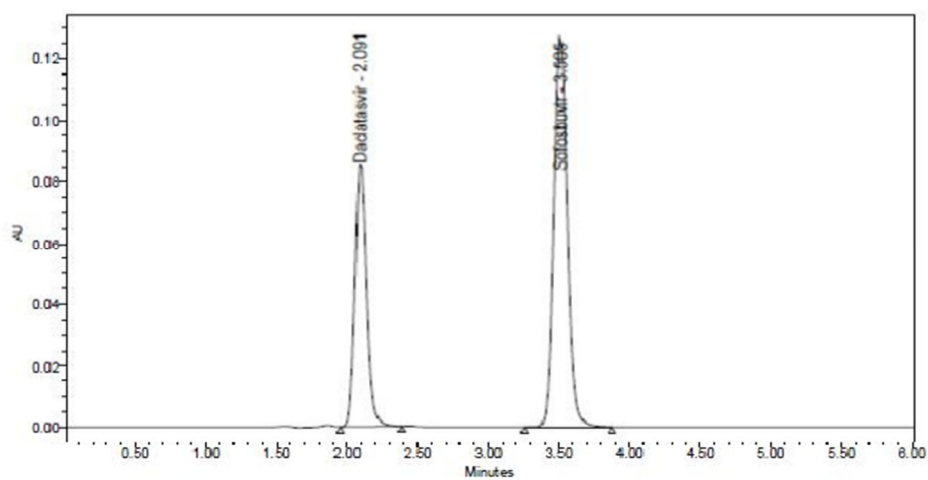


Fig. 4.27: Typical chromatogram for Accuracy 150 %

Table 4.6: Recovery data for Sofosbuvir and Daclatasvir

S. No.	Accuracy level	Injection	Sofosbuvir		Daclatasvir	
			% Recovery	Average	% Recovery	Average
1	50%	1	99.4	99.3%	99.7	99.7%
		2	99.2		99.5	
		3	99.3		99.9	
2	100%	1	99.9	99.4%	99.2	99.4%
		2	99.2		100.0	
		3	99.2		98.9	
3	150%	1	99.1	99.0%	99.0	99.2%
		2	99.3		98.9	
		3	98.8		99.5	

Result: Results of accuracy study are presented in the above table. All the results indicate that the method is highly accurate.

Robustness: Robustness is the measure of a method remain unaffected by small, deliberate changes in method parameters like flow rate and detection wavelength on assay of the analyte of interest. Here the detection wavelength varied ± 2 nm and flow rate was varied ± 0.2 ml/min. The chromatograms were shown in Figures 4.24 and 4.25 and the results were shown in Table 4.7.

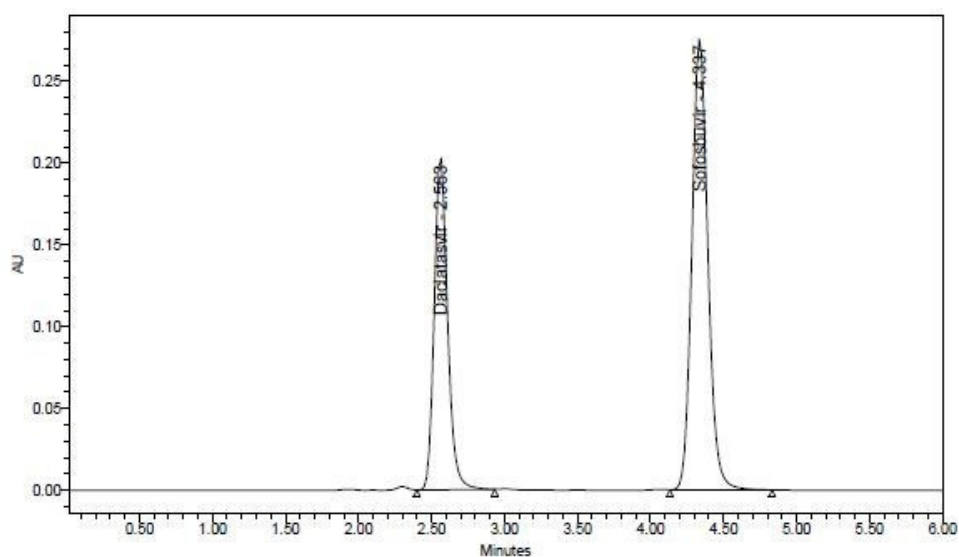


Fig. 4.28: Chromatogram for decreased flow rate

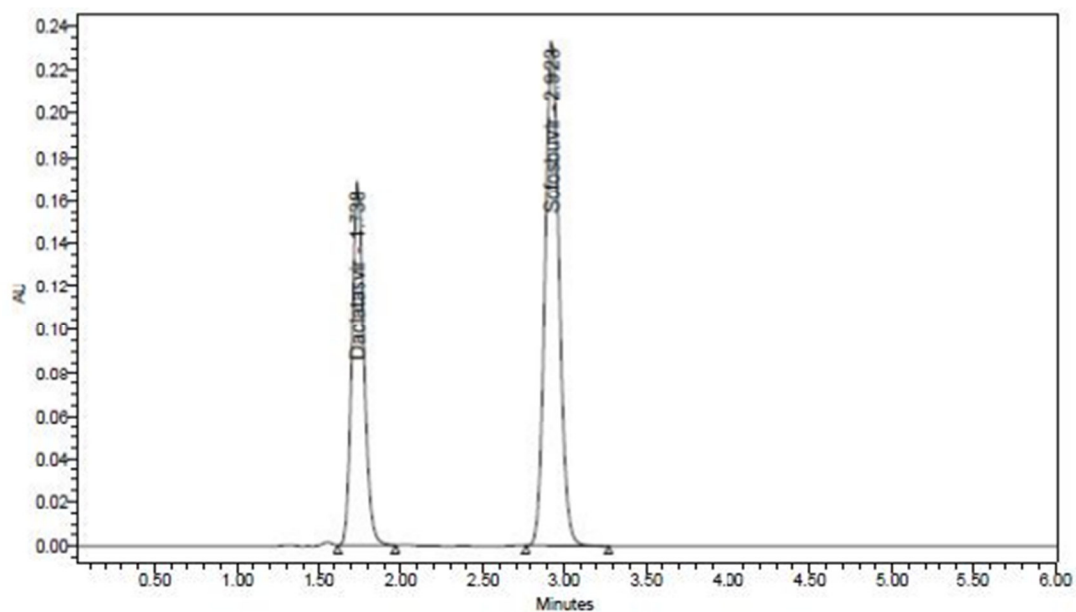


Fig. 4.29: Chromatogram for increased flow rate

Table 4.7: Results of Robustness data for Sofosbuvir and Daclatasvir

parameter	RT (min)		Area	
	Sofosbuvir	Daclatasvir	Sofosbuvir	Daclatasvir
Decreased flow rate (0.9ml/min)	4.337	2.563	2098358	1290171
Increased flow rate (1.1ml/min)	2.923	1.738	1383875	852316

Result: The results of Robustness of the present method had shown that changes made in the Flow and wavelength did not produce significant changes in analytical results which were presented in the above table. As the changes are not significant we can say that the method is Robust.

Limit of detection and limit of quantification: The limit of detection (LOD) and limit of quantification (LOQ) were separately determined based on standard deviation of the y-intercept and the slope of the calibration curve by using the equations (1) and (2), respectively. The data obtained was shown in Table 4.6.

Where,

$$\text{LOD} = 3.3 \sigma / S \dots\dots\dots (1) \quad \text{LOQ} = 10 \sigma / S \dots\dots\dots (2)$$

σ = the standard deviation of the response

S = the slope of the calibration curve

The slope S may be estimated from the calibration curve of the analyte.

Table 4.8: LOD and LOQ

	SOFOSBUVIR (µg)	DACLATASVIR (µg)
LOD	0.005	0.001
LOQ	0.014	0.003

Analysis of Marketed Formulation

Preparation test solution: A total of 20 tablets were accurately weighed and powdered in a mortar. An amount equivalent to one tablet (Containing 400 mg of SFV and 60 mg of DCV) was transferred to 25 mL volumetric flask, 10 mL of methanol was added and content of the flask were ultra-sonicated for 10 min. The solution was filtered through whatmann filter paper No. 41. The sample solution thus prepared was diluted with mobile phase to get the solution containing SFV and DCV in 15 & 6 µg/mL proportion, respectively. The test solution was injected in to HPLC and % assay was calculated.

The validated method was applied for the assay of commercial tablets of daclatasvir and sofosbuvir (400 mg of Sofosbuvir and 60 mg of Daclatasvir). Peak area of the detector response was used to calculate % assay. The % assay was found to be 99.57 % for Daclatasvir and 99.38 % for Sofosbuvir. The results presented good agreement with the labelled content.

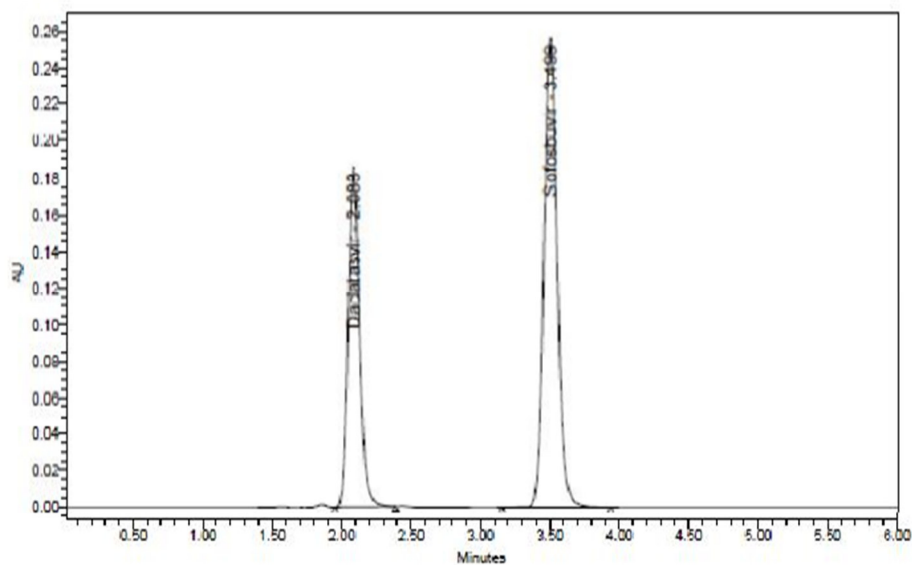
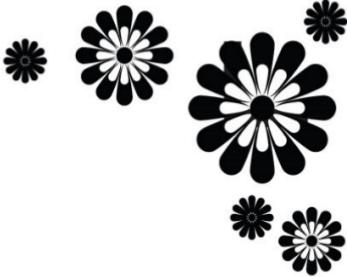


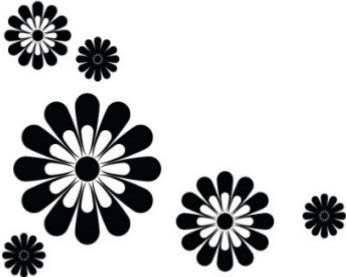
Fig. 4.30: Typical Chromatogram of Sample

Table 4.9: Assay of test sample

Test formulation (Tablet)	Label claimed (mg/tab)	
	SFV	DCV
Sofosbuvir and Daclatasvir	400	60
	Conc found (mg)	
	15	6
	%Assay	
	99.57	99.38



CHAPTER - 5
OMBITASVIR,
PARITAPREVIR AND
RITONAVIR



DRUG PROFILE

Ombitasvir, paritaprevir and ritonavir (Figure: 1.0) drugs were combined in a single dosage form (film-coated tablet) in the brand name of TECHNIVIE for the treatment of hepatitis-C. These three drugs will act against the hepatitis-C virus (HCV) in three different mechanisms.

Ombitasvir, produces its antiviral activity by inhibiting the HCV non-structural protein (NS) 5A. Ombitasvir chemically designated as dimethyl ([[(2S, 5S)-1-(4-tert-butyl phenyl) pyrrolidine-2,5-diyl] bis {benzene -4, 1 diylcarbamoyl (2S) pyrrolidine -2, 1-diyl} [(2S) -3-methyl -1-oxobutane -1, 2-diyl]]) biscarbamate hydrate with molecular weight of 894.11 g/mole (Fig. 1).

Paritaprevir chemically designated as (2R, 6S, 12Z, 13aS,14aR, 16aS)-N-(cyclopropylsulfonyl)-6-[[[(5-methyl-2-pyrazinyl) carbonyl] amino]-5, 16-dioxo-2-(6-phenanthridinyloxy)-1, 2, 3, 6, 7, 8, 9, 10, 11, 13a, 14, 15, 16, 16a-tetradecahydrocyclopropa[e] pyrrolo[1,2-a][1,4] diazacyclopentadecine -14a(5H)-carboxamide with molecular weight of 765.89 g/mole (Fig.1). It is a powerful inhibitor of the NS-3/4A serine protease of HCV. Subsequently, replication of HCV genetic components and translation into a single polypeptide, NS-3, and its activating cofactor NS-4A are accountable for splitting it into the succeeding nonstructural and structural proteins essential for assembly into a mature virus, viz., NS-3, NS-4A, NS-4B, NS-5A, and NS-5B. By inhibiting viral protease NS-3/4A, Paritaprevir, therefore, prevents viral replication and function.

Ritonavir is an anti-retroviral medication utilized along with other medications to treat the human immunodeficiency virus. This combination treatment is known as highly active anti-retroviral therapy (HAART). At low doses of ritonavir, it is utilized with other

protease inhibiting agents and useful in combination with other hepatitis-C medicaments. It is chemically designated as 1, 3-thiazol- 5-ylmethyl N-[(2S, 3S, 5S) -3- hydroxy- 5- [(2S)- 3- methyl -2- {[methyl({[2-(propan-2-yl)-1,3-thiazol-4-yl]methyl}) carbamoyl] amino} butanamido]-1,6 diphenylhexan-2-yl]carbamate with molecular weight of 720.946 g/mole.

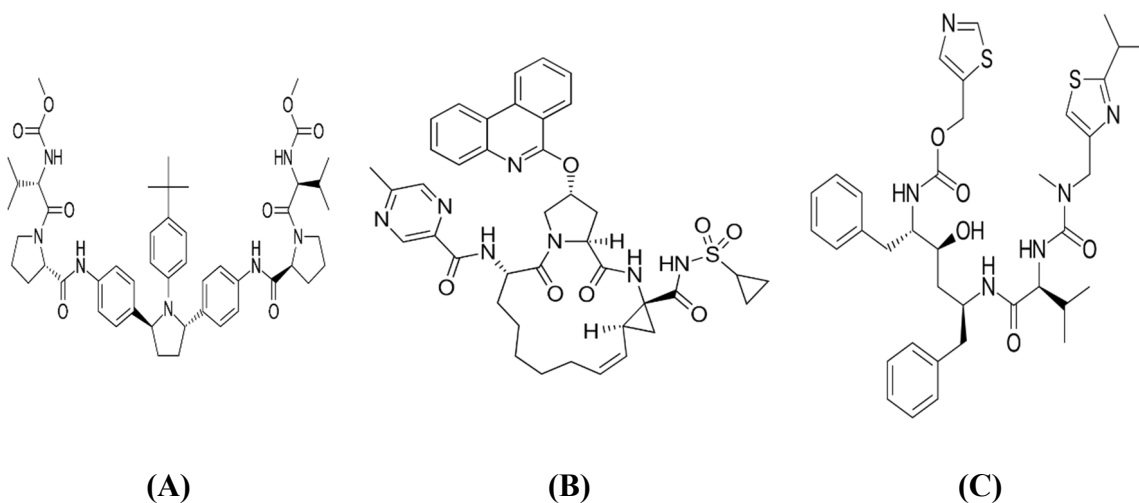


Fig. 5.1: Chemical structures of (A) Ombitasvir (B) Paritaprevir (C) Ritonavir

MATERILAS AND METHODS**Instrumentation:**

The HPLC system (Agilent HPLC 1200 Infinity LC Specifications) consisted of a pump (Agilent LC20AT) programmed with Ezchrom Elite Software and rheodyne Injector was used. The detector consisted of UV/VIS (UV-2489) model was operated at a wavelength of 262 nm. The column used was Inertsil CN- 3 column at ambient temperature

Chemicals and Reagents:

Hetero Aurobindo Pharma Pvt. Ltd, Hyderabad, India kindly supplied the pure working standards of known potency of ombitasvir, paritaprevir and ritonavir as a gift sample. The marketed sample with strength of ombitasvir 25 mg, paritaprevir 150 mg, and ritonavir 100 mg purchased from the local Pharmacy. The reagents like orthophosphoric acid (OPA) of Hi-Media Laboratories Pvt. Ltd, water, methanol, acetonitrile, triethylamine of Merk, potassium dihydrogen phosphate of Thermo Fisher Scientific India Pvt. Ltd were used.

Preparation of Standard Stock Solution:

Each 10mg of ombitasvir, paritaprevir and ritonavir were transferred to 100 ml volumetric flask and dissolved and diluted to the mark with methanol. The stock solutions were further diluted with mobile phase to obtain a solution of 100 µg/ml.

Test sample preparation:

Tablet powder equivalent to 10 mg of ombitasvir, paritaprevir and ritonavir was weighed from a pooled powder of twenty tablets and transferred into a 10 ml volumetric flask, few ml of methanol was added and sonicated for 10 min. The volume was made up to mark with methanol and the sample solution was filtered and used for further dilution.

METHOD DEVELOPMENT

For developing the method, a systematic study of the effect of various factors were undertaken by varying one parameter at a time and keeping all other conditions constant. Method development consists of selecting the appropriate mass parameters and choice of stationary and mobile phases. The following studies were conducted for this purpose.

Trial -1:

Chromatographic conditions :
Mobile Phase : Water: Methanol (60:40, %V/V)
Column : C18, HPLC column, Emerald, 3 μ m, 30.0 \times 3.0 mm
Detection Wavelength : 254 nm

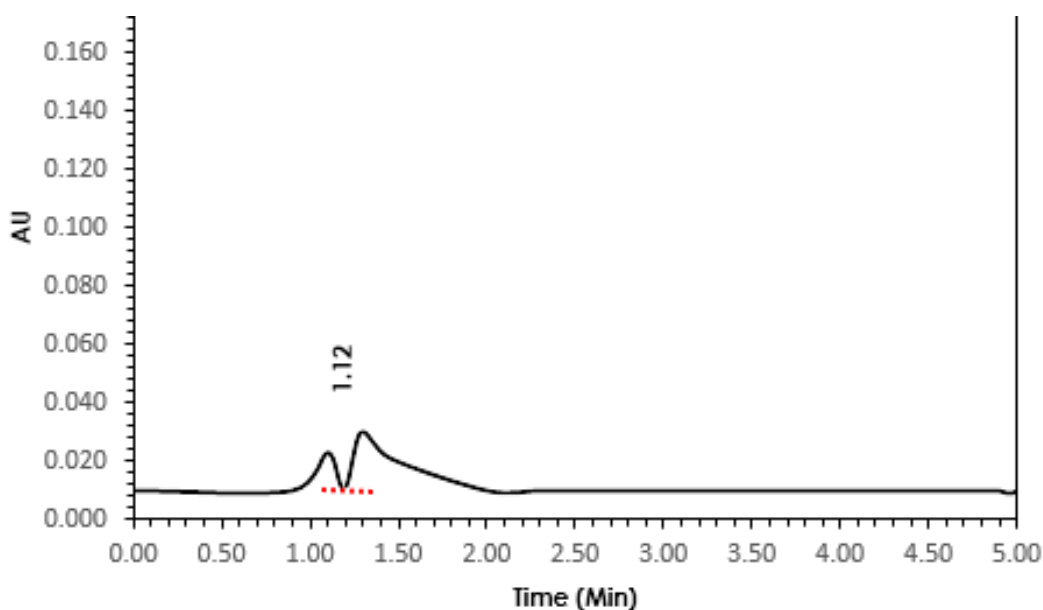


Fig. 5.2: Chromatogram Showing Trial – 1

Trail - 2:

Chromatographic conditions :

Mobile Phase : Water: Acetonitrile: Methanol (80:10: 10, %V/V)

Column : Kinetex 2.6u XB-C18 150x4.60mm

Detection Wavelength : 260 nm

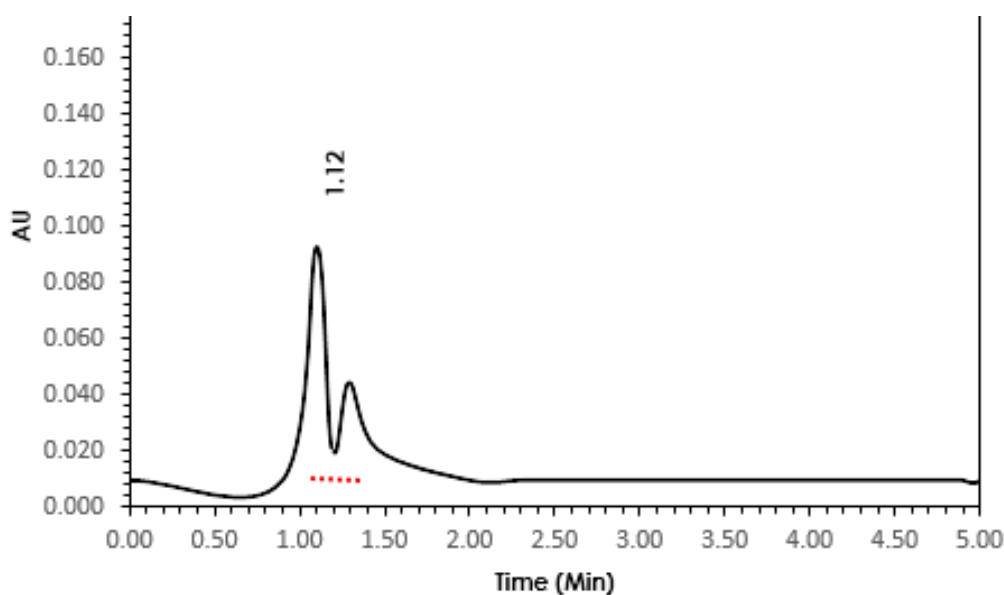


Fig. 5.3: Chromatogram Showing Trial – 2

Conclusion: The components were not separated completely.

Selection of Wavelength (λ_{\max}): 10 mg of the ombitasvir, paritaprevir and ritonavir standard drug is taken in a 10 ml volumetric flask and dissolved in acetonitrile and volume made up to the mark, from this solution 0.1ml is pipetted into 10 ml volumetric flask and made up to the mark with the acetonitrile to give a concentration of 10 $\mu\text{g/ml}$. The above prepared solution is scanned in UV between 200-400 nm using acetonitrile as blank. The λ_{\max} was found to be 262nm.

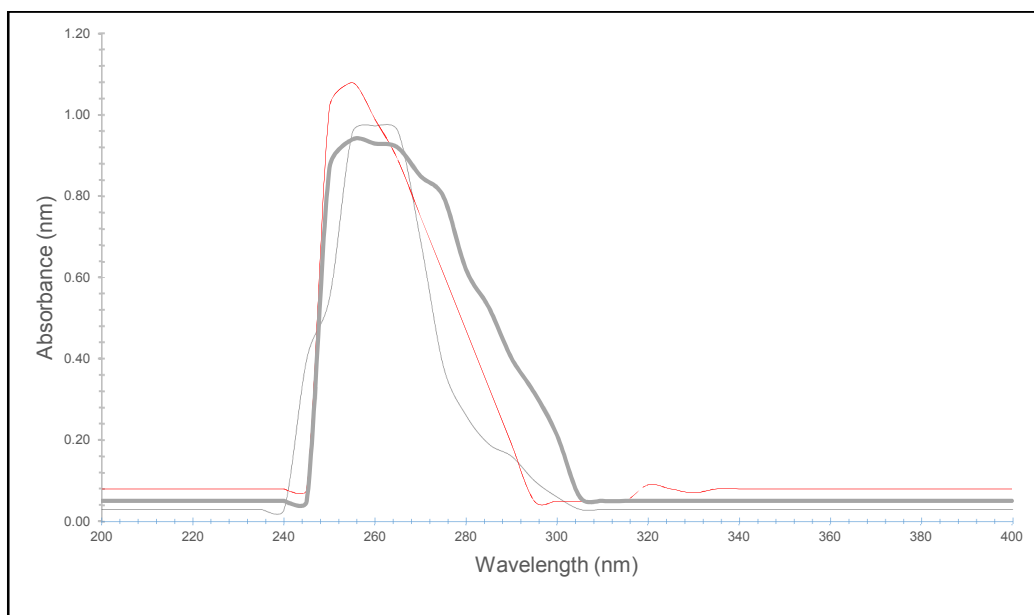


Fig. 5.4: Over lay spectrum UV spectrum of Ombitasvir, Paritaprevir and Ritonavir

Choice of stationary phase

Initially the separation was tried with different columns having different dimensions like diameter and length and pore size. Finally good separation with finest peak shape was achieved with the analytical column Inertsil ODS-C18; 5 μ m (4.6 X 250mm).

Selection of mobile phase

Several systematic test plans were performed to optimize the mobile phase. Different solvents like methanol, water and acetonitrile in different ratios and different pH values of the mobile phase ratios, by using different buffer solutions in order to get sharp peak and base line separation of the components and without interference of the excipients. Satisfactory peak symmetry, resolved and free from tailing was obtained in mobile phase 0.02M phosphate buffer (pH-4.5): acetonitrile: methanol, (50:30:20) (v/v).

Selection of the mobile phase flow rate

Flow rates of the mobile phase were changed from 0.5-1.0 ml/min for optimum separation. A minimum flow rate as well as minimum run time gives the maximum saving on the usage of solvents. It was found from the experiments that 1.0 mL/min flow rate was ideal for the successful elution of the analyte.

Optimized Chromatographic conditions

After series of trials, the chromatographic conditions was accomplished with following parameters.

Buffer	: 0.02M phosphate buffer (pH-4.5)
Mobile Phase	: 0.02M phosphate buffer (pH-4.5): Acetonitrile: Methanol, (50:30:20) (v/v)
Column	: Inertsil ODS-C18; 5 μ m (4.6 X 250mm)
Flow Rate	: 1.0ml/min
Temperature	: Ambient
Injection Volume	: 20 μ l
Detector	: 262nm
Diluent	: Water: Acetonitrile (50:50) column with a mixture of as mobile phase.

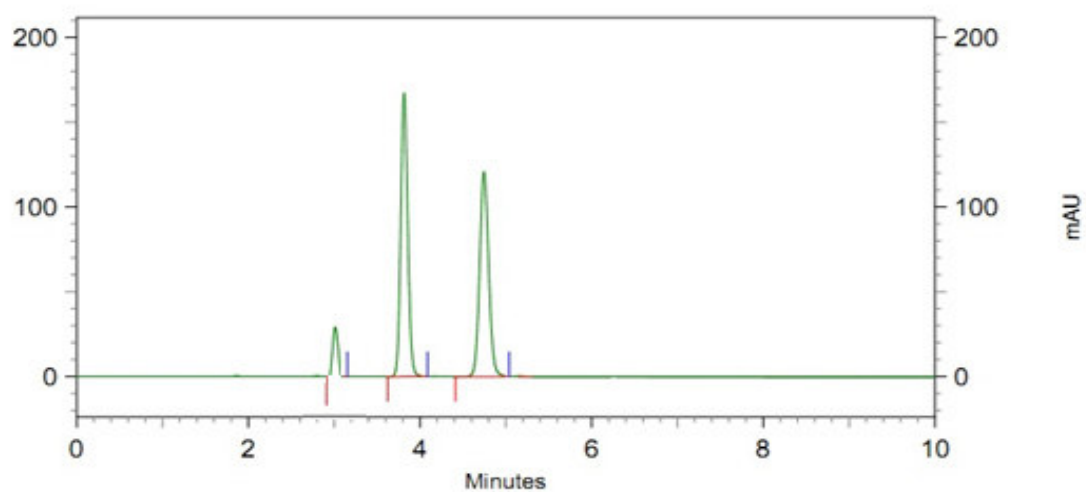


Fig. 5.5: Chromatogram of Ombitasvir, Paritaprevir and Ritonavir

METHOD VALIDATION

After the development of RP-HPLC method for the estimation of drug in a dosage form, validation of the method was performed. This part describes the procedure followed for validation of the developed method.

Specificity

Specificity is the ability of a method to discriminate between the analyte (s) of interest and other components that are present in the sample. A study of placebo interference from excipients was conducted. Equivalent weight of placebo taken as per the test method and placebo interference was conducted in duplicate.

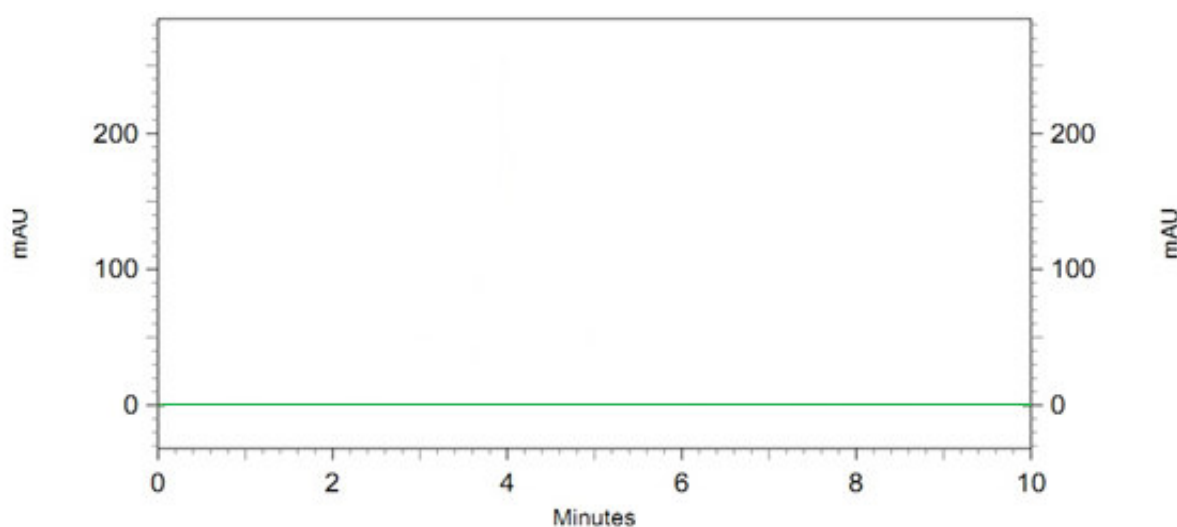


Fig. 5.6: Chromatogram of Blank

System suitability

To verify the system producing the consistent results with the optimized method injected the standard for six times with the criteria of % RSD for retention time and area NMT 2.0%, theoretical plates NLT 3000 plates, tailing factor NMT 1.5 and resolution NLT 4.

Table. 5.1: System suitability parameters

Parameter	Compound	Result
Retention Time	Ombitasvir	3.14 min
	Paritaprevir	3.92 min
	Ritonavir	4.91 min
Peak Area	Ombitasvir	254622
	Paritaprevir	565892
	Ritonavir	431312
Theoretical plates	Ombitasvir	4231
	Paritaprevir	3452
	Ritonavir	1342
Tailing Factor	Ombitasvir	0.12
	Paritaprevir	0.34
	Ritonavir	0.35
Resolution	Ombitasvir	-
	Paritaprevir	3.34
	Ritonavir	4.34

Linearity:

A series of standard solutions (not less than 5 is recommended) were prepared in the range of 15µg/ml-45µg/ml containing ombitasvir, paritaprevir and ritonavir standards and injected. A plot of average peak area versus the concentration in µg/ml or mg/ml is made and from this the correlation coefficient, y-intercept (constant of regression) and slope (coefficient of regression) of the regression line were calculated. The calibration data and calibration curve shown in Table No.02 and Fig No. 2, 3 and 4.

Table 5.2: Linearity data

S.NO	Concentration µg/mL	Area of OT	Concentration µg/mL	Area of PT	Concentration µg/mL	Area of RT
1	15	279425	15	313823	15	414740
2	21	496653	21	467371	21	580637
3	27	684443	27	600918	27	746533
4	33	853368	33	734456	33	912430
5	39	991189	39	867991	39	1059512
6	45	1199969	45	1001530	45	1244222
Concentration range	15-45µg/mL		15-45µg/mL		15-45µg/mL	
Slope (m)	27167		22409		27471	
Correlation coefficient (r ²)	0.9903		0.9996		0.9998	

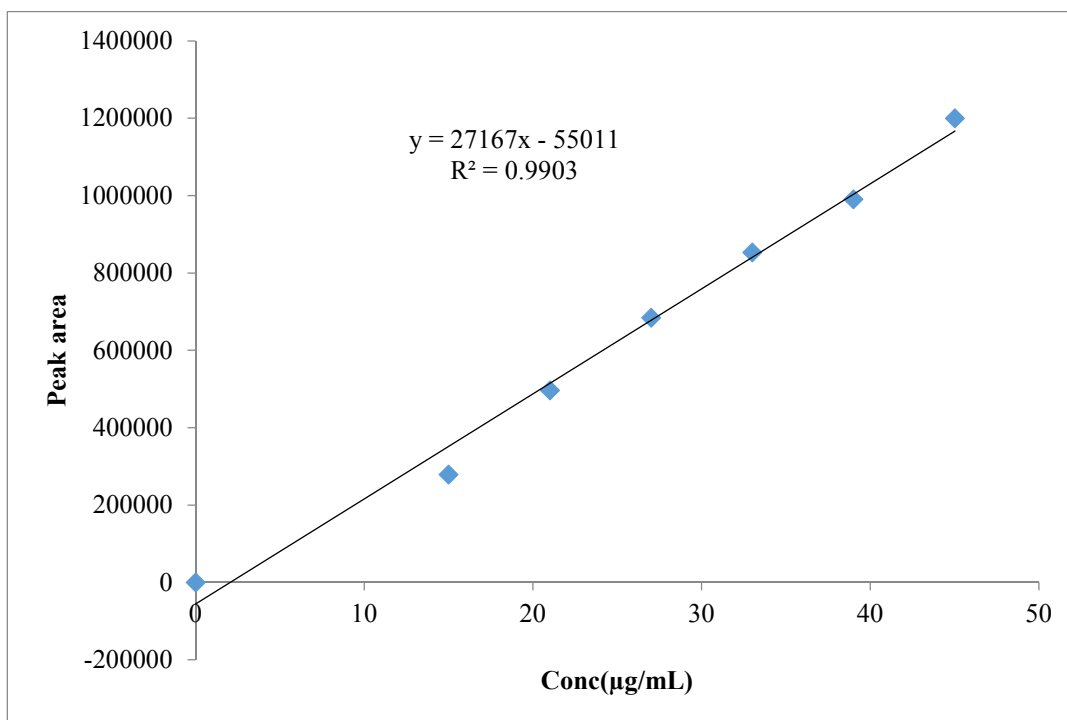


Fig. 5.7: Linearity Plot of Ombitasvir

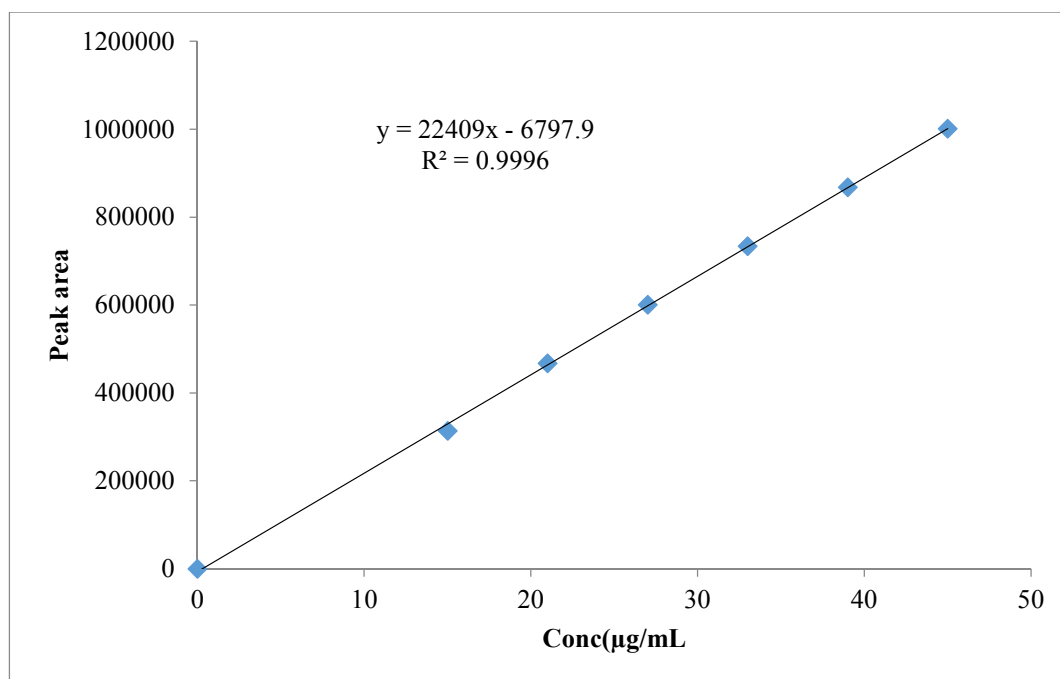


Fig. 5.8: Linearity Plot of Paritaprevir

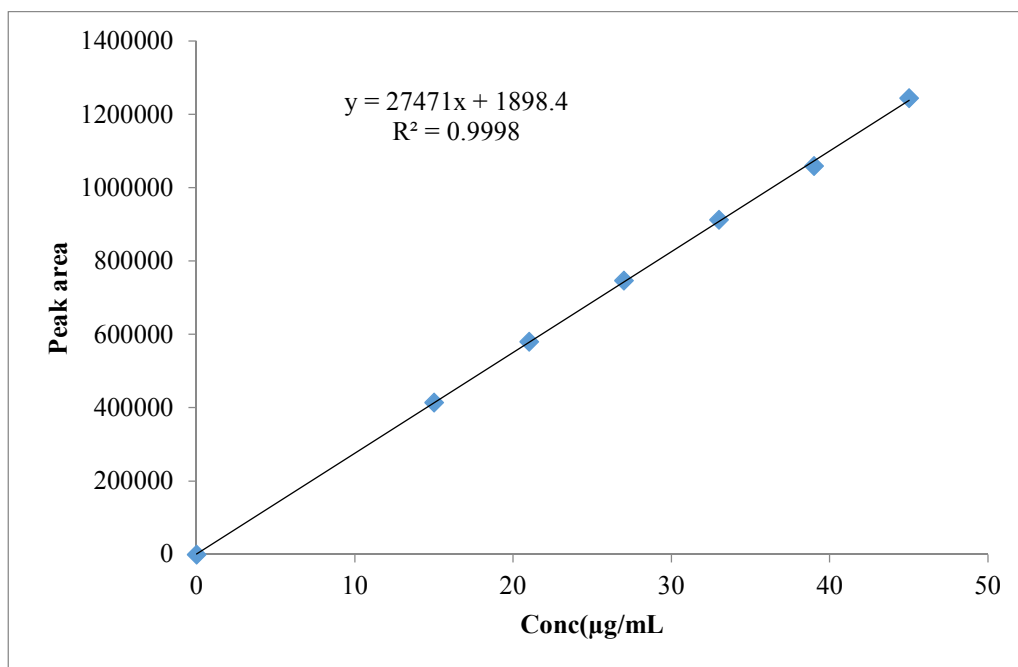


Fig. 5.9: Linearity Plot of Ritonavir

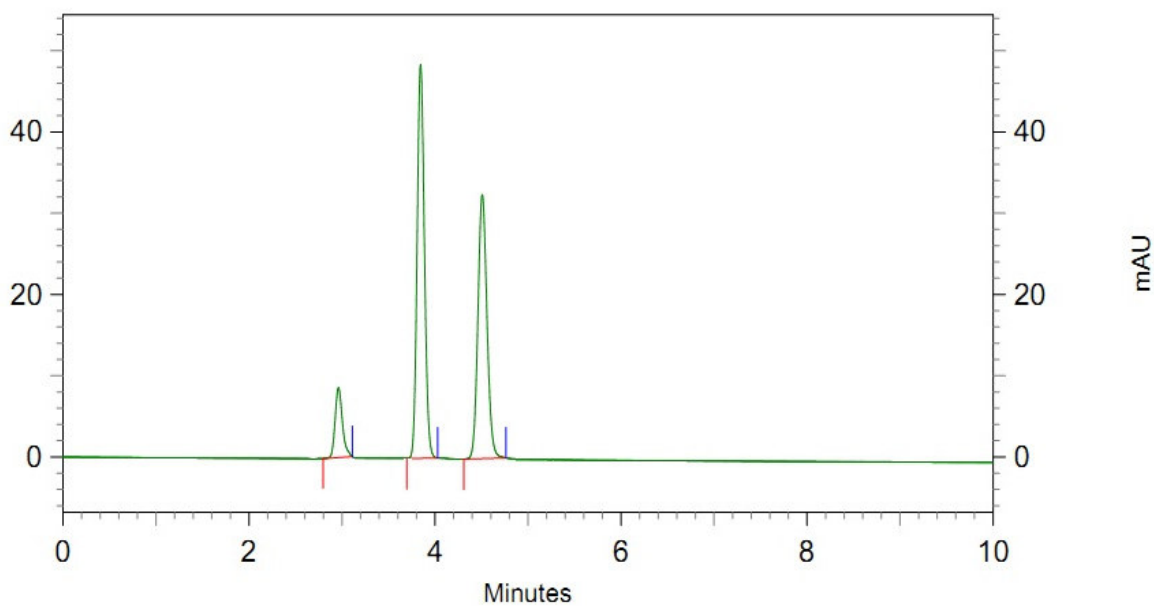


Fig. 5.10: Chromatogram of 5 mcg/ml

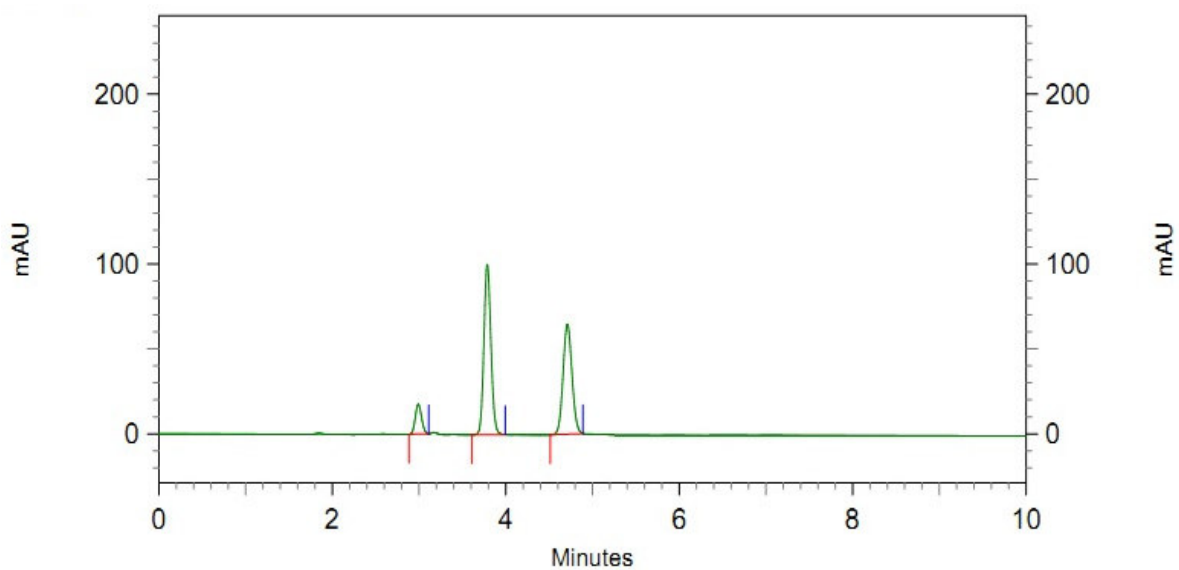


Fig. 5.11: Chromatogram of 10mcg/ml

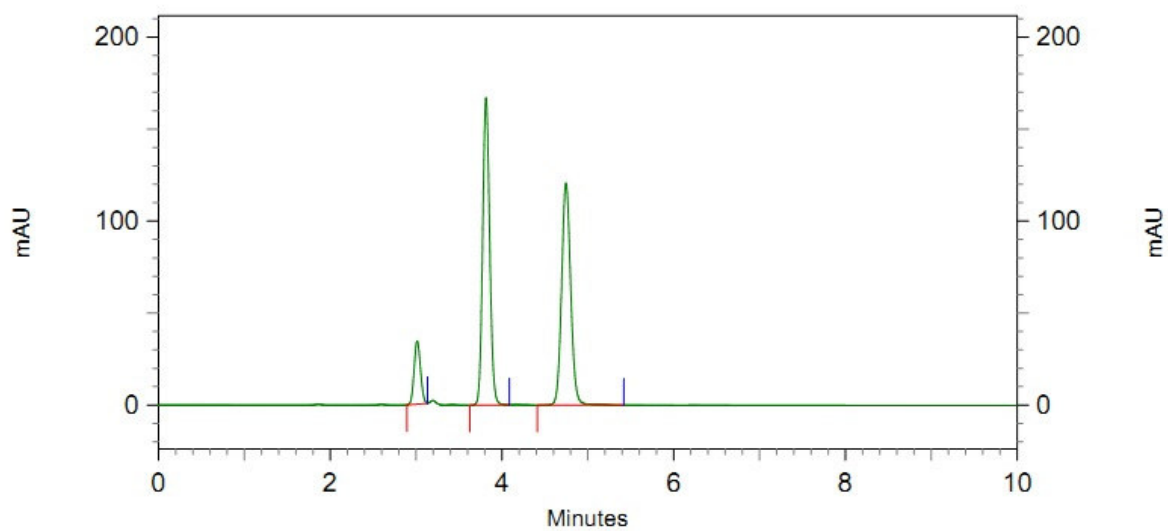


Fig. 5.12: Chromatogram of 15 mcg/ml

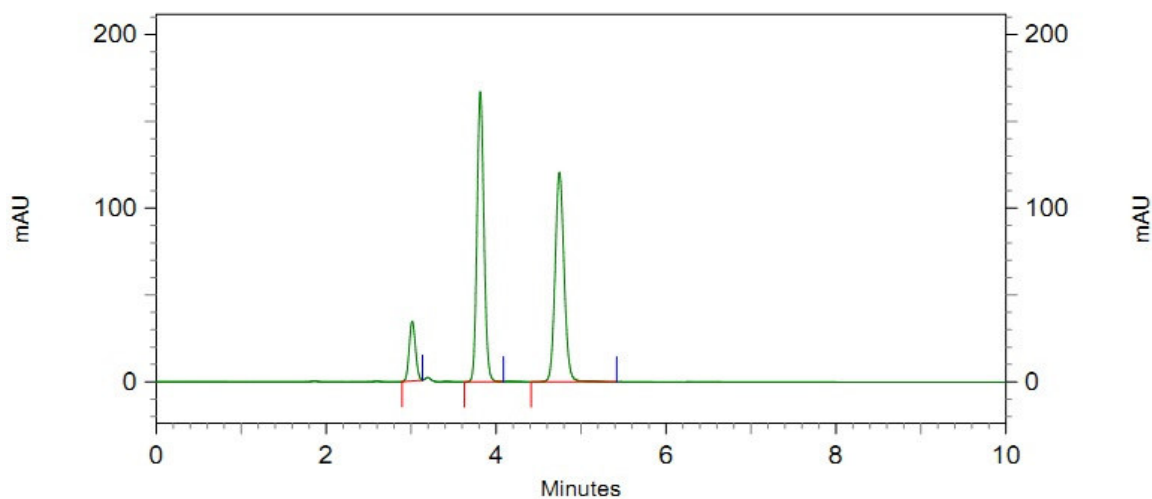


Fig. 5.13: Chromatogram of 20mcg/ml

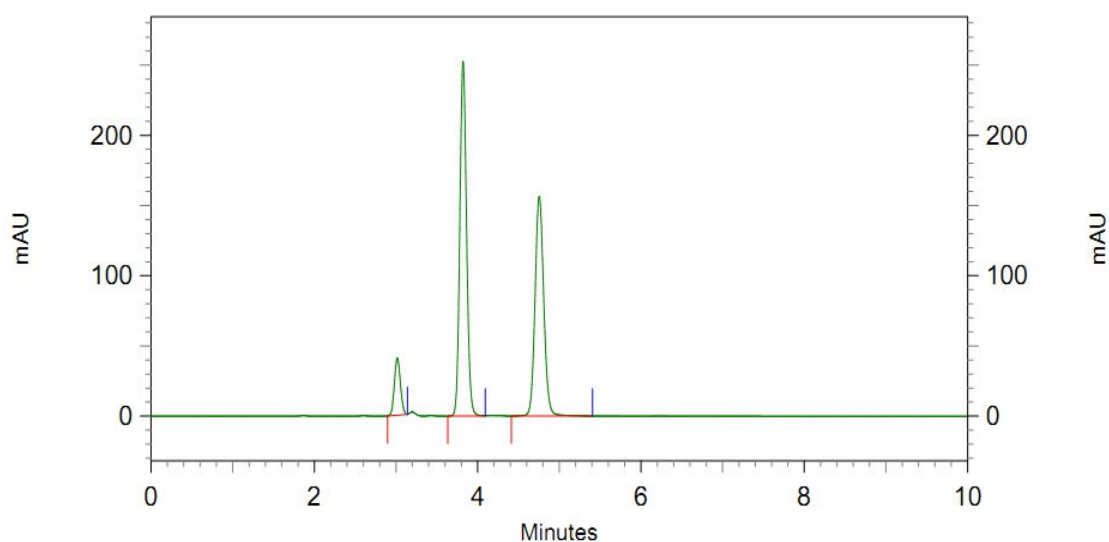


Fig. 5.14: Chromatogram of 25mcg/ml

Precision

The precision of the test procedure was evaluated by injecting the six test solutions (33 $\mu\text{g/ml}$). The Relative Standard Deviation of six injections was calculated. The result of Precision studies is given in Table No.03.

Table 5.3: Precision for Ombitasvir, Paritaprevir and Ritonavir

	Intraday precision					
	Test conc: 33µg/ml					
Sample. No	OT	%Assay	PT	%Assay	RT	%Assay
1	853368	99.45	734451	99.67	912310	98.56
2	821362	98.32	734121	99.54	911321	99.06
3	829356	99.16	734123	97.95	901235	99.16
4	837350	99.94	731243	97.43	904320	97.35
5	845344	99.76	734312	97.43	912349	97.29
6	843338	99.11	733123	97.23	912340	98.54
Mean	838353	99.29	733562	98.21	908979	98.33
SD	11574.29	0.58	1228.86	1.11	4917.51	0.82
% RSD	1.38	0.58	0.17	1.13	0.54	0.83

	Inter-day precision					
	Test conc: 33µg/ml					
Sample. No	OT	%Assay	PT	%Assay	RT	%Assay
1	822371	98.34	732112	98.20	901235	98.56
2	824345	95.91	731033	96.38	904320	96.74
3	837350	98.20	729954	98.56	907405	97.92
4	845344	96.38	728875	96.74	910490	96.10
5	853338	98.61	727796	97.92	913575	98.28
6	861332	99.45	726717	99.10	916660	98.46
Mean	840680	97.82	729415	97.82	908947.50	98
SD	15630.89	1.37	2018.62	1.06	5771.51	1.02
% RSD	1.86	1.40	0.28	1.08	% RSD	1.04

Accuracy

To validate whether the test method can accurately quantify ombitasvir, paritaprevir and ritonavir, prepare samples in three times for higher and lower levels, in triplicate for other levels by spiking ombitasvir, paritaprevir and ritonavir of active material with equivalent amount of placebo and perform CU as per test procedure. Samples were prepared at levels 80% and 120% of the target assay concentration i.e. 100% level. Table No.04 shows the results for accuracy of ombitasvir, paritaprevir and ritonavir.

Table 5.4: Accuracy results of Ombitasvir

Level of % recovery	Target Conc. (µg/mL)	Amount of drug spiked(µg/mL)	Nominal conc (µg/mL)	Drug recovered	%	Mean	SD	%RSD
				(µg/mL)	Recovery			
80	33	27.00	60.00	59.24	98.73	98.54	0.28	0.29
				58.93	98.22			
				59.21	98.68			
100	33	33.00	66.00	66.25	99.62	101.02	1.70	1.68
				66.34	100.52			
				67.92	102.91			
120	33	40.00	73.00	73.25	100.34	100.03	1.08	1.07
				72.15	98.84			
				73.67	100.92			

Table 5.5: Accuracy results of Paritaprevir

Level of % recovery	Target Conc. (µg/mL)	Amount of drug spiked (µg/mL)	Nominal conc (µg/mL)	Drug recovered	%	Mean	SD	%RSD
				(µg/mL)	Recovery			
80	33	27.00	60.00	61.34	102.23	100.41	1.86	1.85
				60.28	100.47			
				59.11	98.52			
100	33	33.00	66.00	64.92	101.66	102.95	1.31	1.28
				68.83	104.29			
				67.92	102.91			
120	33	40.00	73.00	73.13	100.18	99.77	0.42	0.42
				72.84	99.78			
				72.52	99.34			

Table 5.6: Accuracy results of Ritonavir

Level of % recovery	Target Conc. (µg/mL)	Amount of drug spiked (µg/mL)	Nominal conc (µg/mL)	Drug recovered	%	Mean	SD	%RSD
				(µg/mL)	Recovery			
80	33	27.00	60.00	63.22	105.37	103.72	1.57	1.52
				62.13	103.55			
				61.34	102.23			
100	33	33.00	66.00	67.04	98.45	98.88	0.40	0.40
				65.32	98.97			
				65.49	99.23			
120	33	40.00	73.00	73.28	100.38	101.97	1.77	1.73
				74.2	101.64			
				75.83	103.88			

Robustness

Robustness of the method is performed by altering the chromatographic conditions such as pH of the buffer, Wavelength, Mobile phase composition and observed the variation of the results which should be within the acceptance criteria.

Table 5.7: Robustness results

S. No.	Parameter	Condition	OT		PT		RT	
			Area (n=3)	% change	Area (n=3)	% change	Area (n=3)	% change
1	Standard	Standard conditions	837350	0	729954	0	907405	0
2	Mobile Phase composition (±2%)	0.02M phosphate buffer (pH - 4.5): Acetonitrile: Methanol, (v/v), (52:28:20)	834512	0.339	728847	#REF!	917341	-1.095
		0.02M phosphate buffer (pH - 4.5): Acetonitrile: Methanol, (v/v), (48:32:20)	831674	0.340	727740	0.152	927277	-1.083
3	Mobile	2.9	838836	-0.861	726633	0.152	917213	1.085
	phase pH	3.1	835998	0.338	725526	0.152	917149	0.007
4	Wavelength (nm)	248	833160	0.339	724419	0.153	914065	0.336
		252	830322	0.341	723312	0.153	907021	0.771
5	Flow rate (mL) ±0.2mL	1.2	837484	-0.863	722205	0.153	916957	-1.095
		0.8	834646	0.339	721098	0.153	906893	1.098

Limit of Detection (LOD) and Limit of Quantitation (LOQ)

The detection limit of an individual analytical procedure is the lowest amount of analyte in a sample which can be detected but not necessarily quantitated as an exact value.

1. Based on Signal-to-Noise for LOD (3:1), LOQ (10:1)
2. Based on the Standard Deviation of the Response and the Slope

The quantitation limit of an individual analytical procedure is the lowest amount of analyte in a sample which can be quantitatively determined with suitable precision and accuracy.

From the linearity data, the limit of detection and quantitation were calculated using the following formula.

$$\text{LOD} = \frac{3.3 \sigma}{S}, \quad \text{LOQ} = \frac{10 \sigma}{S}$$

σ = standard deviation of the response, S = slope of the calibration curve

LOD and LOQ of Amlodipine, Hydrochlorothiazide and Olmesartan are performed by spiking of known concentrations of the sample into the placebo of formulation and inject the sample.

Table 5.8: Results of LOD and LOQ

Sample	LOD	LOQ
Ombitasvir	1.89859	5.753304
Paritaprevir	0.297175	0.900531
Ritonavir	0.693251	2.100761

ASSAY

Six replicates of the samples solutions were injected for quantitative analysis. The amounts of ombitasvir, paritaprevir and ritonavir estimated were found to 99.52%, 102.00% and 99.02% respectively. A good separation and resolution of both drugs indicate that there were no interference from the excipients commonly present in pharmaceutical formulations. This showed that the estimation of dosage form was accurate within given acceptable level of 95% to 105%. The amount of ombitasvir, paritaprevir and ritonavir per tablet were calculated by extrapolating the value of area from the calibration curve. Analysis procedure was repeated six times with tablet formulation. The result formulation was reported in Table No. 05.

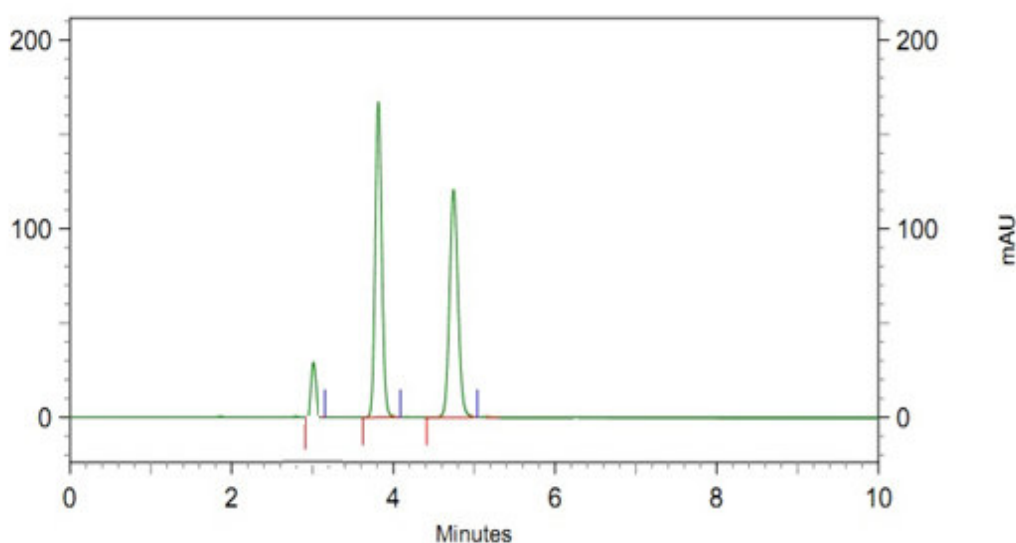
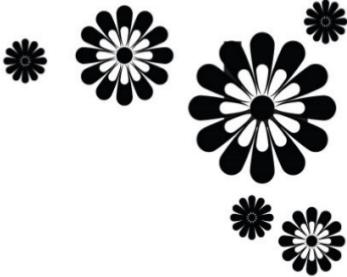


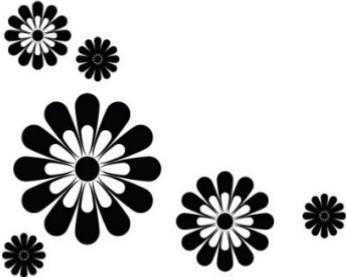
Fig. 5.15: Chromatogram of test sample

Table 5.9: Assay of test sample

Test formulation (Tablet)	Label claimed (mg/tab)		
Ombitasvir, Paritaprevir, Ritonavir tablets	OT	RT	PT
	12.5	75	50
	Conc found (mg)		
	12.44	76.2	49.51
	%Assay		
	99.52	102	99.02



CHAPTER - 6
ABACAVIR,
DOLUTEGRAVIR AND
LAMIVUDINE



DRUG PROFILE

Ombitasvir, paritaprevir and ritonavir (Figure:1.0) drugs were combined in a single dosage form (film-coated tablet) in the brand name of TECHNIVIE for the treatment of hepatitis-C. These three drugs will act against the hepatitis-C virus (HCV) in three different mechanisms.

Ombitasvir, produces its antiviral activity by inhibiting the HCV non-structural protein (NS) 5A. Ombitasvir chemically designated as dimethyl ([[(2S,5S)-1-(4-tert-butyl phenyl) pyrrolidine-2,5-diyl] bis {benzene -4, 1 diylcarbamoyl (2S) pyrrolidine -2, 1-diyl} [(2S) -3-methyl -1-oxobutane -1, 2-diyl]}) biscarbamate hydrate with molecular weight of 894.11 g/mole (Fig. 1).

Paritaprevir chemically designated as (2R, 6S, 12Z, 13aS,14aR, 16aS)-N-(cyclopropylsulfonyl)-6-[[[(5-methyl-2-pyrazinyl) carbonyl] amino]-5, 16-dioxo-2-(6-phenanthridinyloxy)-1, 2, 3, 6, 7, 8, 9, 10, 11, 13a, 14, 15, 16, 16a-tetradecahydrocyclopropa[e] pyrrolo[1,2-a][1,4] diazacyclopentadecine -14a(5H)-carboxamide with molecular weight of 765.89 g/mole (Fig.1). It is a powerful inhibitor of the NS-3/4A serine protease of HCV. Subsequently, replication of HCV genetic components and translation into a single polypeptide, NS-3, and its activating cofactor NS-4A are accountable for splitting it into the succeeding nonstructural and structural proteins essential for assembly into a mature virus, viz., NS-3, NS-4A, NS-4B, NS-5A, and NS-5B. By inhibiting viral protease NS-3/4A, Paritaprevir, therefore, prevents viral replication and function.

Ritonavir is an anti-retroviral medication utilized along with other medications to treat the human immunodeficiency virus. This combination treatment is known as highly active anti-retroviral therapy (HAART). At low doses of ritonavir, it is utilized with other

protease inhibiting agents and useful in combination with other hepatitis-C medicaments. It is chemically designated as 1, 3-thiazol- 5-ylmethyl N-[(2S, 3S, 5S) -3- hydroxy- 5- [(2S)- 3- methyl -2- {[methyl({[2-(propan-2-yl)-1,3-thiazol-4-yl]methyl})carbamoyl] amino} butanamido]-1,6 diphenylhexan-2-yl]carbamate with molecular weight of 720.946 g/mole.

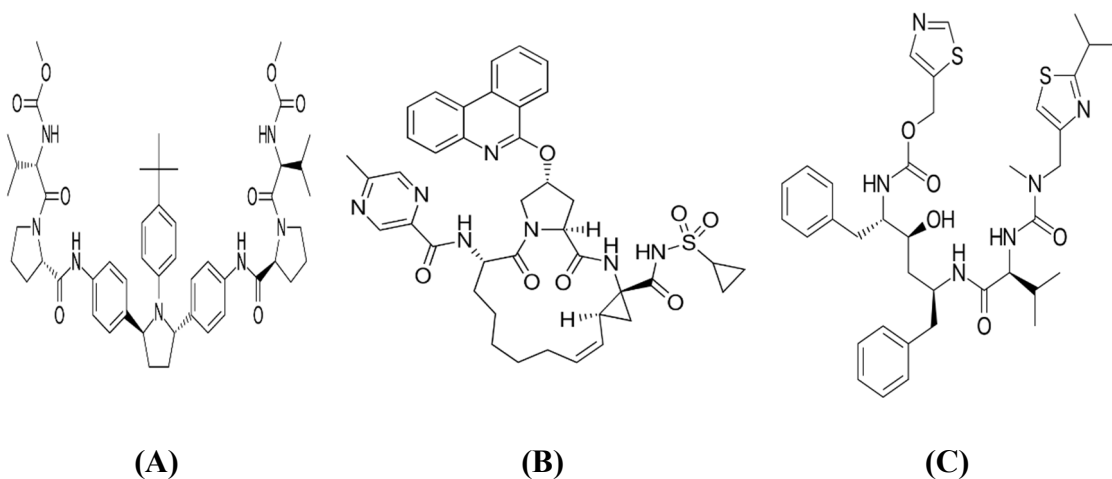


Fig. 6.1: Chemical structures of (A) Ombitasvir (B) Paritaprevir (C) Ritonavir

MATERILAS AND METHODS**Instrumentation:**

The HPLC system (Agilent HPLC 1200 Infinity LC Specifications) consisted of a pump (Agilent LC20AT) programmed with Ezchrom Elite Software and rheodyne Injector was used. The detector consisted of UV/VIS (UV-2489) model was operated at a wavelength of 262 nm. The column used was Inertsil CN- 3 column at ambient temperature

Chemicals and Reagents:

Hetero Aurobindo Pharma Pvt. Ltd, Hyderabad, India kindly supplied the pure working standards of known potency of ombitasvir, paritaprevir and ritonavir as a gift sample. The marketed sample with strength of ombitasvir 25 mg, paritaprevir 150 mg, and ritonavir 100 mg purchased from the local Pharmacy. The reagents like orthophosphoric acid (OPA) of Hi-Media Laboratories Pvt. Ltd, water, methanol, acetonitrile, triethylamine of Merk, potassium dihydrogen phosphate of Thermo Fisher Scientific India Pvt. Ltd were used.

Preparation of Standard Stock Solution:

Each 10mg of ombitasvir, paritaprevir and ritonavir were transferred to 100 ml volumetric flask and dissolved and diluted to the mark with methanol. The stock solutions were further diluted with mobile phase to obtain a solution of 100 µg/ml.

Test sample preparation:

Tablet powder equivalent to 10 mg of ombitasvir, paritaprevir and ritonavir was weighed from a pooled powder of twenty tablets and transferred into a 10 ml volumetric flask, few ml of methanol was added and sonicated for 10 min. The volume was made up to mark with methanol and the sample solution was filtered and used for further dilution.

METHOD DEVELOPMENT

For developing the method, a systematic study of the effect of various factors were undertaken by varying one parameter at a time and keeping all other conditions constant. Method development consists of selecting the appropriate mass parameters and choice of stationary and mobile phases. The following studies were conducted for this purpose.

Trail -1:

Chromatographic conditions :
Mobile Phase : Water: Methanol (60:40, %V/V)
Column : C18, HPLC column, Emerald, 3 μ m, 30.0 \times 3.0 mm
Detection Wavelength : 254 nm

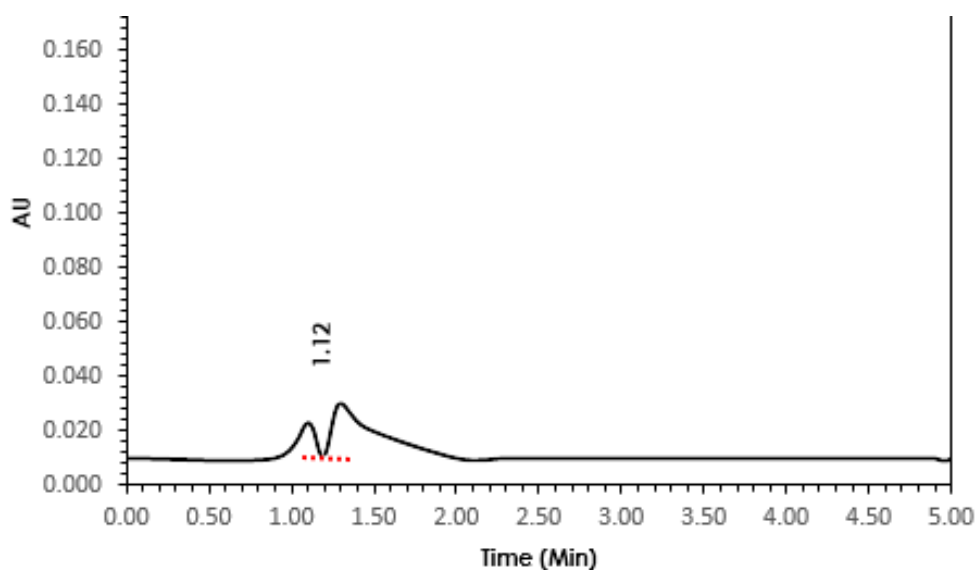


Fig. 6.2: Chromatogram Showing Trial – 1

Conclusion: The baseline and peak shape is not good.

Trail -2:

Chromatographic conditions :

Mobile Phase : Water: Acetonitrile: Methanol (80:10: 10, %V/V)

Column : Kinetex 2.6u XB-C18 150x4.60mm

Detection Wavelength : 260 nm

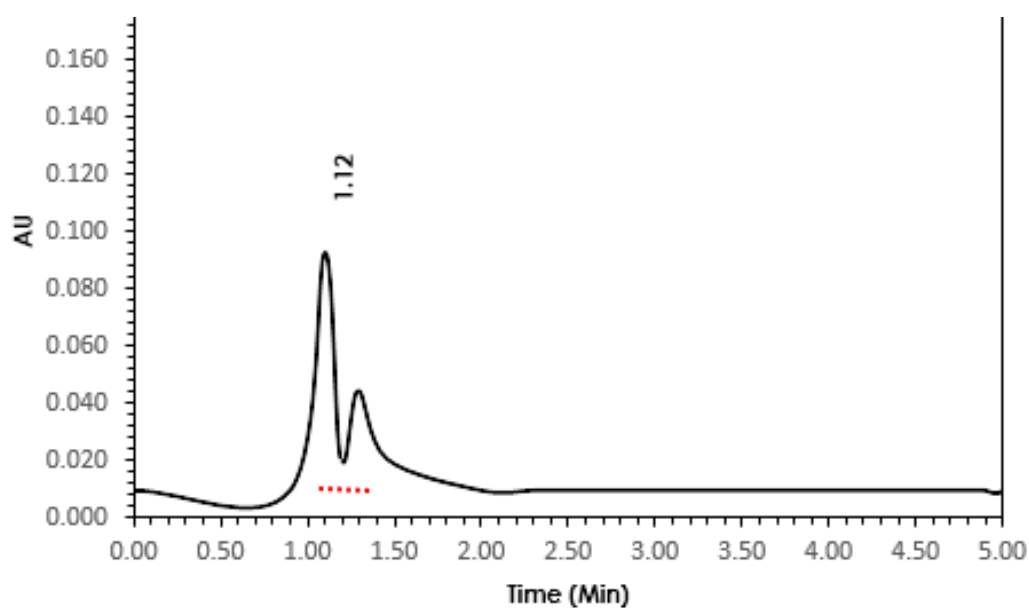


Fig. 6.3: Chromatogram Showing Trial – 2

Conclusion: The components were not separated completely.

Selection of Wavelength (λ_{max}): 10 mg of the ombitasvir, paritaprevir and ritonavir standard drug is taken in a 10 ml volumetric flask and dissolved in acetonitrile and volume made up to the mark, from this solution 0.1ml is pipetted into 10 ml volumetric flask and made up to the mark with the acetonitrile to give a concentration of 10 $\mu\text{g/ml}$. The above prepared solution is scanned in UV between 200-400 nm using acetonitrile as blank. The λ_{max} was found to be 262nm.

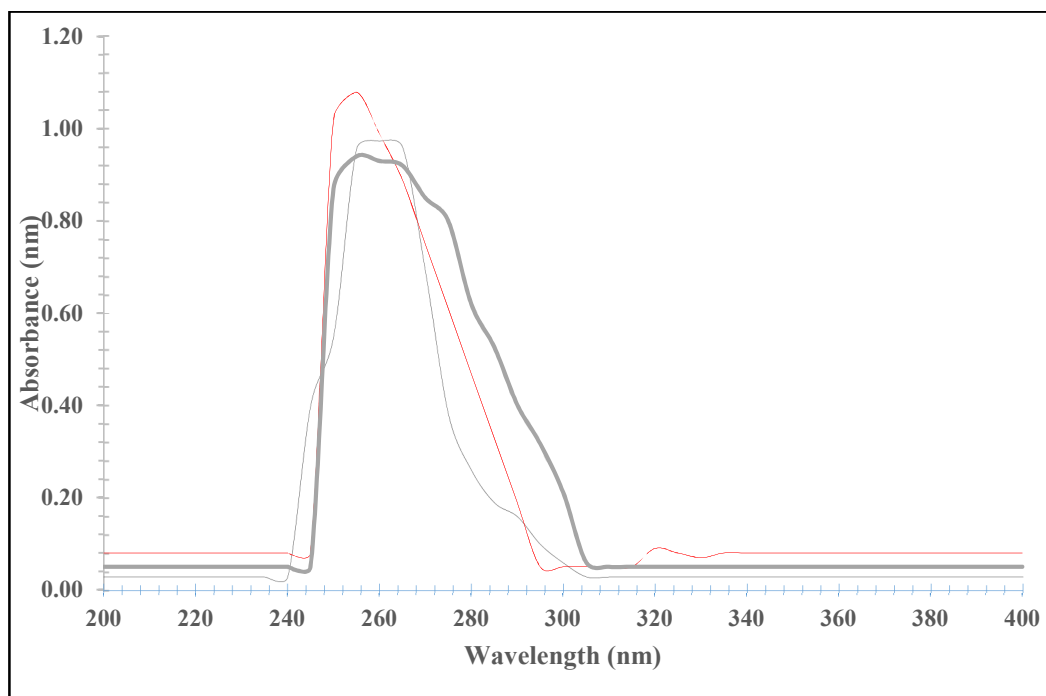


Fig. 6.4: Over lay spectrum UV spectrum of Ombitasvir, Paritaprevir and Ritonavir

Choice of stationary phase

Initially the separation was tried with different columns having different dimensions like diameter and length and pore size. Finally good separation with finest peak shape was achieved with the analytical column Inertsil ODS-C18; 5 μ m (4.6 X 250mm).

Selection of mobile phase

Several systematic test plans were performed to optimize the mobile phase. Different solvents like methanol, water and acetonitrile in different ratios and different pH values of the mobile phase ratios, by using different buffer solutions in order to get sharp peak and base line separation of the components and without interference of the excipients. Satisfactory peak symmetry, resolved and free from tailing was obtained in mobile phase 0.02M phosphate buffer (pH-4.5): acetonitrile: methanol, (50:30:20) (v/v).

Selection of the mobile phase flow rate

Flow rates of the mobile phase were changed from 0.5-1.0 ml/min for optimum separation. A minimum flow rate as well as minimum run time gives the maximum saving on the usage of solvents. It was found from the experiments that 1.0 mL/min flow rate was ideal for the successful elution of the analyte.

Optimized Chromatographic conditions

After series of trials, the chromatographic conditions was accomplished with following parameters.

Buffer	: 0.02M phosphate buffer (pH-4.5)
Mobile Phase	: 0.02M phosphate buffer (pH-4.5): Acetonitrile: Methanol, (50:30:20) (v/v)
Column	: Inertsil ODS-C18; 5 μ m (4.6 X 250mm)
Flow Rate	: 1.0ml/min
Temperature	: Ambient
Injection Volume	: 20 μ l
Detector	: 262nm
Diluent	: Water: Acetonitrile (50:50) column with a mixture of as mobile phase.

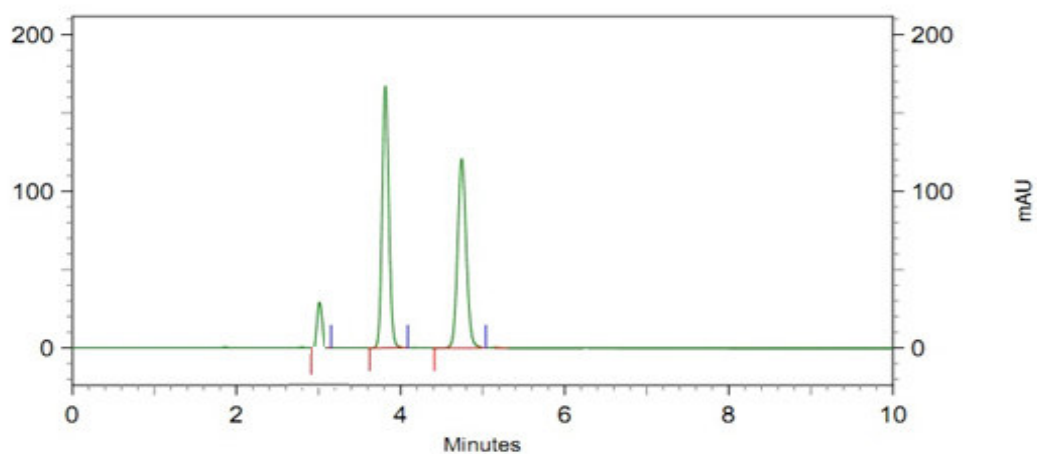


Fig. 6.5: Chromatogram of Ombitasvir, Paritaprevir and Ritonavir

METHOD VALIDATION

After the development of RP-HPLC method for the estimation of drug in a dosage form, validation of the method was performed. This part describes the procedure followed for validation of the developed method.

Specificity

Specificity is the ability of a method to discriminate between the analyte (s) of interest and other components that are present in the sample. A study of placebo interference from excipients was conducted. Equivalent weight of placebo taken as per the test method and placebo interference was conducted in duplicate.

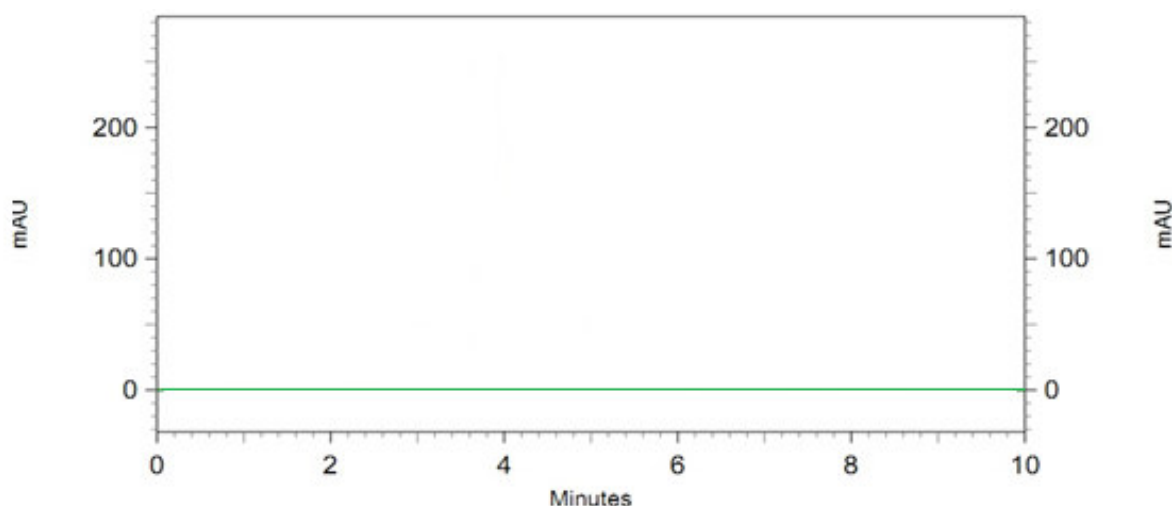


Fig. 6.6: Chromatogram of Blank

System suitability

To verify the system producing the consistent results with the optimized method injected the standard for six times with the criteria of % RSD for retention time and area NMT 2.0%, theoretical plates NLT 3000 plates, tailing factor NMT 1.5 and resolution NLT 4.

Table. 6.1: System suitability parameters

Parameter	Compound	Result
Retention Time	Ombitasvir	3.14 min
	Paritaprevir	3.92 min
	Ritonavir	4.91 min
Peak Area	Ombitasvir	254622
	Paritaprevir	565892
	Ritonavir	431312
Theoretical plates	Ombitasvir	4231
	Paritaprevir	3452
	Ritonavir	1342
Tailing Factor	Ombitasvir	0.12
	Paritaprevir	0.34
	Ritonavir	0.35
Resolution	Ombitasvir	-
	Paritaprevir	3.34
	Ritonavir	4.34

Linearity:

Chapter - 6 ABACAVIR, DOLUTEGRAVIR AND LAMIVUDINE

A series of standard solutions (not less than 5 is recommended) were prepared in the range of 15µg/ml-45µg/ml containing ombitasvir, paritaprevir and ritonavir standards and injected. A plot of average peak area versus the concentration in µg/ml or mg/ml is made and from this the correlation coefficient, y-intercept (constant of regression) and slope (coefficient of regression) of the regression line were calculated. The calibration data and calibration curve shown in Table No.02 and Fig No. 2, 3 and 4.

Table 6.2: Linearity data

S. No.	Concentration µg/mL	Area of OT	Concentration µg/mL	Area of PT	Concentration µg/mL	Area of RT
1	15	279425	15	313823	15	414740
2	21	496653	21	467371	21	580637
3	27	684443	27	600918	27	746533
4	33	853368	33	734456	33	912430
5	39	991189	39	867991	39	1059512
6	45	1199969	45	1001530	45	1244222
Concentration range	15-45µg/mL		15-45µg/mL		15-45µg/mL	
Slope (m)	27167		22409		27471	
Correlation coefficient (r ²)	0.9903		0.9996		0.9998	

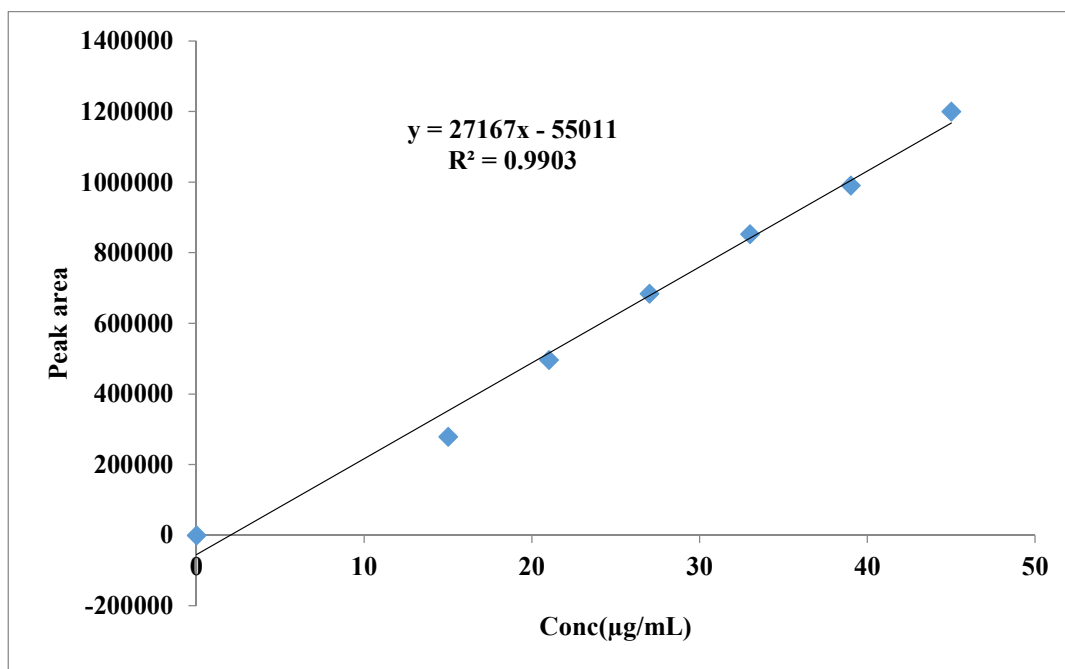


Fig. 6.7: Linearity Plot of Ombitasvir

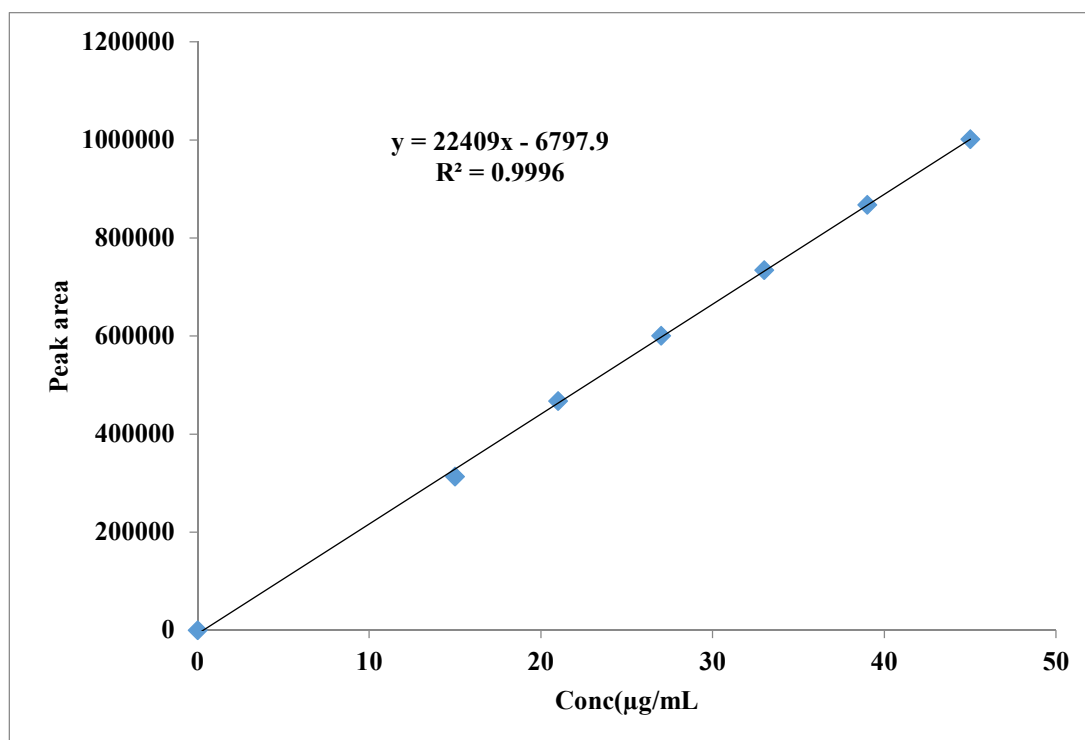


Fig. 6.8: Linearity Plot of Paritaprevir

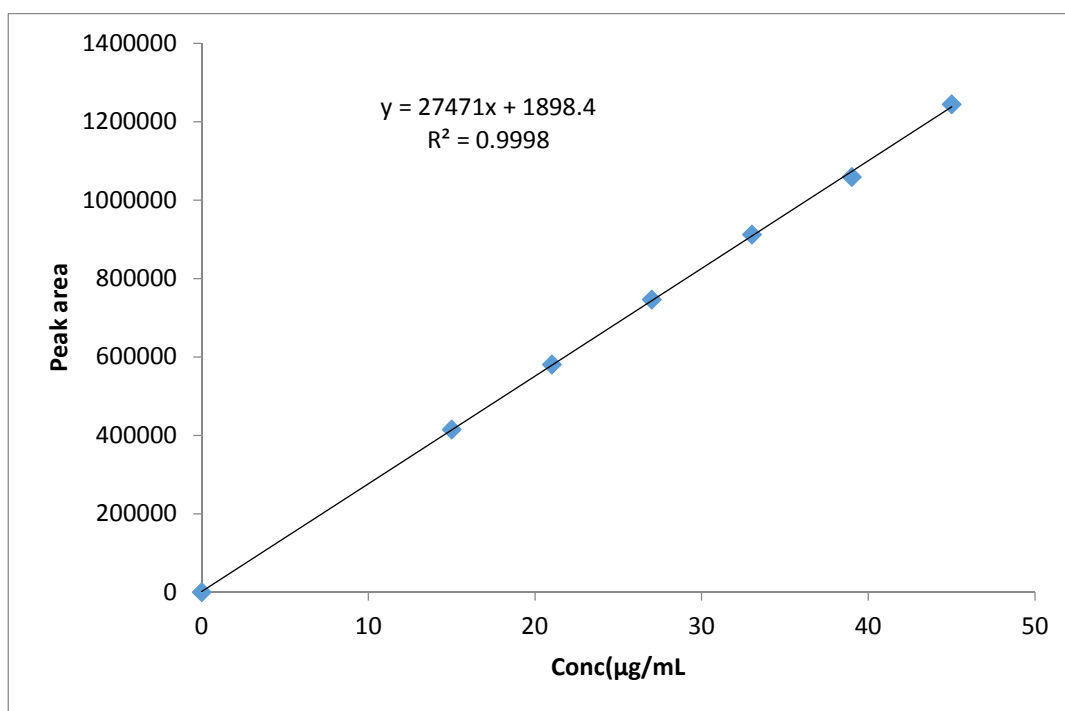


Fig. 6.9: Linearity Plot of Ritonavir

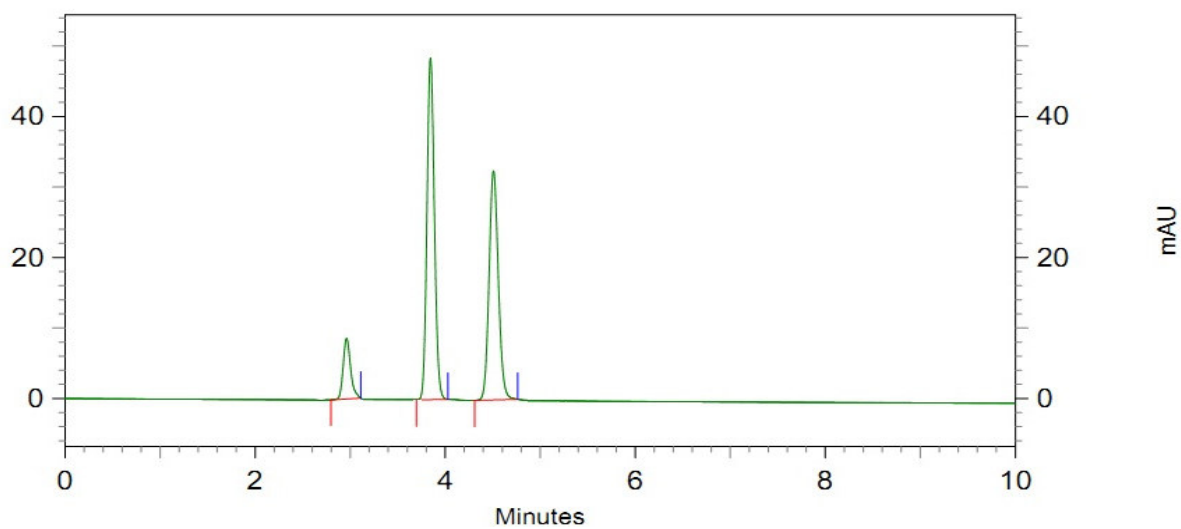


Fig. 6.10: Chromatogram of 5mcg/ml

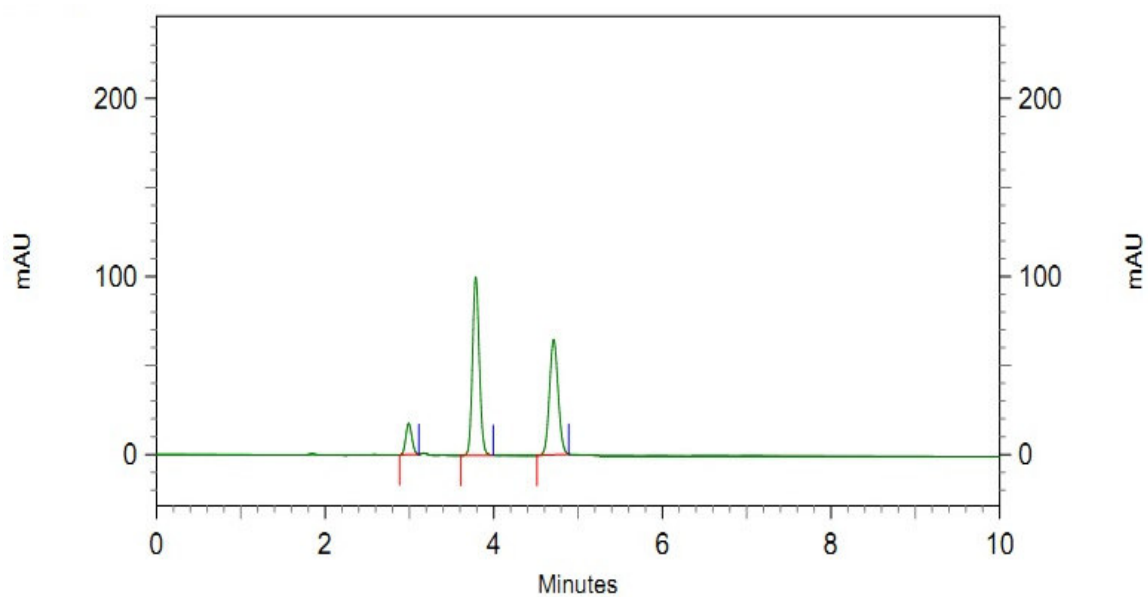


Fig. 6.11: Chromatogram of 10mcg/ml

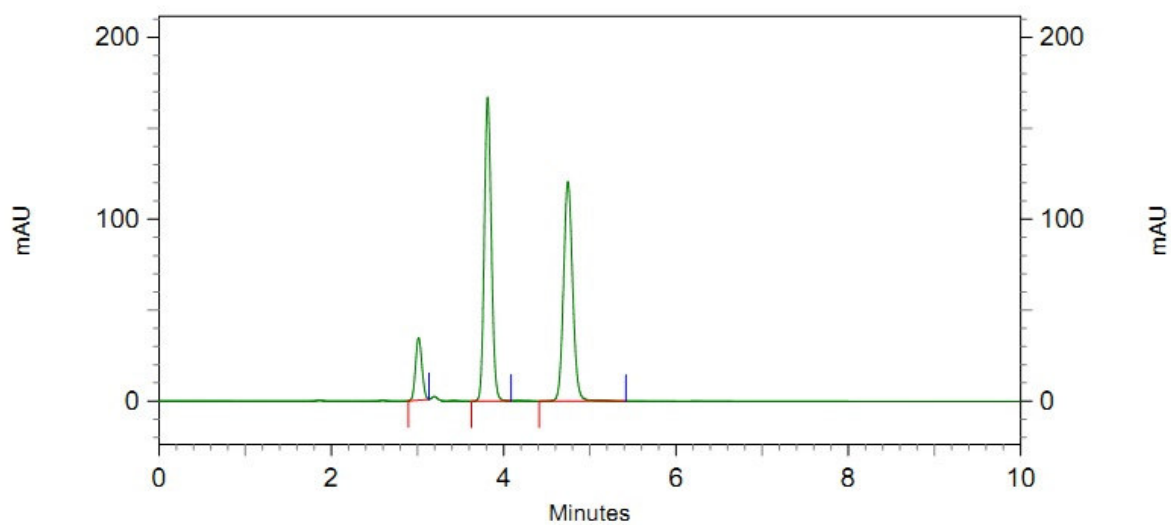


Fig. 6.12: Chromatogram of 15 mcg/ml

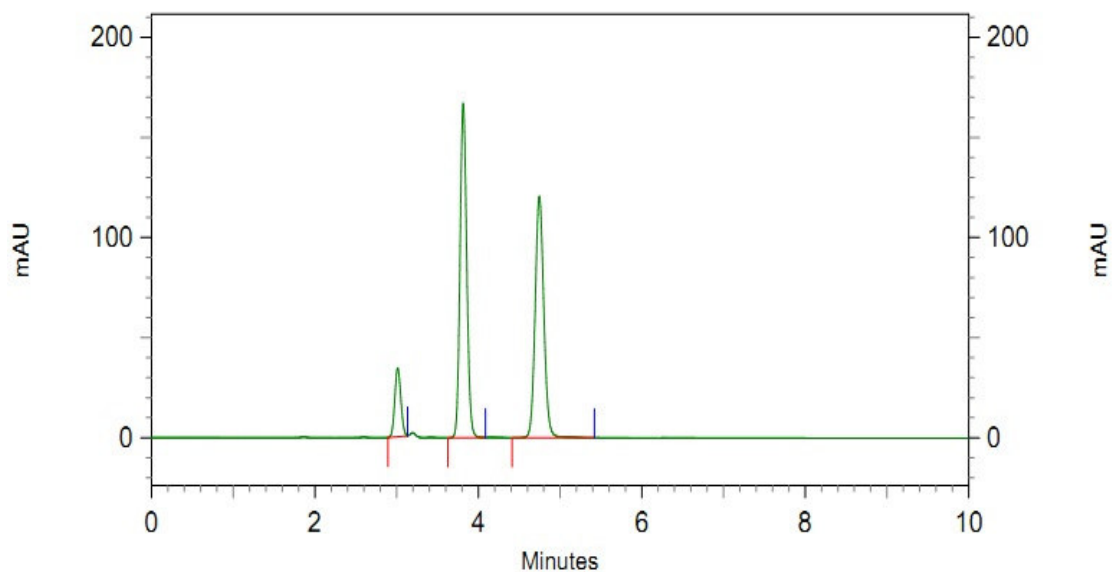


Fig. 6.13: Chromatogram of 20mcg/ml

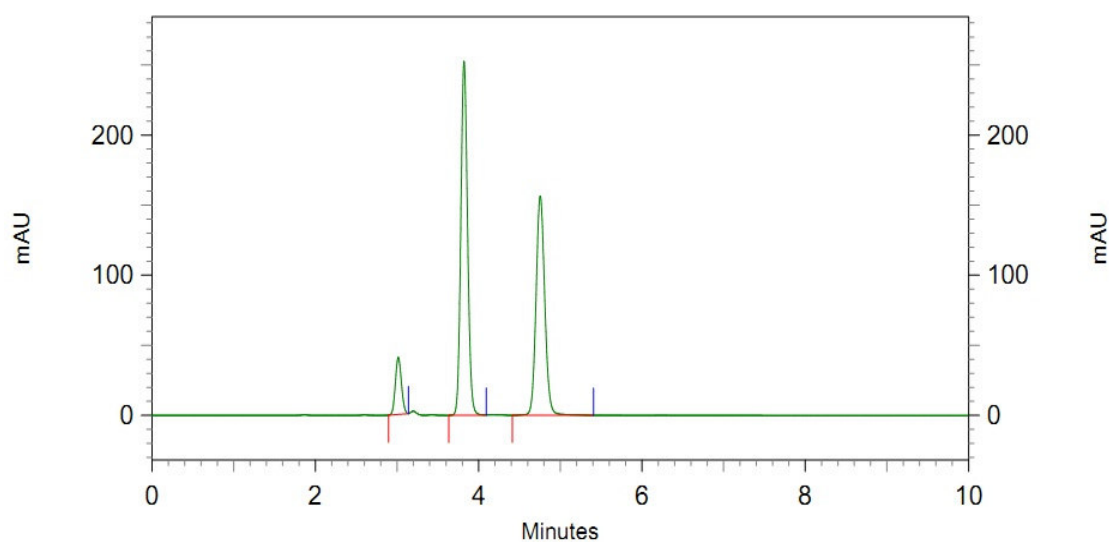


Fig. 6.14: Chromatogram of 25mcg/ml

Precision

The precision of the test procedure was evaluated by injecting the six test solutions (33 $\mu\text{g/ml}$). The Relative Standard Deviation of six injections was calculated. The result of Precision studies is given in Table No.03.

Table 6.3: Precision for Ombitasvir, Paritaprevir and Ritonavir

	Intraday precision					
	Test conc: 33µg/ml					
Sample. No	OT	%Assay	PT	%Assay	RT	%Assay
1	853368	99.45	734451	99.67	912310	98.56
2	821362	98.32	734121	99.54	911321	99.06
3	829356	99.16	734123	97.95	901235	99.16
4	837350	99.94	731243	97.43	904320	97.35
5	845344	99.76	734312	97.43	912349	97.29
6	843338	99.11	733123	97.23	912340	98.54
Mean	838353	99.29	733562	98.21	908979	98.33
SD	11574.29	0.58	1228.86	1.11	4917.51	0.82
% RSD	1.38	0.58	0.17	1.13	0.54	0.83

	Inter-day precision					
	Test conc: 33µg/ml					
Sample. No	OT	%Assay	PT	%Assay	RT	%Assay
1	822371	98.34	732112	98.20	901235	98.56
2	824345	95.91	731033	96.38	904320	96.74
3	837350	98.20	729954	98.56	907405	97.92
4	845344	96.38	728875	96.74	910490	96.10
5	853338	98.61	727796	97.92	913575	98.28
6	861332	99.45	726717	99.10	916660	98.46
Mean	840680	97.82	729415	97.82	908947.50	98
SD	15630.89	1.37	2018.62	1.06	5771.51	1.02
% RSD	1.86	1.40	0.28	1.08	% RSD	1.04

Accuracy

To validate whether the test method can accurately quantify ombitasvir, paritaprevir and ritonavir, prepare samples in three times for higher and lower levels, in triplicate for other levels by spiking ombitasvir, paritaprevir and ritonavir of active material with equivalent amount of placebo and perform CU as per test procedure. Samples were prepared at levels 80% and 120% of the target assay concentration i.e. 100% level. Table No.04 shows the results for accuracy of ombitasvir, paritaprevir and ritonavir.

Table 6.4: Accuracy results of Ombitasvir

Level of % recovery	Target Conc. (µg/mL)	Amount of drug spiked(µg/mL)	Nominal conc (µg/mL)	Drug recovered	%	Mean	SD	%RSD
				(µg/mL)	Recovery			
80	33	27.00	60.00	59.24	98.73	98.54	0.28	0.29
				58.93	98.22			
				59.21	98.68			
100	33	33.00	66.00	66.25	99.62	101.02	1.70	1.68
				66.34	100.52			
				67.92	102.91			
120	33	40.00	73.00	73.25	100.34	100.03	1.08	1.07
				72.15	98.84			
				73.67	100.92			

Table 6.5: Accuracy results of Paritaprevir

Level of % recovery	Target Conc. (µg/mL)	Amount of drug spiked (µg/mL)	Nominal conc (µg/mL)	Drug recovered	%	Mean	SD	%RSD
				(µg/mL)	Recovery			
80	33	27.00	60.00	61.34	102.23	100.41	1.86	1.85
				60.28	100.47			
				59.11	98.52			
100	33	33.00	66.00	64.92	101.66	102.95	1.31	1.28
				68.83	104.29			
				67.92	102.91			
120	33	40.00	73.00	73.13	100.18	99.77	0.42	0.42
				72.84	99.78			
				72.52	99.34			

Table 6.6: Accuracy results of Ritonavir

Level of % recovery	Target Conc. (µg/mL)	Amount of drug spiked (µg/mL)	Nominal conc (µg/mL)	Drug recovered	%	Mean	SD	%RSD
				(µg/mL)	Recovery			
80	33	27.00	60.00	63.22	105.37	103.72	1.57	1.52
				62.13	103.55			
				61.34	102.23			
100	33	33.00	66.00	67.04	98.45	98.88	0.40	0.40
				65.32	98.97			
				65.49	99.23			
120	33	40.00	73.00	73.28	100.38	101.97	1.77	1.73
				74.2	101.64			
				75.83	103.88			

Robustness

Robustness of the method is performed by altering the chromatographic conditions such as pH of the buffer, Wavelength, Mobile phase composition and observed the variation of the results which should be within the acceptance criteria.

Table 6.7: Robustness results

S. No.	Parameter	Condition	OT		PT		RT	
			Area (n=3)	% change	Area (n=3)	% change	Area (n=3)	% change
1	Standard	Standard conditions	837350	0	729954	0	907405	0
2	Mobile Phase composition ($\pm 2\%$)	0.02M phosphate buffer (pH - 4.5): Acetonitrile: Methanol, (v/v), (52:28:20)	834512	0.339	728847	#REF!	917341	-1.095
		0.02M phosphate buffer (pH - 4.5): Acetonitrile: Methanol, (v/v), (48:32:20)	831674	0.340	727740	0.152	927277	-1.083
3	Mobile	2.9	838836	-0.861	726633	0.152	917213	1.085
	phase pH	3.1	835998	0.338	725526	0.152	917149	0.007
4	Wavelength (nm)	248	833160	0.339	724419	0.153	914065	0.336
		252	830322	0.341	723312	0.153	907021	0.771
5	Flow rate (mL) ± 0.2 mL	1.2	837484	-0.863	722205	0.153	916957	-1.095
		0.8	834646	0.339	721098	0.153	906893	1.098

Limit of Detection (LOD) and Limit of Quantitation (LOQ)

The detection limit of an individual analytical procedure is the lowest amount of analyte in a sample which can be detected but not necessarily quantitated as an exact value.

1. Based on Signal-to-Noise for LOD (3:1), LOQ (10:1)
2. Based on the Standard Deviation of the Response and the Slope

The quantitation limit of an individual analytical procedure is the lowest amount of analyte in a sample which can be quantitatively determined with suitable precision and accuracy.

From the linearity data, the limit of detection and quantitation were calculated using the following formula

$$\text{LOD} = \frac{3.3 \sigma}{S}, \quad \text{LOQ} = \frac{10 \sigma}{S}$$

σ = standard deviation of the response, S = slope of the calibration curve

LOD and LOQ of Amlodipine, Hydrochlorothiazide and Olmesartan are performed by spiking of known concentrations of the sample into the placebo of formulation and inject the sample.

Table 6.8: Results of LOD and LOQ

Sample	LOD	LOQ
Ombitasvir	1.89859	5.753304
Paritaprevir	0.297175	0.900531
Ritonavir	0.693251	2.100761

ASSAY

Six replicates of the samples solutions were injected for quantitative analysis. The amounts of ombitasvir, paritaprevir and ritonavir estimated were found to 99.52%, 102.00% and 99.02% respectively. A good separation and resolution of both drugs indicate that there were no interference from the excipients commonly present in pharmaceutical formulations. This showed that the estimation of dosage form was accurate within given acceptable level of 95% to 105%. The amount of ombitasvir, paritaprevir and ritonavir per tablet were calculated by extrapolating the value of area from the calibration curve. Analysis procedure was repeated six times with tablet formulation. The result formulation was reported in Table No. 05.

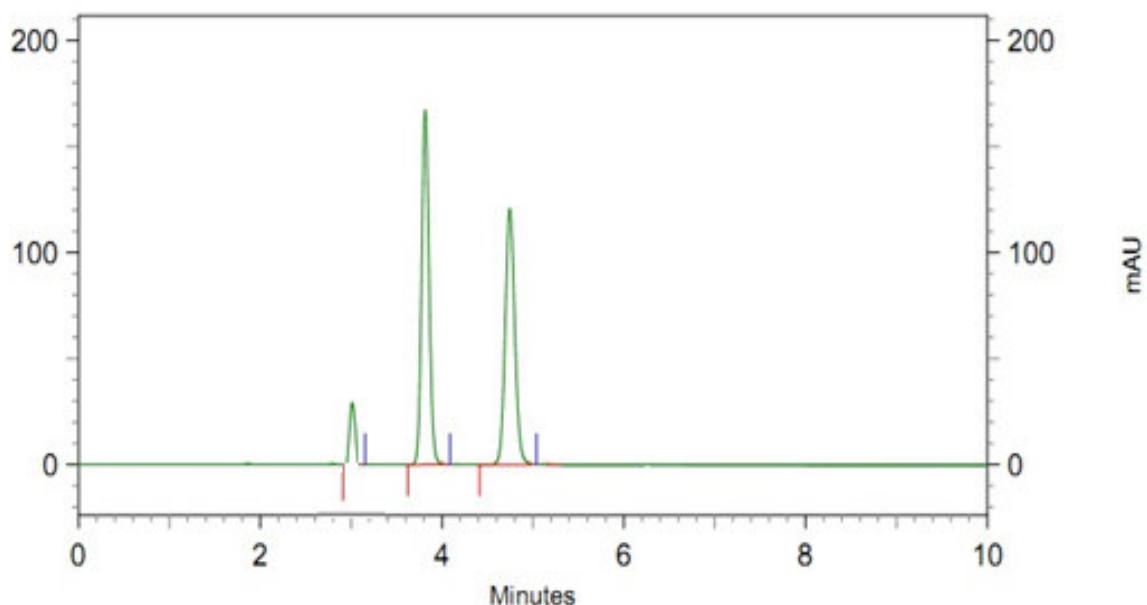
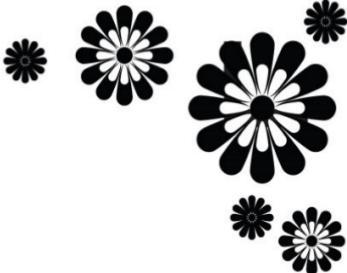


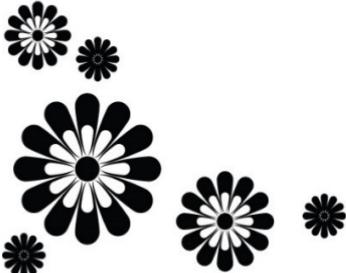
Fig. 6.15: Chromatogram of test sample

Table 6.9: Assay of test sample

Test formulation (Tablet)	Label claimed (mg/tab)		
Ombitasvir, Paritaprevir, Ritonavir tablets	OT	RT	PT
	12.5	75	50
	Conc found (mg)		
	12.44	76.2	49.51
	%Assay		
	99.52	102	99.02



CHAPTER - 7
BICTEGRAVIR,
EMTRICITABINE AND
TENOFOVIR ALAFENAMIDE



Bictegravir:

Bictegravir is a recently approved investigational drug that has been used in trials studying the treatment of HIV-1 and HIV-2 infection. It has been approved for HIV-1 monotherapy combined with 2 other antiretroviral in a single tablet (50 mg bictegravir, 200 mg emtricitabine, 25 mg tenofovir alafenamide).

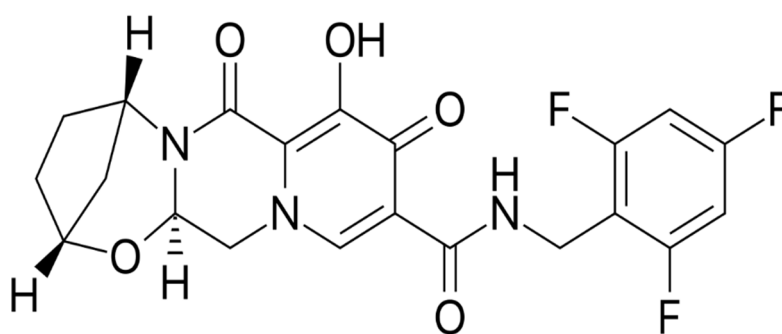
Structure:

Fig. 7.1: Structure of Bictegravir

IUPAC Name	: (1S,11R,13R)-5-hydroxy-3,6-dioxo-N-[(2,4,6-trifluorophenyl)methyl] -12-oxa-2,9-diazatetracyclo[11.2.1.02,11.04,9]hexadeca-4,7-diene-7-carboxamide
Molecular formula	: C ₂₁ H ₁₈ F ₃ N ₃ O ₅
Molecular Weight	: 449.386
Solubility	: Soluble in ACN, Water, and Methanol
p_{ka}	: 9.81

Emtricitabine:

Emtricitabine, a drug approved by FDA for the treatment of HIV-1 and sold under the brand name Emtriva, a nucleoside reverse transcriptase inhibitor (NTRI). It is used both for adults and children with HIV infection.

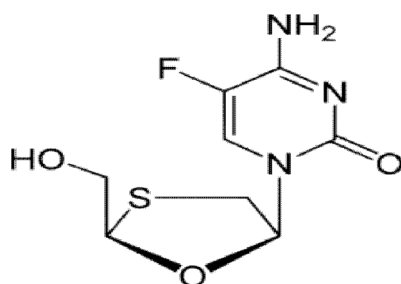
Structure:

Fig. 7.2: Structure of Emtricitabine

IUPAC Name	: 4-amino-5-fluoro-1-[(2R, 5S)-2-(hydroxymethyl)-1, 3-oxathiolan-5-yl]-1, 2-dihydropyrimidin-2-one
Molecular formula	: C ₈ H ₁₀ FN ₃ O ₃ S
Molecular Weight	: 247.248 g/mol
Solubility	: Soluble in ACN, Water, and Methanol
p_{ka}	: 14.29

Tenofovir Alafenamide:

Tenofovir Alafenamide, sold under the brand name Vemlidy, a Nucleoside reverse transcriptase inhibitor (NTRI) and a prodrug of tenofovir. It was approved by FDA in 2016. It is used for the medical treatment of HIV infection along with Hepatitis-B, in the form of Tenofovir disoproxil fumarate (TDF). High antiviral activity and better distribution into lymphoid tissues can be seen by tenofovir alafenamide.

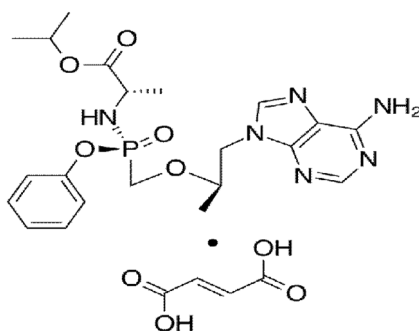
Structure:

Fig. 7.3: Structure of Tenofovir alafenamide fumarate

IUPAC Name	: Isopropyl (2S)-2-[[[(1R)-2-(6-aminopurin-9-yl)-1-methylethoxy] methyl-phenoxy-phosphoryl] amino] propanoate
Molecular formula	: C ₂₁ H ₂₉ N ₆ O ₅ P
Molecular Weight	: 476.466 g/mol
Solubility	: Soluble in methanol, Acetonitrile and water
P_{ka}	: 11.36

MATERIALS AND METHODS

Table 7.1: List of chemicals and reference standards

S. No	Chemical/reagents	Grade make	Make
1	Orthophosphoric Acid (OPA)	HPLC	HiMedia Laboratories Pvt. Ltd
2	Water	HPLC	Merk
3	Acetonitrile	HPLC	Merck
4	Triethylamine	HPLC	Merck
5	Potassium dihydrogen phosphate	AR	Thermo Fisher Scientific India Pvt.
6	Torsemide	Reference standard	Lupin labs Ltd
7	Spironolactone	Reference standard	Lupin labs Ltd

Table 7.2: List of instrument details

S. No	Instruments	Make/model
1	HPLC	Agilent Equipped with a UV-Visible detector
2	Column	Phenomenex Luna ODS (250 x 4.6 mm), 5µm
3	Pump	LC20AT
4	Detector	UV-2489
5	Analytical Balance	Shimadzu
6	pH meter	Range from 0-14 (Labindia 352)
7	Water bath Sonicator	Loba Life

PREPARATION OF SOLUTIONS:**Preparation of 0.1% Ortho phosphoric acid:**

Pipette out 0.1 mL⁻¹ of ortho phosphoric acid in 100 mL⁻¹ of volumetric flask and make up the volume to 100 mL⁻¹ with HPLC water.

PREPARATION OF STANDARD STOCK SOLUTION:**Preparation of bictegravir standard stock solution:**

10 mg of bictegravir was weighed accurately and transferred into 10 mL⁻¹ volumetric flask and dissolved in distilled water and then made up the remaining volume to 10 mL⁻¹ with HPLC water to get the concentration of 1000 µg/ mL⁻¹.

Preparation of emtricitabine standard stock solution:

Weighed accurately 10 mg of emtricitabine and transferred into 10 mL⁻¹ volumetric flask and dissolved in HPLC water and made up the volume with HPLC water to get the concentration of 1000µg/ mL⁻¹.

Preparation of tenofovir alafenamide standard stock solution:

Weighed accurately 10 mg of tenofovir alafenamide and transferred into 10 mL⁻¹ volumetric flask and dissolved in HPLC water and made up with HPLC water to get the concentration of 1000 µg/ mL⁻¹.

PREPARATION OF WORKING STANDARD SOLUTION:**Preparation of bictegravir working standard solution:**

Pipetted out 1 mL⁻¹ of bictegravir standard stock solution into a 10 mL⁻¹ volumetric flask. Diluted up to 10 mL⁻¹ volume with mobile phase and mixed well. From working standard solutions, further dilutions were made up to get concentrations up to 5-160 µg/ mL⁻¹.

Preparation of emtricitabine working standard solution:

Pipetted out 1 mL⁻¹ of emtricitabine standard stock solution into a 10 mL⁻¹ volumetric flask. Diluted up to 10 mL⁻¹ volume with mobile phase and mixed well. From working standard solutions, further dilutions were made up to 5-160 µg/ mL⁻¹.

Preparation of tenofovir alafenamide standard solution:

Pipetted out 1 mL⁻¹ of tenofovir alafenamide standard stock solution into a 10 mL⁻¹ volumetric flask. Diluted up to 10 mL⁻¹ volume with mobile phase and mixed well. From working standard solutions, further dilutions were made up to 5-160 µg/ mL⁻¹.

Preparation of sample solution:

The formulation containing bictegravir 200 mg, emtricitabine 100 mg and tenofovir alafenamide 25mg. Weighed 20 tablets and determined the average weight and crushed to fine powder. Weighed accurately tablet powder equivalent to 31.6 mg of bictegravir, 35 mg of emtricitabine and 1.5 mg of tenofovir alafenamide transferred into 10 mL⁻¹ volumetric flask, and dissolve it in HPLC water and make the final volume to 10 mL⁻¹ with water. From the solution 1 mL⁻¹ was pipetted out into a 10 mL⁻¹ volumetric flask and made up to 10 mL⁻¹ with mobile phase and used for further dilutions.

METHOD DEVELOPMENT AND OPTIMIZATION

Method development involves in the evaluation and optimization of the various stages of sample preparation, chromatographic separation, qualification and quantification. Optimization of various parameters was performed in order to develop a selective and sensitive method for analysis on HPLC using UV detection.

Trial 1:**Chromatographic conditions:**

Mobile phase	: Methanol and orthophosphoric acid taken in the ratio 75:25
Flow rate	: 1.0 mL ⁻¹ /min
Column	: Phenomenex, ODS 250 x 4.6 mm 5μm.
Detector wave length	: 280nm
Column temperature	: 30°C
Injection volume	: 20μg/ mL ⁻¹
Run time	: 20 min
Observation	: The peaks were not resolved

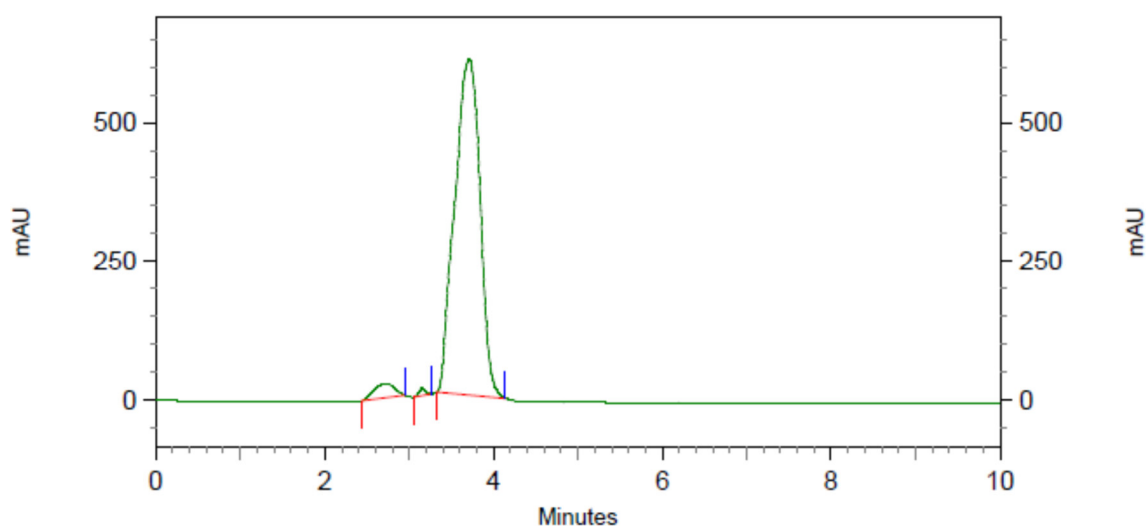
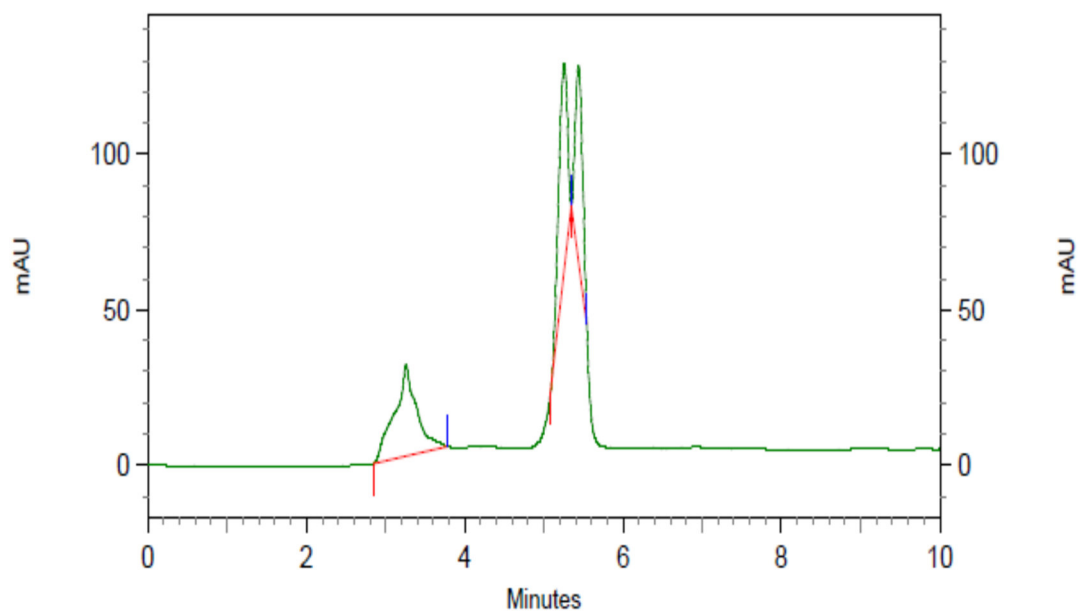


Fig. 7.4: Chromatogram for Trail-I

Trial 2:**Chromatographic conditions :**

Mobile phase	: Acetonitrile and ortho phosphoric acid taken in the ratio 60:40
Flow rate	: 0.8 mL ⁻¹ /min
Column	: Phenomenex, ODS 150 x 4.6 mm 5 μ m.
Detector wave length	: 270 nm
Column temperature	: 30°C
Injection volume	: 20 μ g/ mL ⁻¹
Run time	: 10 min
Observation	: three peaks are resolved with splitting

**Fig. 7.5: Chromatogram for trail-II**

Trial 3:

Chromatographic conditions : Acetonitrile and ortho phosphoric acid taken in the ratio 60:40

Mobile phase : Acetonitrile and ortho phosphoric acid taken in the ratio 60:40

Flow rate : 0.8 mL⁻¹ /min

Column : Phenomenex, ODS 150 x 4.6 mm 5 μ m.

Detector wave length : 270nm

Column temperature : 30°C

Injection volume : 20 μ L⁻¹

Run time : 10 min

Observation : Peaks tailing and fronting were observed for three drugs

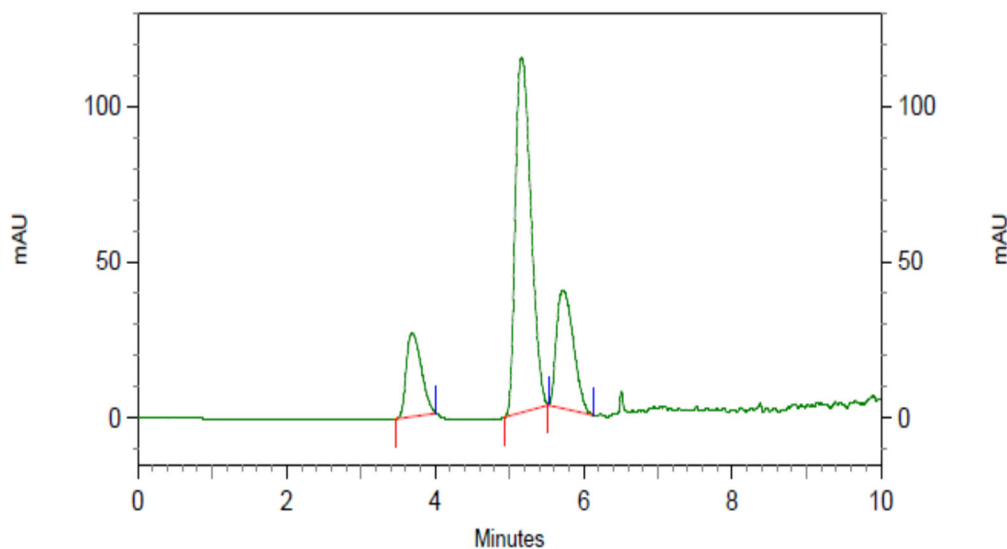


Fig. 7.6: Chromatogram for trail-III

Trial 4:**Chromatographic conditions:** :

Mobile phase : Acetonitrile and ortho phosphoric acid in the ratio of 70:30

Flow rate : 0.8 mL⁻¹ /min

Column : Phenomenex, ODS 150 x 4.6 mm 5 μ m.

Detector wave length : 270 nm

Column temperature : 30°C

Injection volume : 20 μ g/ mL⁻¹

Run time : 10 min

Observation : Three peaks are resolved fronting and tailing are observed for levodopa and carbidopa

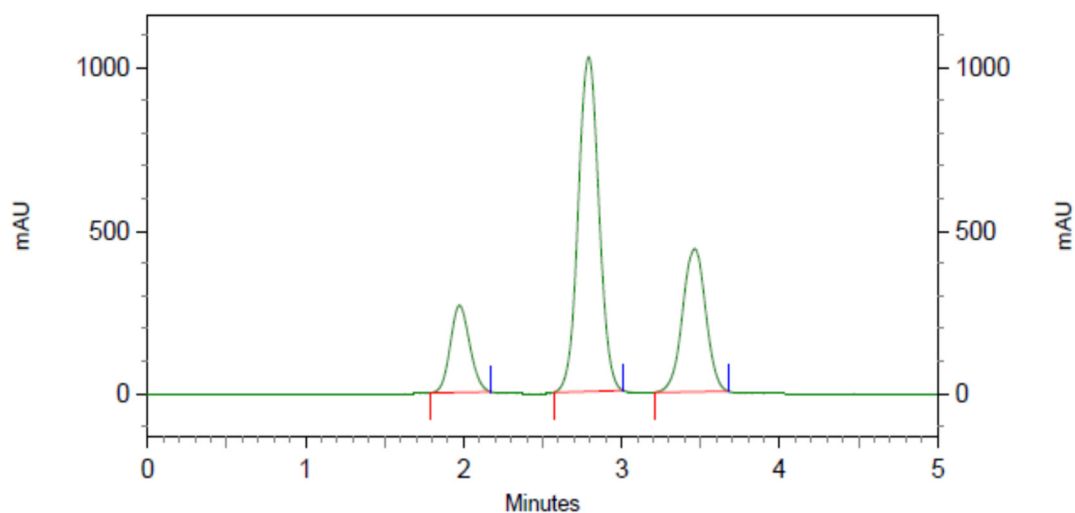


Fig. 7.7: Chromatogram for trail-IV

Choice of stationary phase

Initially the separation was tried with different columns having different dimensions like diameter and length and pore size. Finally good separation with finest peak shape was achieved with the analytical column Inertsil ODS-C₁₈; 5 μ m (4.6 X 250mm).

Chromatographic conditions: :

Mobile phase	: Acetonitrile and 0.1% Ortho phosphoric acid in the ratio of 50:50 (v/v/)
Flow rate	: 1.0 mL ⁻¹ /min
Column	: Phenomenex, ODS 150 x 4.6 mm 5 μ m.
Detector wave length	: 270 nm
Column temperature	: 30°C
Injection volume	: 20 μ L ⁻¹
Run time	: 5 min
Observation	: Peaks tailing and fronting were not observed for three drugs

Selection of mobile phase

Several systematic test plans were performed to optimize the mobile phase. Different solvents like methanol, water and acetonitrile in different ratios and different pH values of the mobile phase ratios, by using different buffer solutions in order to get sharp peak and base line separation of the components and without interference of the excipients. Satisfactory peak symmetry, resolved and free from tailing was obtained in mobile phase acetonitrile: methanol: 0.1% triethylamine buffer (pH-3.0) 25:35:40 (v/v/v).

Selection of the mobile phase flow rate

Flow rates of the mobile phase were changed from 0.5-1.0 ml/min for optimum separation. A minimum flow rate as well as minimum run time gives the maximum saving on the usage of solvents. It was found from the experiments that 1.0 mL/min flow rate was ideal for the successful elution of the analyte.

Optimized Chromatographic onditions: After series of trials, the chromatographic conditions was accomplished with following

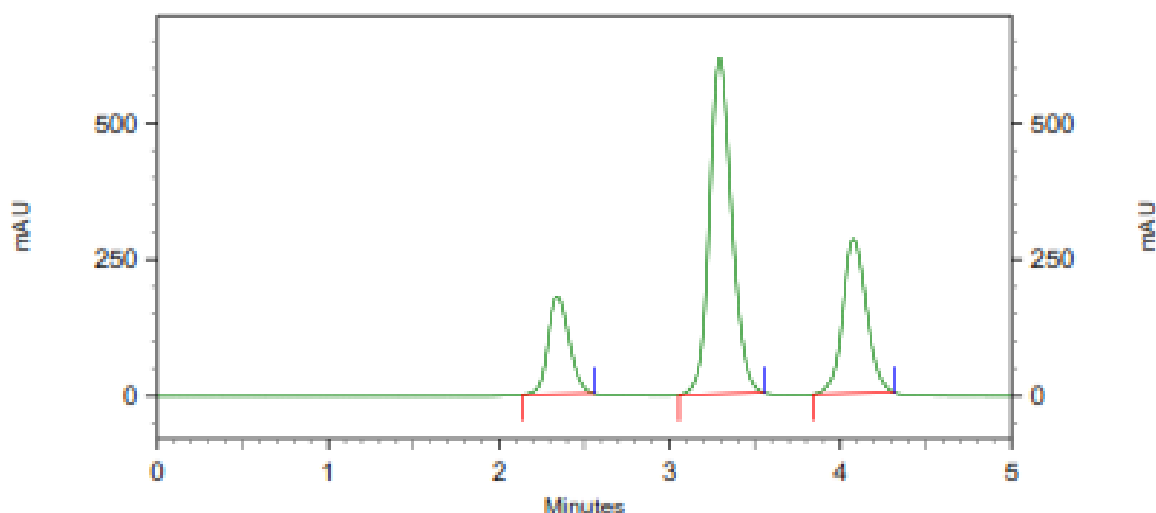


Fig. 7.8: Optimized chromatogram of Bictegravir, Emtricitabine and Tenofovir alafenamide

Discussion: Bictegravir, Emtricitabine and Tenofovir alafenamide were eluted at 2.56 min, 3.57 min and 3.503 min respectively with good resolution. Plate count and tailing factor was very satisfactory, so this method was optimized and to be validated.

METHOD VALIDATION

After the development of RP-HPLC method for the estimation of drug in a dosage form, validation of the method was performed. This section describes the procedure followed for validation of the developed method.

Specificity

Interference was not observed with the standard peaks and the chromatograms of Standard and Sample were identical with same retention time.

Acceptance Criteria:

No Interference should be observed at the retention time of standard peaks in the blank.

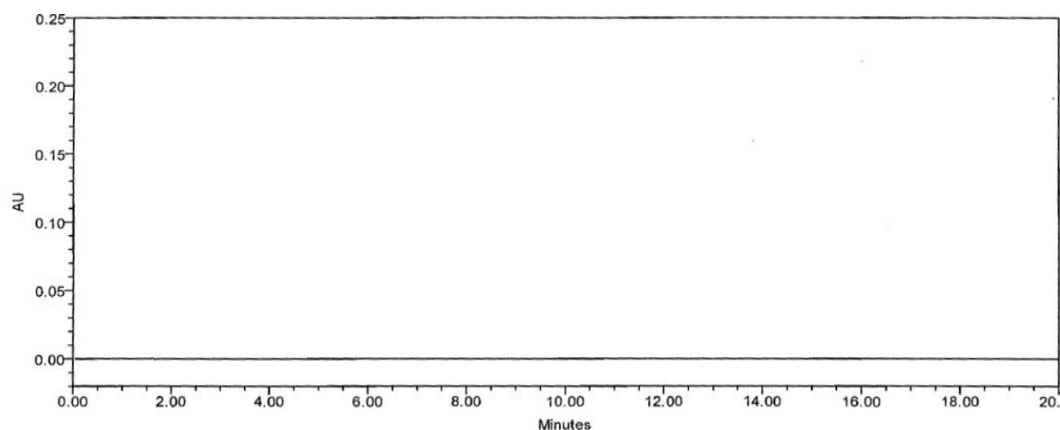


Fig. 7.9: Chromatogram of blank

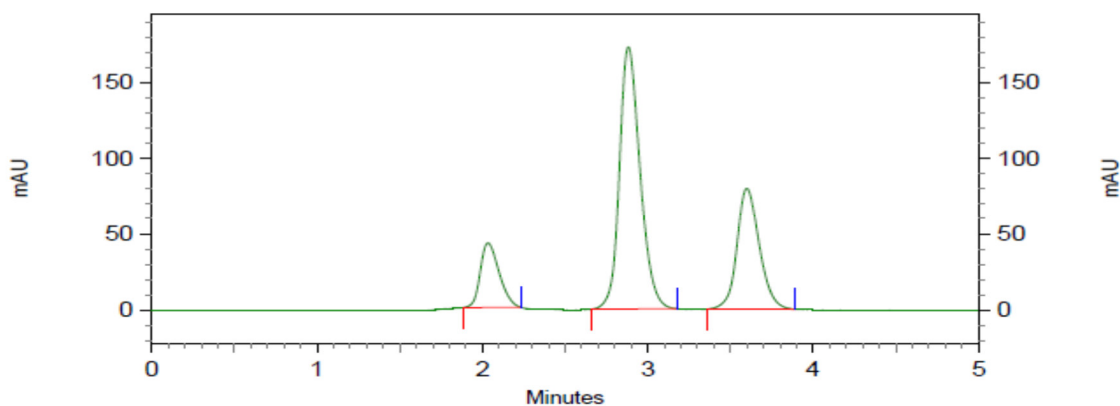
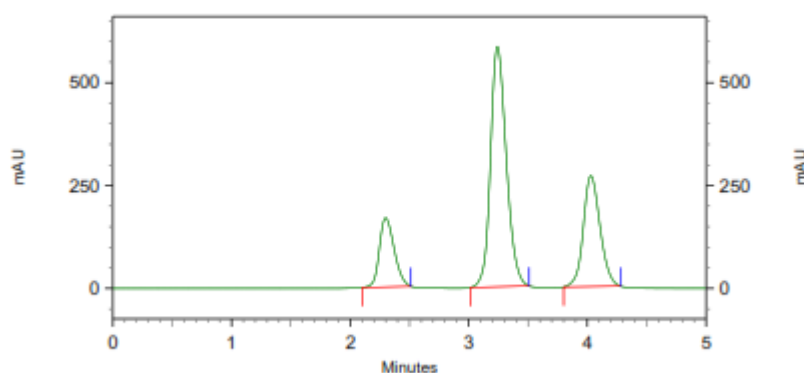


Fig. 7.10: Chromatogram of Standard

System Suitability

System suitability of %Relative standard deviation of individual area response of six replicate injections for bictegavir, emtricitabine and tenofovir alafenamide was found to be 0.42 and 0.84 respectively. The %Relative standard deviation of areas of six replicate injections for bictegavir, emtricitabine and tenofovir alafenamide were found to be within limits. The tailing factor for bictegavir, emtricitabine and tenofovir alafenamide peaks was found to be 1.19 and 0.97 respectively. The tailing factor for bictegavir, emtricitabine and tenofovir alafenamide peaks was found to be within limits. The number of theoretical plates for bictegavir, emtricitabine and tenofovir alafenamide were found to be 2227 and 3036 respectively. The resolution was found to be 4.6 respectively which are well within the limits.



**Fig. 7.11: Standard chromatogram of bictegavir, emtricitabine and tenofovir
alafenamide**

Table 7.3: System suitability Data

S. No	Injection Number	Peak area for Bictegravir	Peak area for Emtricitabine	Peak area for Tenofovir alafenamide	Acceptance criteria	
1	01	24712691	91156709	45245759	The % RSD of peak areas of Bictegravir and Emtricitabine and Tenofovir alafenamide should not be more than 2.0	
2	02	24463324	91701339	44503119		
3	03	24246704	91987990	45054196		
4	04	24549943	90158178	44649392		
5	05	24711992	91329540	45123345		
6	06	24230160	91947369	45049506		
Mean		24485802.33	91380187.5	44937552.83		
%RSD		0.875502296	0.748647825	0.650627105		
System suitability parameters				Observed value		
			Bictegravir	Emtricitabine	Tenofovir alafenamide	Acceptance criteria
The Tailing for bictegravir, emtricitabine and tenofovir alafenamide in standard solution			1.2	1.24	0.94	NMT 2.0
Theoretical plates for bictegravir, emtricitabine and tenofovir alafenamide in standard solution			2125	2338	2832	NLT 2000
Retention time (min)			2.56	3.57	4.42	NA

Precision

The precision of the Relative standard deviation of individual area of bictegrovir, emtricitabine and tenofovir alafenamide were found to be within limits.

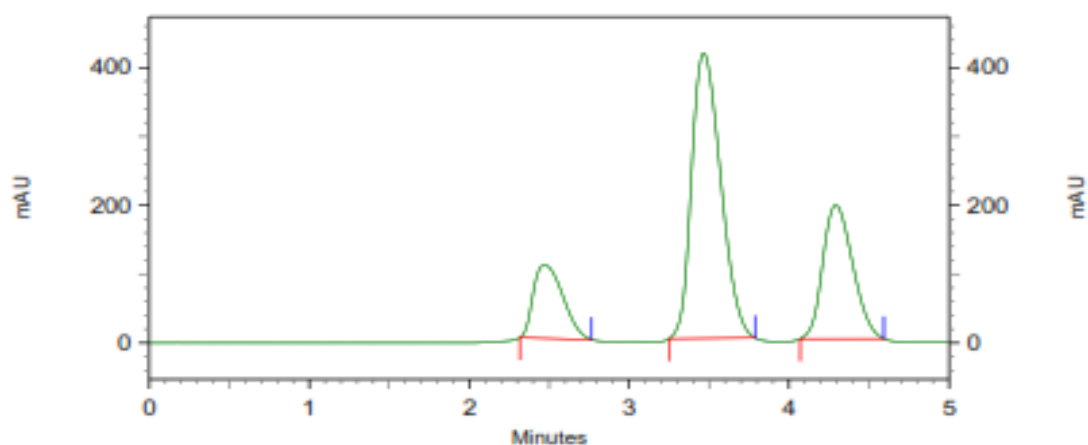


Fig. 7.12: Chromatogram of 20µg/ml

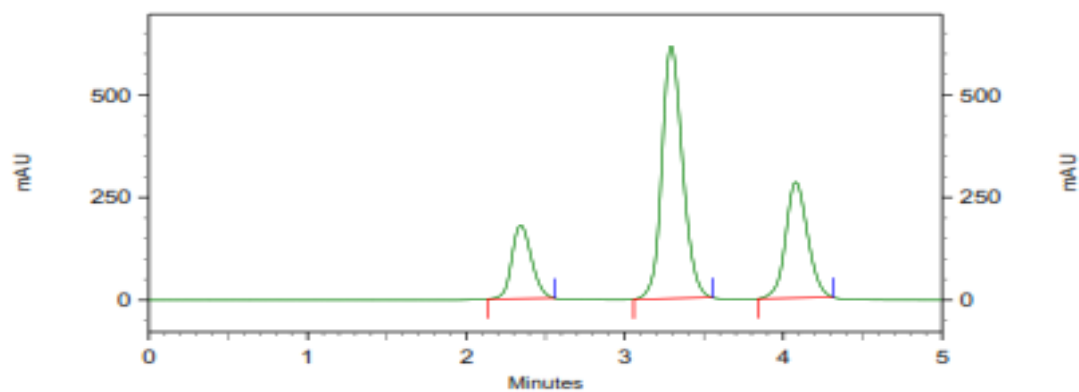


Fig. 7.13: Chromatogram of 40µg/ml

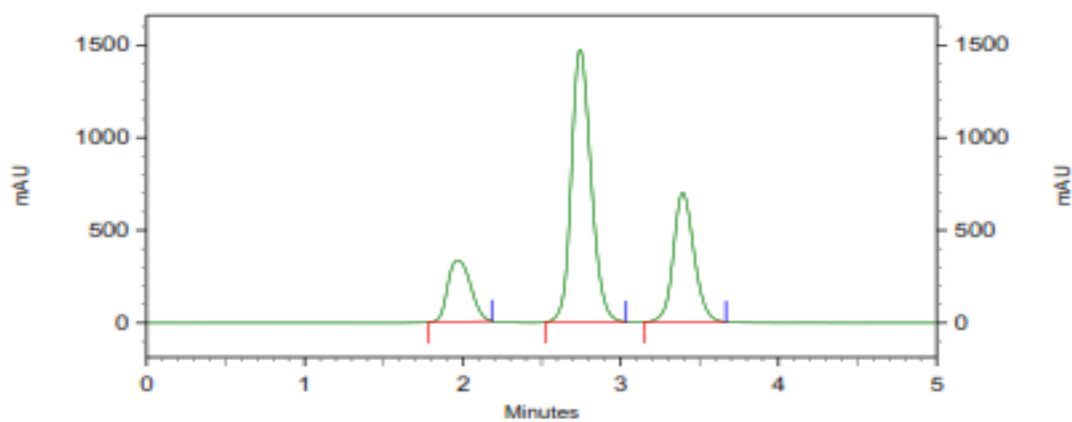


Fig. 7.14: Chromatogram of 80µg/ml

Intra-day Precision

Table 7.4: Intra-day Precision for bictegravir, emtricitabine and tenofovir
alafenamide

S. No.	Injection Number	Peak area for Bictegravir	Peak area for Emtricitabine	Peak area for Tenofovir alafenamide
1	Standard 1	12721084	45439179	21306671
2	Standard 2	12489075	46463093	22042369
3	Standard 3	12556257	46757807	21895016
4	Standard 4	12412413	46606277	22066512
5	Standard 5	12617309	47179161	21478451
6	Standard 6	12899981	46894191	21620082
Mean		12616019.83	46556618	21734850.2
%RSD		1.385726869	1.28942453	1.44393721

Inter-day Precision

Table 7.5: Inter-day Precision for bictegavir, emtricitabine and tenofovir
alafenamide

S. No.	Injection Number	Peak area for Bictegavir	Peak area for Emtricitabine	Peak area for Tenofovir alafenamide
1	Standard 1	12412413	46606277	22066512
2	Standard 2	11917309	45179161	21478451
3	Standard 3	11998917	46094191	21620082
4	Standard 4	12099845	46285956	21487812
5	Standard 5	12189456	45923107	21885217
6	Standard 6	12090459	45964490	21927085
Mean		12118067	46008863.67	21744193.17
%RSD		1.416538	1.037234331	1.143239276

Acceptance Criteria:

The Relative standard deviation of individual area of bictegavir, emtricitabine and tenofovir alafenamide from six standard preparations should be not more than 2.0%.

Ruggedness: The method was performed. It was found to be rugged and % of RSD (less than 2) indicating ruggedness of the method.

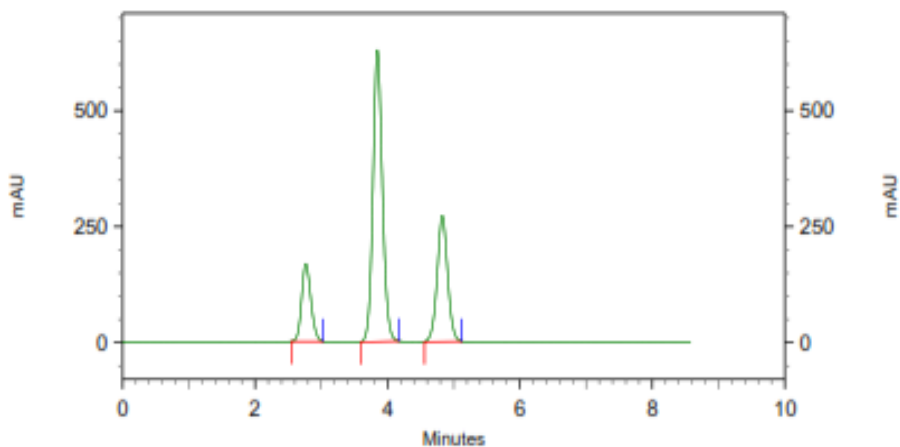


Fig. 7.15: Chromatogram of 40µg/ml

Table 7.6: Report of Ruggedness

S. No.	Concentration ($\mu\text{g}/\text{mL}^{-1}$) of Bictegravir, Emtricitabine and Tenofovir alafenamide	Peak area of Bictegravir	Peak area of Emtricitabine	Peak area of Tenofovir alafenamide
1	40	19683049	78152534	37799115
2		19243134	79688983	38748417
3		18954123	78687933	37806560
4		19589796	76992656	37799664
5		19430985	78813195	38020838
6		19794762	77793090	37273195
		Avg: 19449308	Avg: 78354732	Avg: 37907965
		Std Dev: 310406.78	Std Dev: 928764.10	Std Dev: 480810.4
		%RSD: 1.60	%RSD: 1.19	%RSD: 1.275

Linearity: For linearity, Six linear concentrations of bictegravir, emtricitabine and tenofovir alafenamide (5-160 $\mu\text{g}/\text{ml}$) were injected in a triplicate manner. Average areas were mentioned above and linearity equations obtained for bictegravir, was $y = 641469x$, emtricitabine was $y = 2E+06x$ and Tenofovir alafenamide was $y = 1E+06x$. Correlation coefficient obtained for tenofovir alafenamide 0.9992, for Emtricitabine was 0.9998 and for Tenofovir alafenamide 0.9983.

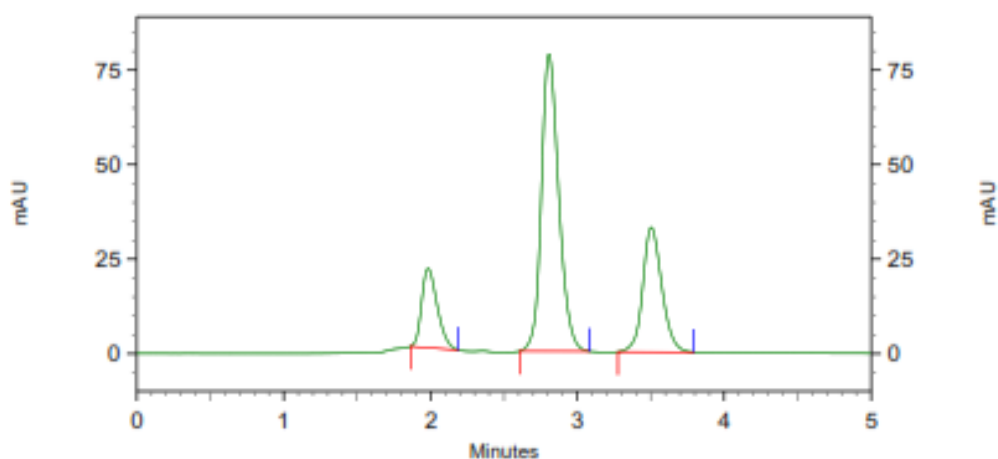


Fig. 7.16: Chromatogram of 5 µg/ml

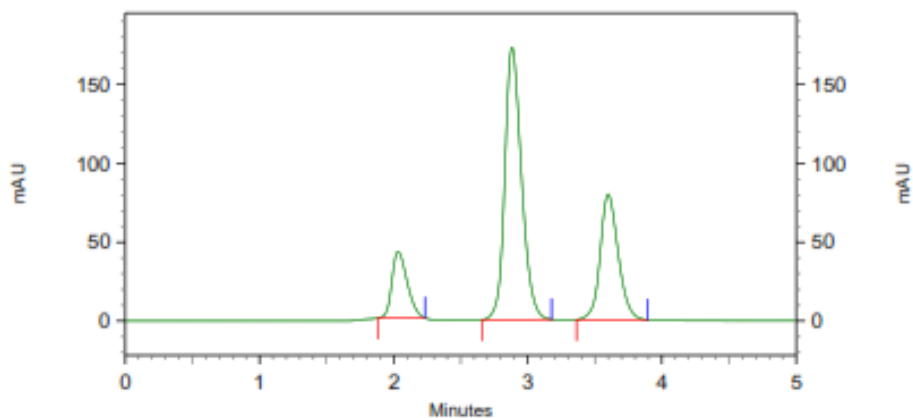


Fig. 7.17: Chromatogram of 10 µg/ml

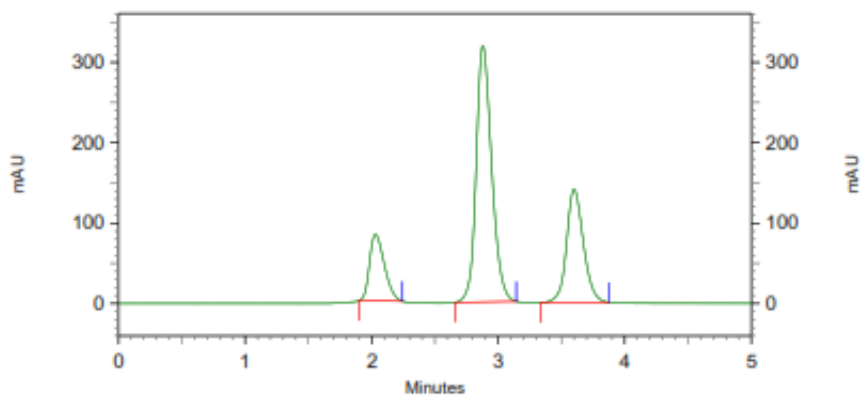


Fig. 7.18: Chromatogram of 20 µg/ml

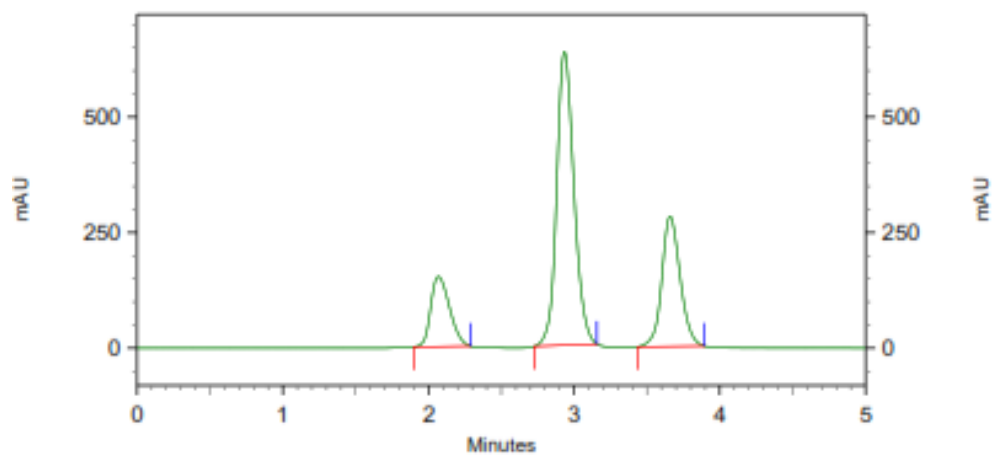
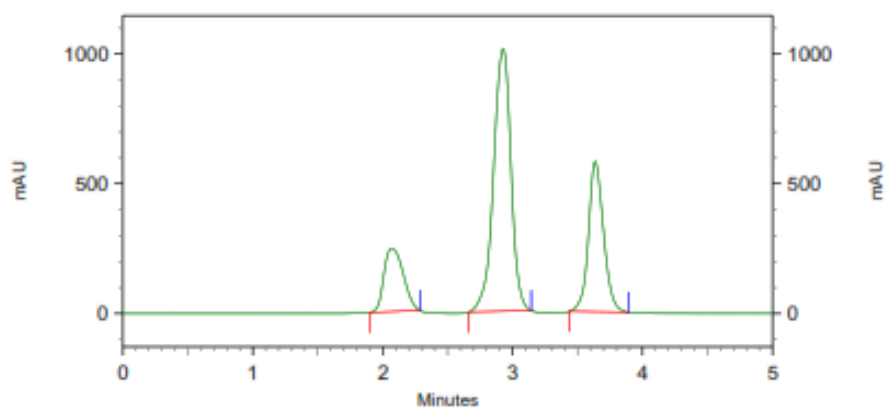
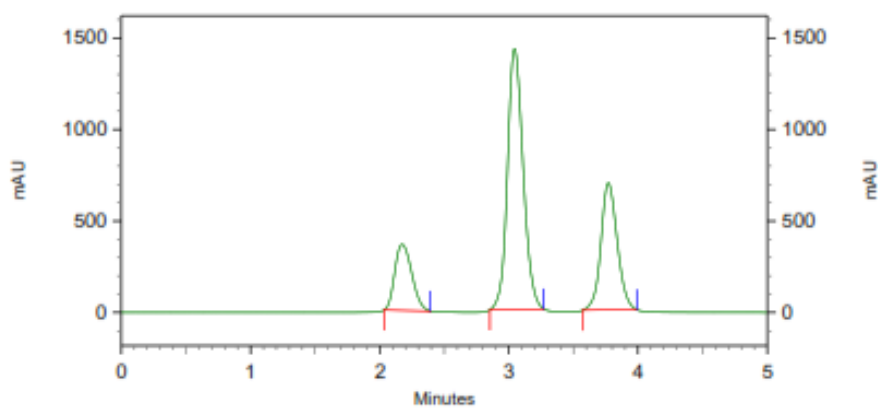
**Fig. 7.19: Chromatogram of 40 µg/ml****Fig. 7.20: Chromatogram of 80 µg/ml****Fig. 7.21: Chromatogram of 160 µg/ml**

Table 7.7: Linearity data

Standard concentration (µg/ml)	Area of Bictegavir	Standard concentration (µg/ml)	Area of Emtricitabine	Standard concentration (µg/ml)	Area of Tenofovir alafenamide
5	2603223	5	10853436	5	5002571
10	5807380	10	25098862	10	12524809
20	12333608	20	47564490	20	21927085
40	25769809	40	87897778	40	41351588
80	49232355	80	178592405	80	95516553
160	103768711	160	359415503	160	192814527
Regression $R^2 = 0.9992$		Regression $R^2 = 0.9998$		Regression $R^2 = 0.9983$	

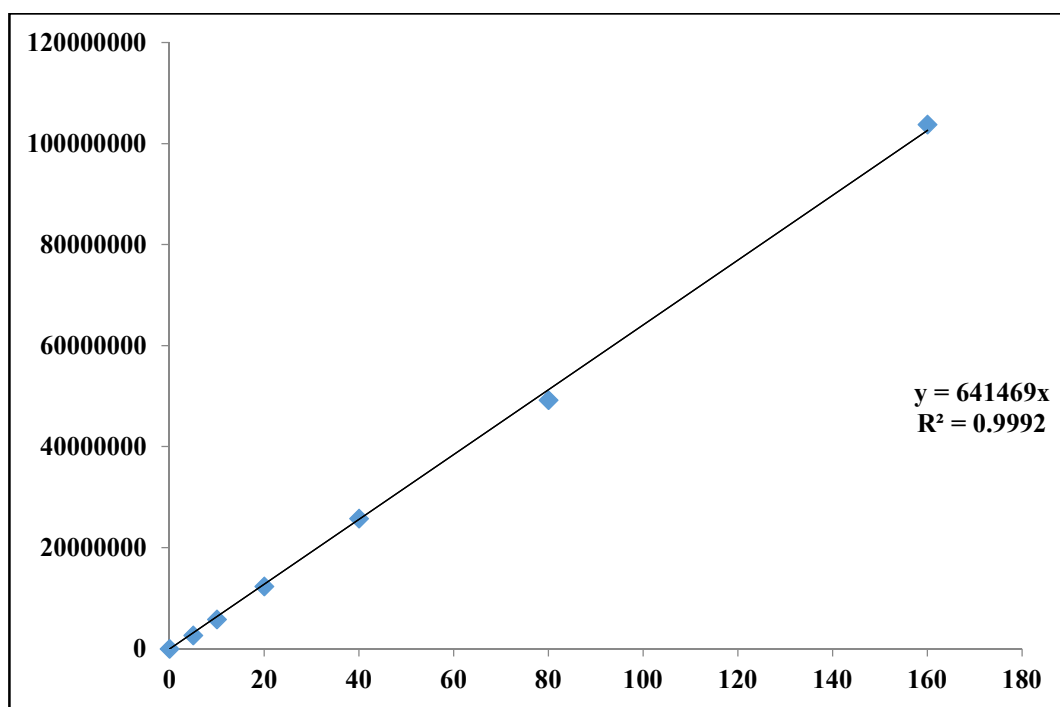


Fig. 7.22: Calibration curve for Bictegavir

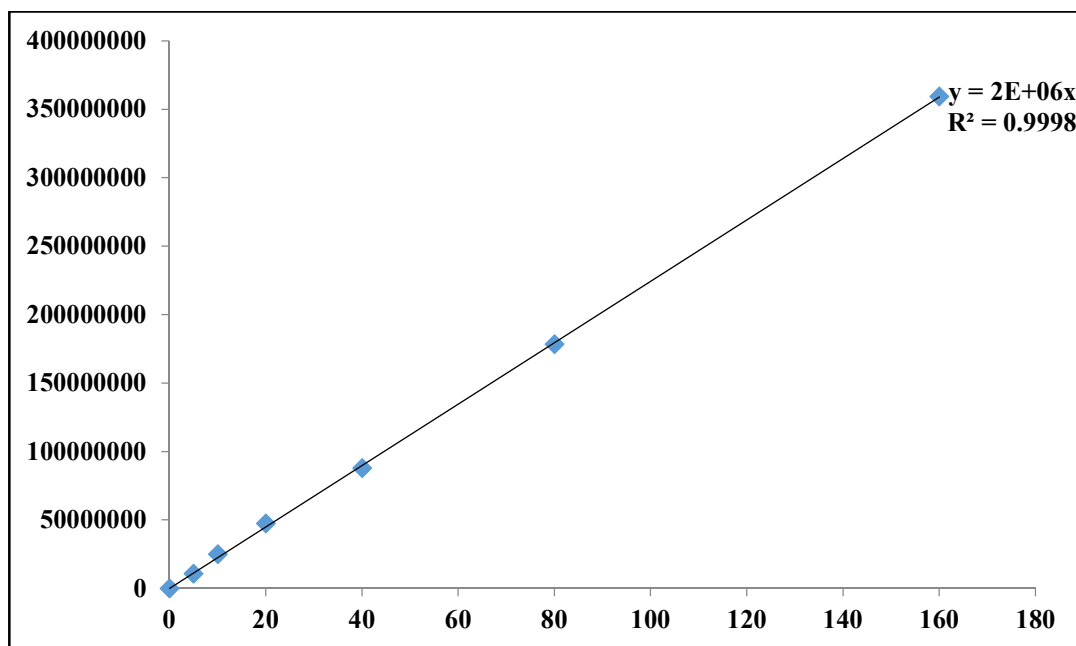


Fig. 7.23: Calibration curve for Emtricitabine

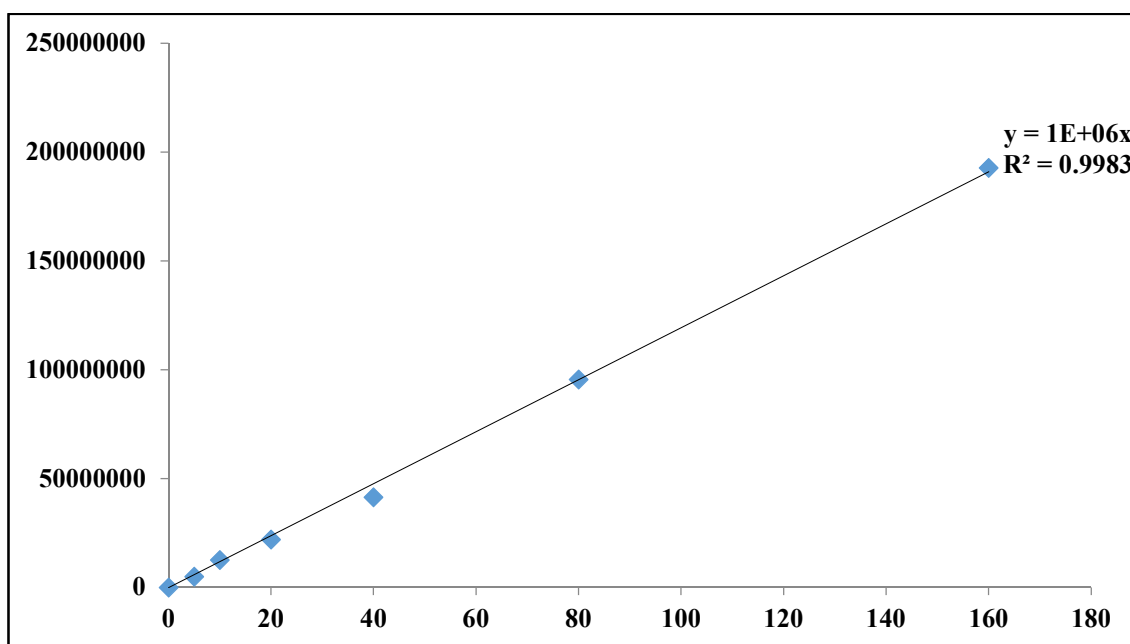


Fig. 7.24: Calibration curve for Tenofovir alafenamide

Acceptance Criteria: The Correlation coefficient should not be less than 0.997.

LOD and LOQ

LOD and LOQ of bictegravir, emtricitabine and tenofovir alafenamide were found be 0.89, 1.32, 1.03 and 2.72, 4.00, 3.13, respectively.

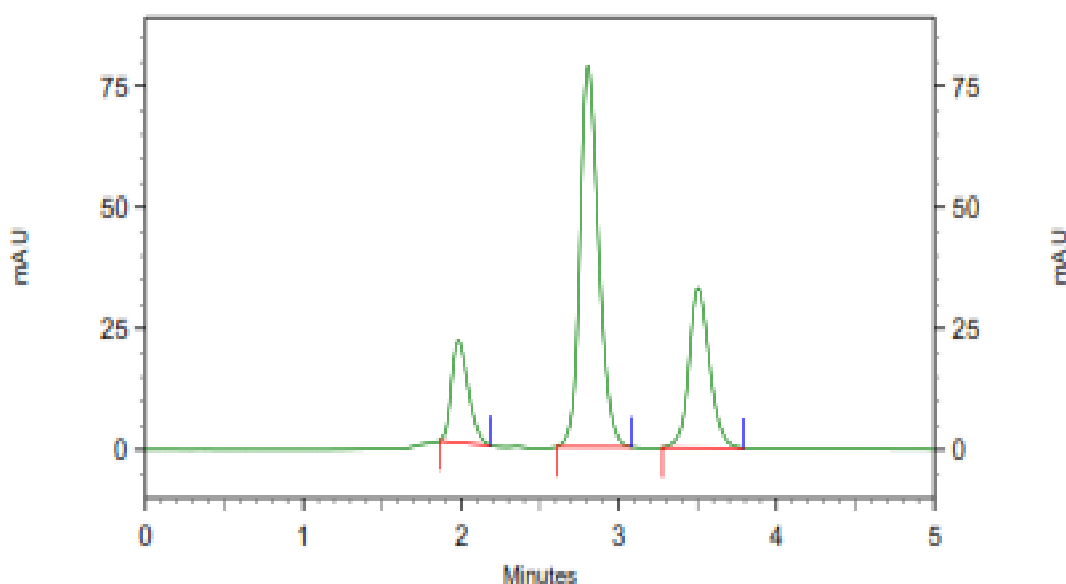


Fig. 7.25: Chromatogram of LOQ

Table 7.8: Report of LOD and LOQ

S. No.	Drugs	LOD ($\mu\text{g/ml}$)	LOQ ($\mu\text{g/ml}$)
1	Bictegravir	0.89	2.72
2	Emtricitabine	1.32	4.00
3	Tenofovir alafenamide	1.03	3.13

Accuracy:

The mean % Recovery of were found be 0.89, 1.32, 1.03 and 2.72, 4.00, 3.13, respectively, were found to be within limits at each level.

The % RSD of recovery of were found be 0.89, 1.32, 1.03 and 2.72, 4.00, 3.13, respectively, from the three sample preparations was found to be 0.40 and 1.22 at 50% level and 0.39 and 0.54 at 150% level respectively.

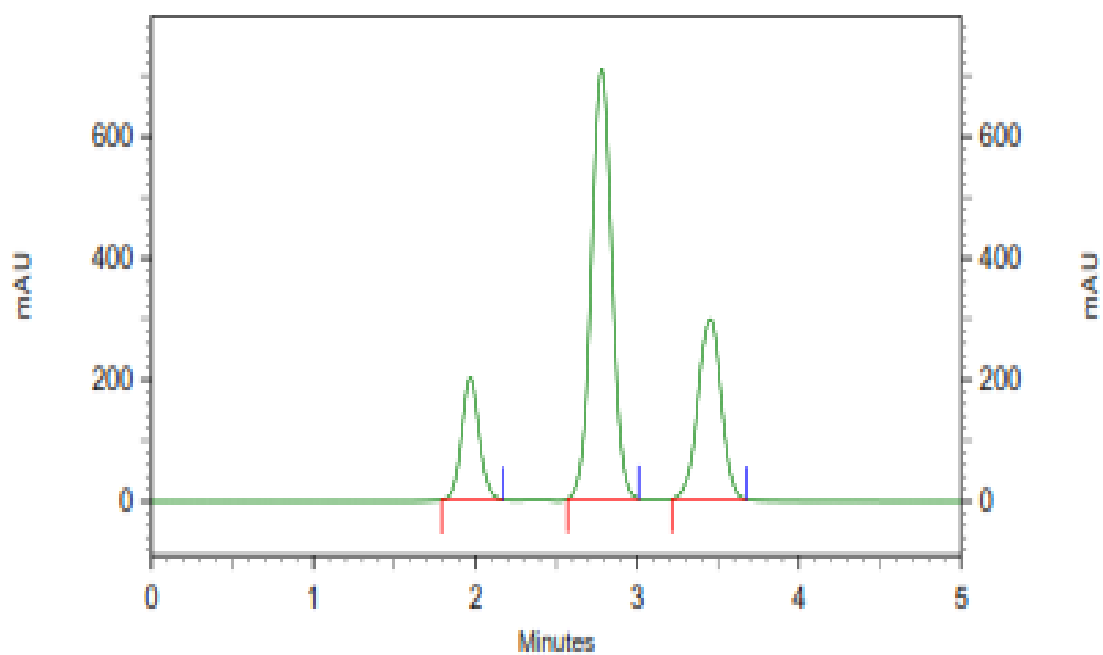


Fig. 7.26: Chromatogram of 60µg/ml

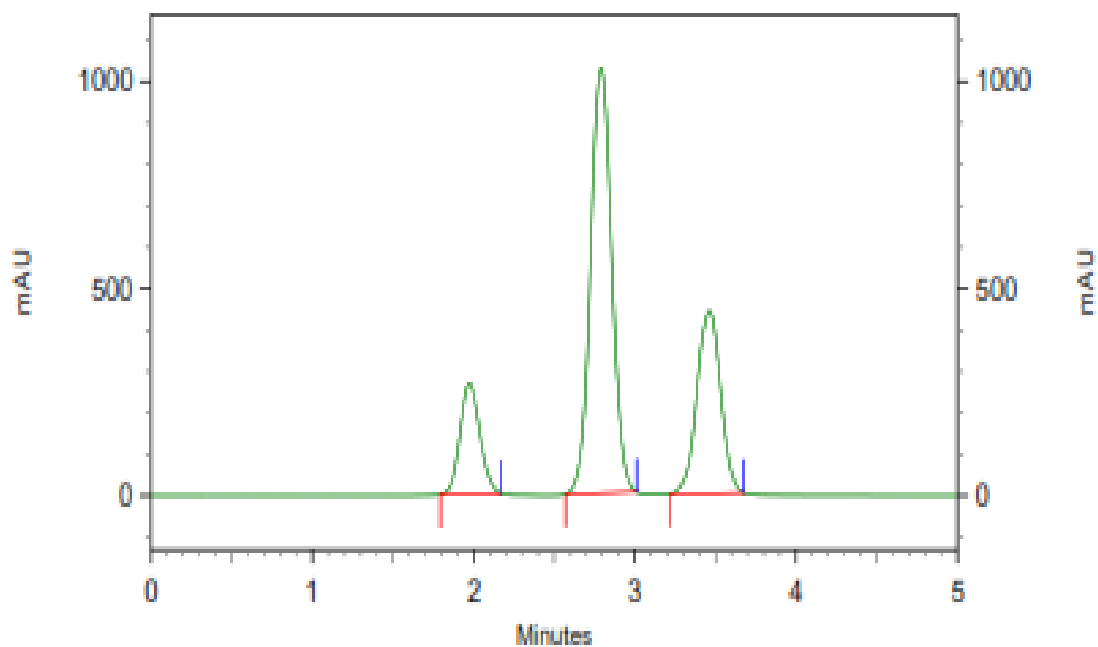


Fig. 7.27: Chromatogram of 80µg/ml

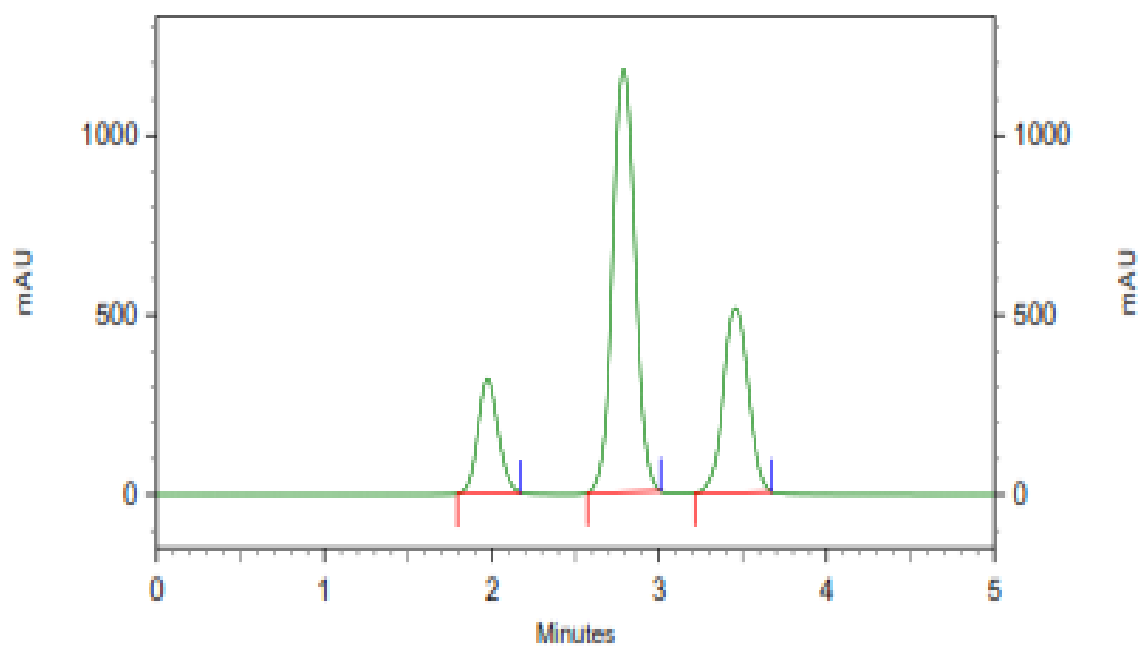


Fig. 7.28: Chromatogram of 100 μ g/ml

Table 7.9: Accuracy data

% Recovery	Target conc (µg/mL ⁻¹)	Spiked conc (µg/mL ⁻¹)	Final conc (µg/mL ⁻¹)	Conc obtained in Bictegravir	% of Assay in Bictegravir	Conc obtained in Emtricitabine	% of Assay in Emtricitabine	Con obtained in Tenofovir alafenamide	% of assay in Tenofovir alafenamide
50	40	20	60	59.985	99.97	59.545	99.24	59.994	99.99
	40	20	60	59.825	99.71	59.549	99.25	59.966	99.94
	40	20	60	60.556	100.93	60.008	100.01	60.097	100.16
100	40	40	80	80.262	100.33	80.000	100.00	80.010	100.01
	40	40	80	80.058	100.07	80.459	100.57	79.308	99.13
	40	40	80	80.106	100.13	80.918	101.15	79.334	99.17
150	40	60	100	99.236	99.24	100.000	100.00	100.000	100.00
	40	60	100	99.379	99.38	104.052	104.05	100.107	100.11
	40	60	100	99.334	99.33	99.465	99.47	100.436	100.44

Acceptance Criteria:

The mean % Recovery of were found be 0.89, 1.32, 1.03 and 2.72, 4.00, 3.13, respectively, at each level should be not less than 95.0% and not more than 105.0%.

The %RSD of recovery of were found be 0.89, 1.32, 1.03 and 2.72, 4.00, 3.13, respectively, from the three sample preparations at 50% and 150% levels should not be more than 5.0%.

Robustness:

From the obtained values %RSD was found to be within the range of 0.6%-1.2% which states the method is acceptable.

Table 7.10: Report of Robustness – Bictegravir

S. No.	Parameter	Condition	System suitability results		
			% RSD	USP tailing	USP Plate Count
1	Flow rate by \pm 10%	1.2 ml	0.94	0.99	2878
		1.0 ml	1.05	0.83	2695
		1.4 ml	1.10	1.01	2308
2	Column Oven temperature by \pm 5°C	20°C	1.00	1.02	2603
		25°C	0.95	1.11	3256
		30°C	0.82	1.23	3968
3	Wavelength of analysis \pm 5nm	275 nm	0.59	1.10	2965
		270 nm	0.66	1.14	2664
		265 nm	0.80	1.01	2723
4	Organic composition of mobile phase by \pm 5%	55:55	0.65	1.23	2527
		50:50	0.78	1.14	2692
		45:55	0.85	1.12	3052

Table 7.11: Report of Robustness – Levodopoa

S. No.	Parameter	Condition	System suitability results		
			% RSD	USP tailing	USP Plate Count
1	Flow rate by \pm 10%	1.2 ml	1.05	1.21	3638
		1.0 ml	1.11	1.23	3410
		1.4 ml	1.20	1.50	2308
2	Column Oven temperature by \pm 5°C	20°C	0.96	1.24	2603
		25°C	0.85	1.22	2850
		30°C	0.86	1.04	2652
3	Wavelength of analysis \pm 5nm	275 nm	0.99	0.91	2921
		270 nm	0.81	0.96	3652
		265 nm	0.79	0.86	2121
4	Organic composition of mobile phase by \pm 5%	55:55	0.69	0.83	2542
		50:50	0.58	0.86	2721
		45:55	0.72	0.79	2533

Table 7.12: Report of of Robustness - Carbidoipoa

S. No.	Parameter	Condition	System suitability results		
			% RSD	USP tailing	USP Plate Count
1	Flow rate by $\pm 10\%$	1.2 ml	1.18	0.55	2531
		1.0 ml	1.05	0.68	2456
		1.4 ml	1.15	0.70	3210
2	Column Oven temperature by $\pm 5^{\circ}\text{C}$	20°C	1.22	1.32	2900
		25°C	1.14	1.21	2533
		30°C	1.17	1.17	2411
3	Wavelength of analysis $\pm 5\text{nm}$	275 nm	0.56	0.86	2865
		270 nm	0.72	0.84	2456
		265 nm	0.65	0.79	2741
4	Organic composition of mobile phase by $\pm 5\%$	55:55	0.79	0.76	2648
		50:50	0.73	0.68	2315
		45:55	0.75	0.82	2145

Acceptance criteria:

% RSD should not be more than 2%. Theoretical plates should not less than 2000. Tailing factor should not more than 2.0.

ASSAY:

The commercial marketed formulation containing 200 mg of Bictegravir, and 100 mg Emtricitabine and 25 mg of Tenofovir alafenamide. The sample solution was treated same as standard solution. The solutions were injected into HPLC.

Six replicates of the samples solutions were injected for quantitative analysis. The amounts of bictegravir, emtricitabine and tenofovir alafenamide estimated were found to 99.5% and

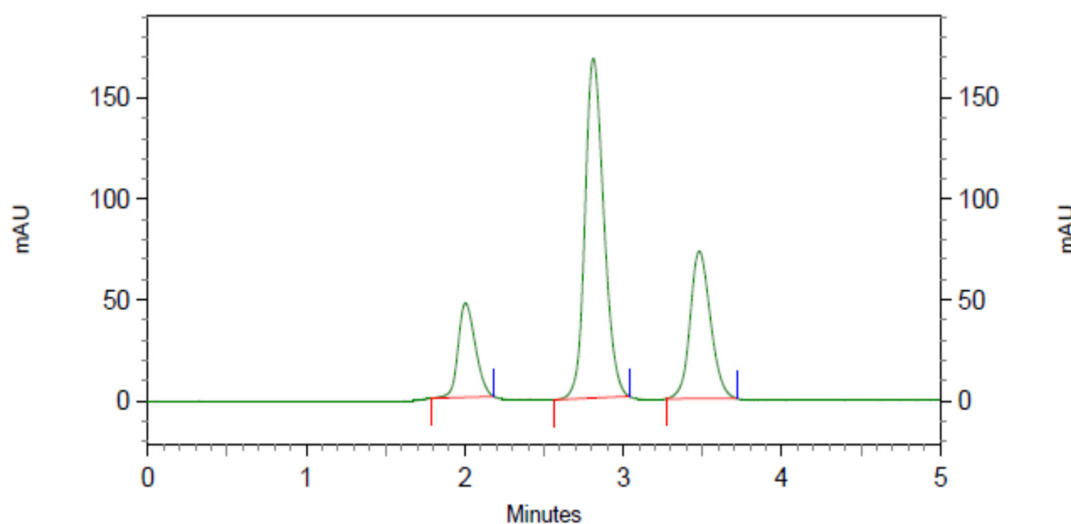
99.98% and 99.68% respectively. A good separation and resolution of all the drugs indicates that there were no interference from the excipients commonly present in pharmaceutical formulations. This showed that the estimation of dosage form was accurate within given acceptable level of 95% to 105%.

Amount found in tablet =

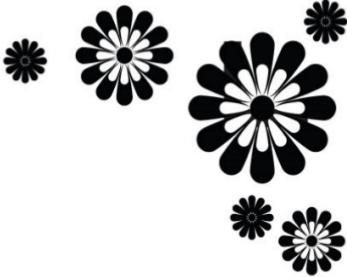
$$\frac{\text{Concentration found from graph} \times \text{Dilution factor}}{\text{Weight of tablet powder} \times 1000} \times \text{Average weight of tablet}$$

Table 7.13: Assay results

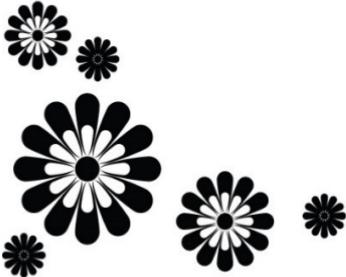
Tablet Sample	Label Claim (mg)	Amount Present	Assay%
Bictegravir	200	199.08	99.54%
Emtricitabine	100	99.98	99.98%
Tenofovir alafenamide	25	24.62	99.68%



**Fig. 7.29: Sample (Test) Chromatogram of Bictegravir, Emtricitabine and
Tenofovir alafenamide**



CHAPTER - 8
SUMMARY AND
CONCLUSION



SUMMARY

It was concluded that there were few methods reported for the simultaneous estimation of the selected multi component dosage forms, which promote to pursue the present work. The present work aimed to assess the applicability of High performance liquid chromatography coupled with UV detector (RP-HPLC with UV) for analysis of different classes of drugs in pharmaceutical formulations. The dissertation described the research work was composed of **7 chapters**.

In **Chapter 1** a general introduction and research on analysis of drugs using chromatographic techniques.

In **Chapter 2** discussed about the objectives and plan of the research work for the selected drugs, namely simultaneous estimation of sofosbuvir and daclatasvir, ombitasvir, paritaprevir, ritonavir and abacavir, dolutegravir, lamivudine and bictegravir, emtricitabine, tenofovir alafenamide in APIs by using RP-HPLC-UV detection to explore the applicability of HPLC.

In **Chapter 3** discussed are about the review of literature related to research work.

In **Chapter 4** discussed about the validated method for simultaneous estimation of A simple, rapid reversed-phase high performance liquid chromatographic method had been developed and validated for estimation of daclatasvir and sofosbuvir in tablet dosage form. The estimation was carried out on Inertsil ODS-C₁₈ column (250 x 4.6 mm, 5 μ) column with a mixture of acetonitrile: methanol: 0.1% triethylamine buffer (pH-3.0) 25:35:40 (v/v/v) as mobile phase. UV detection was performed at 250 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 2.09 and 3.50 min for daclatasvir and sofosbuvir respectively and total run time was 6.0min at a flow rate of 1.0 mL/ min. The calibration curve was linear over the concentration range of 5.0-25.0 µg/ mL for Daclatasvir and 2.0-10.0 µg/ mL for Sofosbuvir. The LOD and LOQ values were found to be 0.313 and 0.948 µg/ mL for daclatasvir and 0.021 and 0.065 µg/mL for sofosbuvir, respectively. The high percentage of recovery and low percentage coefficient of variance confirm the suitability of the method for the simultaneous estimation of daclatasvir and Sofosbuvir in tablet dosage form.

In Chapter 5 discussed about the validated method for simultaneous estimation of The purpose of this work is to develop an accurate and precise HPLC method for the determination of ombitasvir, paritaprevir, ritonavir in tablet. Separation of drug was achieved on an Inertsil ODS-C18; 5µm (4.6 X 250mm) column using a mobile phase consisting of 0.02M phosphate buffer (pH-4.5): acetonitrile: methanol, (50:30:20) (v/v). The retention time was 2.81 and 7.42 min for ombitasvir, ritonavir, paritaprevir, RP-HPLC, ICH guide lines respectively and total run time was 10 min. at a flow rate of 1.0 mL/ min and the detection wavelength was 262 nm. The linearity was observed in the range of 15-45 ppm for ombitasvir, paritaprevir, ritonavir with a correlation coefficient of 0.9903, 0.9996 and 0.9998 respectively. The proposed method was validated for its linearity, accuracy, precision and robustness. This method can be employed for routine quality control analysis of ombitasvir, paritaprevir and ritonavir in tablet. The LOD and LOQ values were found to be 1.8, 0.29 and 0.69 µg/ mL and 5.7, 0.90 and 2.10 µg/ mL for ombitasvir, paritaprevir and ritonavir, respectively. The high percentage of recovery and low percentage coefficient of variance confirm the suitability of the method for the simultaneous estimation of ombitasvir, paritaprevir and ritonavir by RP-HPLC as per ICH guidelines in tablet dosage form.

In Chapter 6 discussed about the validated method for simultaneous estimation of Selective and novel method has been optimized for evaluation of abacavir, dolutegravir and lamivudine in bulk and formulation by HPLC. The principle analytes were eluted with the conditions of mobile phase having the ethanol: ethyl acetate (80:20, % v/v) using the Lichrosphere RP C8 column (Phenomenex, USA (250 x 4.6 mm, 5 μ) analytical column with the 1.0 ml/min flow rate and 10 μ l sample volume at 260 nm in UV detector. The retention times of abacavir, dolutegravir and lamivudine were 2.31 min, 3.120 min and 4.59 min with the total run time of 6 min. The curve indicates correlation coefficient (r^2) was superior by having the value nearer to 1.000 with linear range of 40 μ .g/m.l-130.0 μ .g/m.l for abacavir, dolutegravir and lamivudine. The correlation coefficient (r^2) 0.9971 for abacavir, 0.9979 for dolutegravir and 0.9947 for lamivudine were found. The LOD and LOQ for the abacavir, dolutegravir and lamivudine were found 1.40 μ .g/m.l, 3.01 μ .g/m.l, 5.84 μ .g/m.l and 4.25 μ .g/m.l, 9.12 μ .g/m.l and 17.71 μ .g/m.l. The developed method was applied for the bulk and formulation.

In Chapter 7 discussed about the validated method for simultaneous estimation of Selective and novel method has been optimized for evaluation of Bictegravir, emtricitabine and tenofovir alafenamide in bulk and formulation by HPLC. The principle analytes were eluted with the conditions of mobile phase having the acetonitrile and 0.1% Ortho phosphoric acid in the ratio of 50:50 (v/v/) using the Phenomenex, ODS 150 x 4.6 mm 5 μ m analytical column with the 1.0 ml/min flow rate and 20 μ l sample volume at 270 nm in UV detector. The retention times of bictegravir, emtricitabine and tenofovir alafenamide were 2.56 min, 3.57 min and 3.503 min with the total run time of 5 min. The curve indicates correlation coefficient (r^2) was superior by having the value nearer to 1.000 with linear range of 40 μ .g/m.l-130.0 μ .g/m.l for bictegravir, emtricitabine and tenofovir alafenamide. The correlation coefficient (r^2) 0.9992 for bictegravir, 0.9998 for emtricitabine and 0.9983

for tenofovir alafenamide were found. The LOD and LOQ for the bictegravir, emtricitabine and tenofovir alafenamide were found 0.89 $\mu\text{g}/\text{m.l}$, 1.32 $\mu\text{g}/\text{m.l}$, 1.03 $\mu\text{g}/\text{m.l}$ and 2.72 $\mu\text{g}/\text{m.l}$, 4.00 $\mu\text{g}/\text{m.l}$ and 3.13 $\mu\text{g}/\text{m.l}$. The developed method was applied for the bulk and formulation.

Hence, the developed chromatographic methods free from matrix interference and useful in the analysis of drugs in pharmaceutical formulation.

Precision of the developed methods was studied under intra and inter day precision. The % RSD values for precision was found to be within the acceptable limit, which revealed that the developed method was precise. The developed method was found to be robust. The % RSD values for percentage recovery was found to be within the acceptable criteria. The result indicates satisfactory accuracy of method for simultaneous estimation of the selected drugs.

CONCLUSION

The present work compiled with initial research objectives and successfully demonstrated the applicability of RP-HPLC for pharmaceutical analysis of different classes of drugs namely sofosbuvir and daclatasvir, ombitasvir, paritaprevir, ritonavir and abacavir, dolutegravir, lamivudine and bictegravir emtricitabine, tenofovir alafenamide by RP-HPLC with UV detection. The developed and validated methods shown high degree of sensitivity, selectivity, reproducibility and good recovery, stability with negligible matrix effects when compared with previously reported method.

This research work has contributions in **four** important scientific fields.

From an **analytical research and development (AR&D) point of view**, useful for analytical research scientists, particularly developing new analytical methods for these selected drugs in different biological samples are useful.

From a **formulation research and development (FR&D) point of view**, useful for formulation research scientists, particularly working on these selected drugs in developing new formulations and pharmacokinetic parameter calculations in different biological samples.

From a **drug regulatory point of view**, generated data meeting regulatory standards (bioanalytical and pharmacokinetic data) and it is acceptable for regulatory submission.

From **GLP (Good laboratory practices) point of view**, all bioanalytical lab instruments and methods were calibrated and validated before performing bioanalysis for acquiring of precise and accurate results.

The tremendous potential of RP-HPLC for pharmaceutical analysis is evident and will unquestionably expand future research capabilities in terms of shorter runtime, high rugged and reproducible analytical methods with high precision and accuracy.



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PUBLISHED RESEARCH PAPERS





Simultaneous Estimation of Ombitasvir, Paritaprevir and Ritonavir in Tablet Dosage Form by Reverse Phase High-Performance Liquid Chromatography

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Abstract: Ombitasvir/paritaprevir/ritonavir are the newest medicines approved for use in the treatment of hepatitis C virus (HCV) and are available in tablet form as an oral combination. Specifically, these agents are indicated in the treatment of HCV in patients with genotype 1 infection. Due to the therapeutic importance and increased use of Viekira Pak, proper methods for its determination in bulk and pharmaceutical formulations must be developed. The purpose of this work is to develop an accurate and precise RP-HPLC method for the determination of ombitasvir, paritaprevir and ritonavir in bulk and pharmaceutical preparations. Drugs were separated using Inertsil ODS-C18; 5 μ m (4.6 X 250mm) column using a mobile phase consisting of 0.02M phosphate buffer (pH-4.5): Acetonitrile: Methanol, (50:30:20) (v/v). The retention time was 2.98, 3.77 and 4.70 min for Ombitasvir, Paritaprevir, Ritonavir respectively and total run time was 10 min. at a flow rate of 1.0 mL/ min and the detection wavelength was 262 nm. The linearity was observed in the range of 15-45 μ g/ mL for ombitasvir, paritaprevir and ritonavir with a correlation coefficient of 0.9903, 0.9996 and 0.9998 respectively. The proposed method was validated for its linearity, accuracy, precision and robustness. This method can be employed for routine quality control analysis of Ombitasvir, Paritaprevir and Ritonavir in tablets. The LOD and LOQ values were found to be 1.8, 0.29 and 0.69 μ g/ mL and 5.7, 0.90 and 2.10 μ g/ mL for Ombitasvir, Paritaprevir and Ritonavir, respectively. The proposed method was successfully applied for the determination of ombitasvir/paritaprevir/ritonavir 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.

Keywords: Hepatitis C virus, Dosage forms, RP- HPLC; Validation, Quality control

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I. INTRODUCTION

More than 170 million people worldwide are infected with Hepatitis-C virus (HCV). The leap forward in HCV treatment wasn't until the discovery of directly acting antiviral drugs (DAADs) few years ago. According to the American Association for the Study of Liver Diseases and Infectious Diseases Society of America (AASLD-IDS), the estimated average cost of regimens using DAADs ranges from 26,400 to 94,500USD per patient¹. For example, the lowest identified price for treatment course for sofosbuvir alone in a developing country was \$ 900 in Egypt¹. This can demonstrate the massive production size of HCV regimens all over the world which will require development of simple, fast and economic methods of analysis for determination of HCV drugs in pharmaceutical quality control (QC) and research laboratories. Technivie® (paritaprevir, ritonavir plus ombitasvir) was approved by FDA in 2015, as single dose combination therapy². Ombitasvir (OMB) is a hepatitis C virus NS5A inhibitor, paritaprevir (PAR) is a hepatitis C virus NS3/4A protease inhibitor, while ritonavir (RIT) is a CYP3A inhibitor. This combination showed activity against HCV genotype-4 with sustained virologic response of 100%³. HCV genotype-4 has been considered the most difficult-to-treat genotype which accounts for more than 90% of the HCV infections in Egypt alone (about 14.1% of Egyptian population)³. Ombitasvir, paritaprevir and ritonavir (Figure:1.0) drugs were combined in a single dosage form (film-coated tablet) in the brand name of TECHNIVIE for the treatment of Hepatitis-C. These three drugs will act against the hepatitis-C virus (HCV) in three different mechanisms. Ombitasvir, produces its antiviral activity by inhibiting the HCV nonstructural protein (NS) 5A. Ombitasvir chemically designated as dimethyl ((2S,5S)-1-(4-tert-butyl phenyl) pyrrolidine-2,5-diyl) bis (benzene -4, 1 diylcarbamoyl (2S) pyrrolidine -2, 1-diy l[(2S) -3-methyl -1-oxobutane -1, 2-diyl])) biscarbamate hydrate with molecular weight of 894.11 g/mole (Fig. 1)¹⁻⁴. Paritaprevir chemically designated as

(2R, 6S, 12Z, 13aS,14aR, 16aS)-N-(cyclopropylsulfonyl)-6-[[[(5-methyl-2-pyrazinyl) carbonyl] amino]-5, 16 -dioxo-2-(6-phenanthridinyloxy) -1, 2, 3, 6, 7, 8, 9, 10, 11, 13a, 14, 15, 16, 16a -tetradecahydrocyclopropa[e] pyrrolo[1,2-a] [1,4] diazacyclopentadecine -14a(5H)-carboxamide with molecular weight of 765.89 g/mole (Fig. 1). TECHNIVIE inhibits the NS-3/4A serine protease of HCV. Subsequently, replication of HCV genetic components and translation into a single polypeptide, NS-3, and its activating cofactor NS-4A are accountable for splitting it into the succeeding nonstructural and structural proteins essential for assembly into a mature virus, viz., NS-3, NS-4A, NS-4B, NS-5A, and NS-5B. By inhibiting viral protease NS-3/4A, PRTR, therefore, prevents viral replication and function¹⁻⁴. Ritonavir RTNR is an anti-retroviral medication utilized along with other medications to treat the human immunodeficiency virus. This combination treatment is known as highly active antiretroviral therapy (HAART). At low doses of RTNR, it is utilized with other protease inhibiting agents and useful in combination with other Hepatitis-C medications. It is chemically designated as 1, 3-thiazol- 5-ylmethyl N- [(2S, 3S, 5S) -3- hydroxy- 5-[(2S)- 3- methyl -2-[[methyl]([2-(propan-2-yl)-1,3-thiazol-4-yl] methyl)) carbamoyl] amino} butanamido]-1,6 diphenylhexan-2-yl] carbamate with molecular weight of 720.946 g/mole⁴. Literature review reveals that few chromatographic methods were reported for simultaneous determination of Ombitasvir, Paritaprevir and Ritonavir. As per literature review, combination of drugs was estimated by chromatographic techniques like HPLC^{3-9, 12, 18, 20-25}, UPLC¹⁷, HPTLC^{13, 21}, LC-MS/MS^{14-16, 19, 24}, Capillary electrophoresis^{26, 27} have been reported for the estimation of Ombitasvir, Paritaprevir and Ritonavir. To our knowledge, only four research methods were reported using HPLC. The purpose of this research reported here was to develop a new RP-HPLC for separation and quantification of three drugs in bulk and formulations.

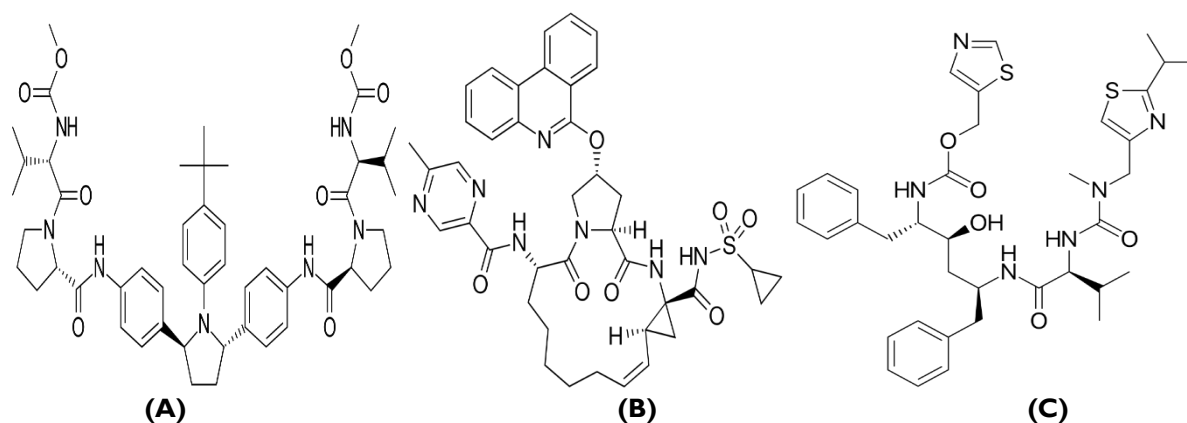


Fig.1: Chemical structures of (A) Ombitasvir (B) Paritaprevir (C) Ritonavir

2. MATERIALS AND METHODS

2.1 Chemicals and Reagents

Hetero Pharma. Pvt. Ltd., Hyderabad, India, graciously sent a gift sample of pure Working Standards of known efficacy of Ombitasvir, Paritaprevir, and Ritonavir. The marketed sample contained ombitasvir 25 mg, paritaprevir 150 mg, and ritonavir 100 mg and was obtained from a local pharmacy. Orthophosphoric Acid (OPA) from HI Media Laboratories

Pvt. Ltd., Water, Methanol, Acetonitrile, Trimethylamine from Merk, and Potassium dihydrogen phosphate from Thermo Fisher Scientific India Pvt. Ltd. were used as reagents.

2.2 Instrumentation

The HPLC system (Agilent HPLC 1200 Infinity LC Specifications) had a pump (Agilent LC20AT) configured with Ezchrom Elite Software and a rheodyne injector. The

detector was made up of a UV/VIS (UV-2489) type that worked at a wavelength of 262 nm. At room temperature, an Inertsil CN-3 column was employed.

2.3 Preparation of Standard Stock Solution

Each 10mg of Ombitasvir, Paritaprevir and Ritonavir were transferred to 100 ml volumetric flask, dissolved and diluted to the mark with methanol. The stock solutions were further diluted with mobile phase to obtain a solution of 100 µg/mL.^{7,8}

2.4 Test Sample Preparation

Tablet powder equivalent to 10mg of Ombitasvir, Paritaprevir and Ritonavir was weighed from a pooled powder of twenty tablets and transferred into a 10 ml volumetric flask, few ml of methanol was added and sonicated for 10 min. The volume was made up to mark with

methanol and the sample solution was filtered and used for further dilution.

2.5 Optimization of HPLC Method

The HPLC procedure was optimized with a view to develop a simultaneous assay method for Ombitasvir, Paritaprevir and Ritonavir, respectively. The mixed standard stock solution (100 µg/mL) was injected. For RP-HPLC method optimization of different ratios of methanol and water were tried, but it was found that drugs were separated using Inertsil ODS-C₁₈; 5µm (4.6 X 250mm) column using a mobile phase consisting of 0.02M phosphate buffer (pH-4.5): Acetonitrile: Methanol, (50:30:20) (v/v). The retention time was 2.98, 3.77 and 4.70 min for Ombitasvir, Paritaprevir, Ritonavir, and total run time was 10 min at a flow rate of 1.0 mL/ min and the detection wavelength was 262 nm gives ideal system suitability parameters like retention time, Peak area, USP plate count, USP Tailing factor, resolution, were depicted in Table 1.

Table 1. System suitability parameters

Name of Peak	Retention time(min)	*PeakArea	*USP plate count	*USP Tailing	Resolution
Ombitasvir	2.980	787012	7533	1.019	-
Paritaprevir	3.779	11706368	11323	1.126	3.54
Ritonavir	4.701	14965799	9856	1.096	2.54
Acceptancecriteria	-	NA	More than 2000	Less than 2	More than 2

*n=6 (Average of six determinations)

3. METHOD VALIDATION

After the development of RP-HPLC method for the estimation of drug in a dosage form, validation of the method was performed as per ICH guidelines.²⁸⁻³⁰

3.1 System Suitability Parameters

System suitability tests are used to verify that the resolution and reproducibility of the system are adequate. Several suitability parameters, including the capacity factor, selectivity, efficiency, resolution and tailing factor were

calculated, as shown in Table 1. The peaks obtained were sharp and showed clear baseline separation.

3.2 Linearity

A series of standard solutions were prepared in the range of 15µg/mL-45µg/mL containing Ombitasvir, Paritaprevir and Ritonavir standards and injected. A plot of average peak area versus the concentration (r^2) in µg/mL is made and from this the correlation coefficient, y-intercept and slope of the regression line were calculated. The calibration data and calibration curve shown in Table No.2 and Fig No. 2, 3 and 4.

Table 2: Linearity data of Detector Response

Concentration range	15-45µg/mL	15-45µg/mL	15-45µg/mL
Slope (m)	27167	22409	27471
Analyte name	Ombitasvir	Paritaprevir	Ritonavir
Correlation coefficient (r^2)	0.9903	0.9996	0.9998

*n=6 (Average of six determinations)

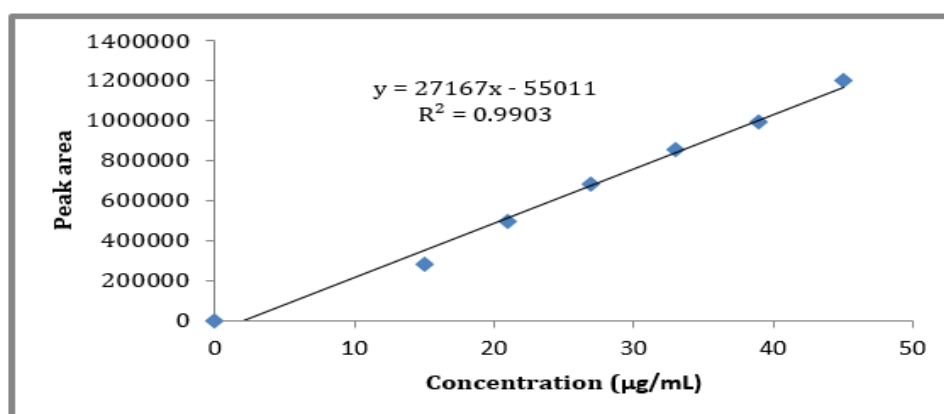


Fig 2: Linearity Plot of Ombitasvir

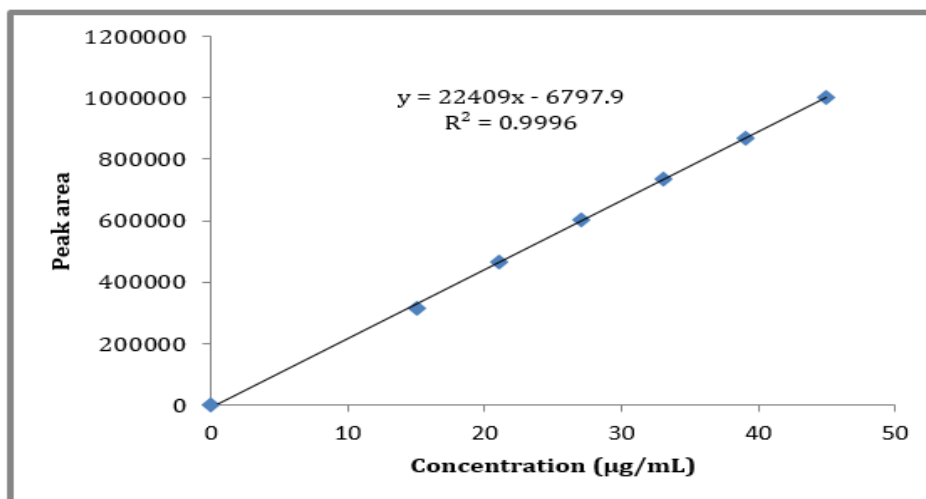


Fig 3: Linearity Plot of Paritaprevir

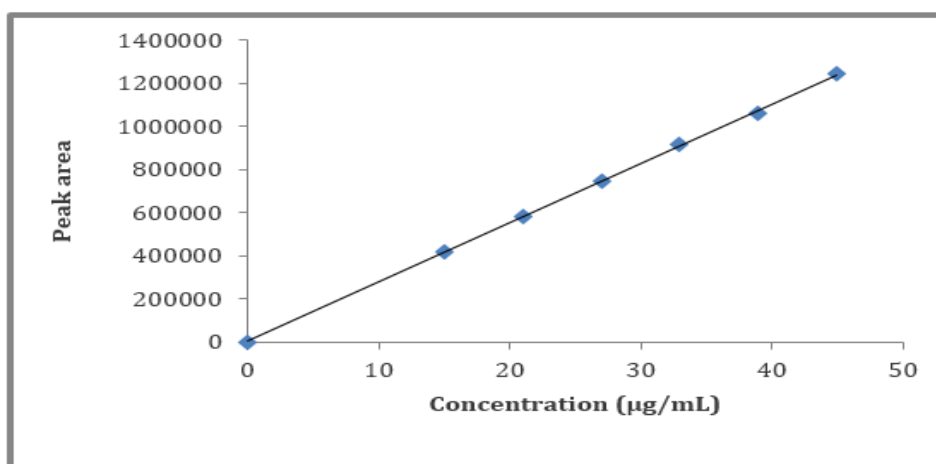


Fig 4: Linearity Plot of Ritonavir

3.3 Precision

The precision of the test procedure was evaluated by injecting the six test solutions (33 µg/ml). The Relative Standard Deviation of six injections was calculated. The result of Precision studies is given in Table No.03.

Table 3 Precision for Ombitasvir, Paritaprevir and Ritonavir						
Intraday precision						
Test conc: 33 µg/mL						
Mean	838353	99.29	733562	98.21	908979	98.33
SD	11574.29	0.58	1228.86	1.11	4917.51	0.82
% RSD	1.38	0.58	0.17	1.13	0.54	0.83
Inter day precision						
Mean	840680	97.82	729415	97.82	908947.50	98
SD	15630.89	1.37	2018.62	1.06	5771.51	1.02
% RSD	1.86	1.40	0.28	1.08	0.63	1.04

*SD= Standard deviation, *RSD= Relative standard deviation

3.4 Specificity

Specificity is the ability of a method to discriminate between the analyte (s) of interest and other components that are present in the sample. A study of placebo interference from excipients was conducted. Equivalent weight of placebo taken as per the test method and placebo interference was conducted in duplicate²⁸⁻³⁰.

3.5 Accuracy

To validate whether the test method can accurately quantify Ombitasvir, Paritaprevir and Ritonavir, prepare samples in three times for higher and lower levels, in triplicate for other levels by spiking Ombitasvir, Paritaprevir and Ritonavir of active material with equivalent amount of placebo and perform CU as per test procedure. Samples were prepared at levels 80% and 120% of the target assay concentration i.e. 100% level. Table no 4 shows the results for accuracy of Ombitasvir, Paritaprevir and Ritonavir.

Table 4: Accuracy results									
Ombitasvir			Paritaprevir				Ritonavir		
Mean	SD	%RSD	Mean	Mean	SD	%RSD	Mean	SD	%RSD
98.54	0.28	0.29	98.54	100.41	1.86	1.85	103.72	1.57	1.52
101.02	1.70	1.68	101.02	102.95	1.31	1.28	98.88	0.40	0.40
100.03	1.08	1.07	100.03	99.77	0.42	0.42	101.97	1.77	1.73

*SD= Standard deviation, *RSD= Relative standard deviation

3.6 Limit of Detection (LOD) And Limit of Quantitation (LOQ)

The limit of detection and limit of quantitation were determined by diluting known concentrations of each drug until signal to noise ratios of approximately 3:1 and 10:1 were obtained, respectively. The LOD and LOQ of

Ombitasvir, Paritaprevir and Ritonavir, which represent the capability of the method to detect and quantify low concentrations, were 1.8, 0.29 and 0.69 µg/ mL and 5.7, 0.90 and 2.10 µg/ mL, respectively. This result indicates the capability of the method to detect and quantify low concentrations. The results are summarized in Table 2.

Table 5: LOD and LOQ			
Analyte name	Ombitasvir	Paritaprevir	Ritonavir
LOD	1.899	0.297	0.693
LOQ	5.753	0.901	2.101

4. ASSAY

Six replicates of the sample solutions were injected for quantitative analysis. The amounts of Ombitasvir, Paritaprevir and Ritonavir estimated were found to be 99.52, 102.0 and 99.02 respectively. A good separation and resolution of both drugs indicate that there was no interference from the excipients commonly present in pharmaceutical formulations.

This showed that the estimation of dosage form was accurate within a given acceptable level of 95% to 105%. The amount of Ombitasvir, Paritaprevir and Ritonavir per tablet were calculated by extrapolating the value of area from the calibration curve. Analysis procedure was repeated six times with tablet formulation. The result formulation was reported in Table 5.

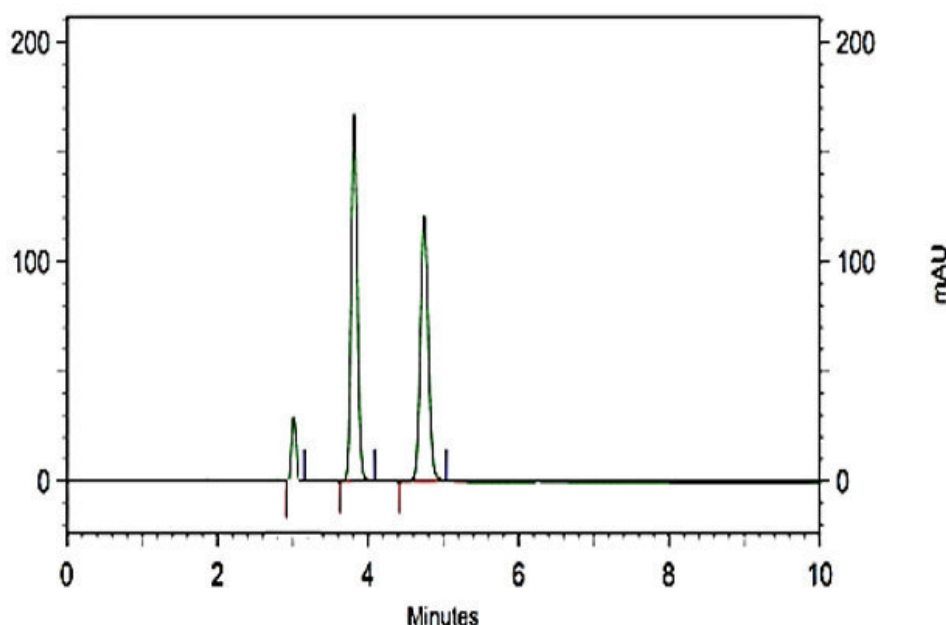


Fig 5: Chromatogram of test sample

5. DISCUSSION

When we compare the proposed HPLC method to other reported methods; HPTLC^{13, 21}, LC-MS/MS^{14-16, 19, 24}, Capillary electrophoresis^{26, 27} have lower solvents and energy consumptions than the conventional HPLC methodologies. However, UPLC¹⁷ methods have higher maintenance costs due to the shorter column life-time and requirement of special instrumentation especially those coupled with MS-MS detectors^{14-16, 19, 24} and due to this UHPLC is used in economic establishments like

pharmaceutical quality control laboratories and is limited and not widespread.

5.1 Method development and optimization of chromatographic conditions

The method was developed based upon the experience obtained from the HPLC method previously developed for the analysis of Ombitasvir/paritaprevir/ritonavir. The previous experiment was performed using a mobile phase consisting of acetonitrile and phosphate buffer (pH 3) at a

ratio of 60:40, v/v. For the separation of analytes from mixtures containing Ombitasvir/paritaprevir/ritonavir, methanol and acetonitrile were used as organic modifier, peak symmetry and optimum pressure was obtained by using acetonitrile. Various ratios of acetonitrile and phosphate buffer solutions and different mobile phase pH values were tested using a C18 (150 × 4.5 mm, 3.5 µm) column, higher acetonitrile ratio resulted in shorter retention times of drugs. Using this mobile phase ratio best results were obtained in terms of peak symmetry, selectivity and analysis time for drugs and the results are shown in Fig. 2. The pKa values of the studied drugs are reported in the literature as 2.8 for ritonavir, 2.5 for Ombitasvir, and 4.6 and for paritaprevir, which has two pKas. Therefore, the pH of the mobile phase was adjusted to 4.5. A wavelength of 262 nm was selected for the simultaneous determination of three analytes with high sensitivity. Moreover, the strength of the phosphate buffer solution (10–100 mM) was evaluated. Good resolution and reasonable retention times were observed for all of the drugs when 0.02M phosphate buffer (pH-4.5): Acetonitrile: Methanol, (50:30:20) (v/v) was delivered at a flow rate of 1 ml/min.

5.2 Method validation

The aim of the present work was to develop a rapid, precise, accurate and cost effective HPLC method for simultaneous estimation of Ombitasvir, Paritaprevir and Ritonavir in its pharmaceutical tablet formulation, using the reverse phase (RP) C18 column with UV detection and validate develop method as per US FDA guideline and ICH guideline²⁸⁻³⁰. The method was found to be specific. There was no peak found in blank sample chromatogram at Ombitasvir, Paritaprevir and

Ritonavir peak retention times. The Correlation Coefficient, r^2 of Ombitasvir, Paritaprevir and Ritonavir was found to be 0.9903, 0.9996 and 0.9998 respectively. The Percentage Relative standard deviation of individual area response of six replicate injections for Ombitasvir, Paritaprevir and Ritonavir was found to be 1.38, 0.17 and 0.54 respectively. The Percentage Relative standard deviation of areas of six replicate injections for Ombitasvir, Paritaprevir and Ritonavir standard were found to be within limits. The tailing factor for Ombitasvir, Paritaprevir and Ritonavir peaks was found to be 1.01, 1.12 and 1.09 respectively. The tailing factor for Ombitasvir, Paritaprevir and Ritonavir peaks was found to be within limits (Less than 2). The number of theoretical plates for Ombitasvir, Paritaprevir and Ritonavir were found to be 7533, 11323 and 9856 respectively. The resolution was found to be 3.54 and 2.54 respectively (Table-I). Interference was not observed with the standard peaks and the chromatograms of Standard and Sample were identical with the same retention time. The mean % Recovery of Ombitasvir, Paritaprevir and Ritonavir were found to be within limits at each level. The % RSD of recovery of Ombitasvir, Paritaprevir and Ritonavir from the three sample preparations was found to be 1.01, 1.18 and 1.21. The proposed HPLC method has several advantages compare to published methods^{3-9, 12, 18, 20-25}. Firstly, the same mobile phase could be stored and used several times for several elutions (i.e. Recycled). Secondly, the simultaneous processing of sample and standard under the exact same conditions gave rise to improved analytical precisions and accuracies. Additionally, the low cost of HPLC method encourages its use as an analytical tool. No interference was observed from the co-formulated substances compare to reported methods by HPLC.^{3-9, 12, 18, 20-25}

Table 5: Assay of test sample			
Test formulation (Tablet)	Label claimed (mg/tab)		
	OT	RT	PT
Ombitasvir, Paritaprevir, Ritonavir tablets	12.5	75	50
	Conc found (mg)		
	12.44	76.2	49.51
	%Assay		
	99.52	102	99.02

6. CONCLUSION

RP-HPLC method was developed and validated as per ICH guidelines. It can be concluded that the method is specific for the estimation of Ombitasvir, Paritaprevir, and Ritonavir in the pharmaceutical dosage form. The high accuracies and precisions of the assays obtained, taken together with the low solvent consumption and replacing hazardous solvents made this method eligible for use in different research and pharmaceutical quality control laboratories for the determination of these drugs. The method has a linear response in the stated range and is accurate and precise. Statistical analysis proves that the method is suitable for the analysis of Ombitasvir, Paritaprevir, and Ritonavir as bulk drugs drug and in the pharmaceutical formulation without any interference from the excipients.

7. ACKNOWLEDGEMENTS

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8. AUTHORS CONTRIBUTION STATEMENT

Gollapalli Nagaraju carried out the experiment and wrote the manuscript with support from Ritesh Agarwal, Rama Rao Nadendla. Prof. Rama Rao Nadendla helped supervise the project. Ritesh Agarwal, Rama Rao Nadendla conceived the original idea. Rama Rao Nadendla supervised the project.

9. CONFLICT OF INTEREST

Conflict of interest declared none.

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A NOVEL QUANTITATIVE ESTIMATION OF DACLATASVIR AND SOFOSBUVIR IN TABLET DOSAGE FORM BY RP-HPLC METHOD

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ABSTRACT

Objective: A Simple, accurate, specific and rugged reverse phase liquid chromatographic method was developed and validated for the simultaneous estimation of Daclatasvir and Sofosbuvirin bulk and tablet dosage form. **Method:** A reverse phase gradient program has been developed to separate the all four active ingredients using a mixture of Acetonitrile: Methanol: 0.1% Triethylamine buffer(pH-3.0) 25:35:40 (v/v/v) as mobile phase. Aisocratic elution has been developed and validated, on a reverse phase Inertsil ODS-C₁₈ column (250 x 4.6mm,5μ)with a flow rate of 1 mL/min by monitoring at 250 nm of wavelength. **Results:** The mean retention times of Daclatasvir and Sofosbuvirwere found to be 2.09 and 3.50 min respectively. Linearity of Daclatasvir and Sofosbuvirwas found to be 5-25μg/mL and 2-10μg/mL respectively. The accuracy of the proposed method was determined by performing recovery studies and was found to be

between 98-102%. The repeatability testing for both sample and standard solutions was found as % RSD<2.0% which is within the acceptable limits showing that the method is precise as well. The LOD and LOQ values were found to be 0.313 and 0.948 μg/ mL for Daclatasvir and 0.021 and 0.065 μg/mL for Sofosbuvir, respectively. **Conclusion:** The proposed method was validated in terms of linearity, range, accuracy, precision, specificity, robustness and

stability studies and the method is successfully applied for the estimation of Daclatasvir and Sofosbuvir in combined tablet dosage form.

KEYWORDS: Sofosbuvir, Daclatasvir; RP- HPLC; Validation, Chromatography.

INTRODUCTION

Daclatasvir, Methyl [(2S)-1-{(2S)-2-[4-(4'-{2-[(2S)-1-{(2S)-2-[(methoxycarbonyl) amino]-3-methylbutanoyl}-2-pyrrolidinyl]-1H-imidazol-4-yl}-4-biphenyl)-1H-imidazol-2-yl]-1-pyrrolidinyl}-3-methyl-1-oxo-2-butanyl] carbamate, is a nucleotide analogue NS5A polymerase inhibitor.^[1-6]

Sofosbuvir, (S)-Isopropyl 2-((S)-(((2R,3R,4R,5R)-5-(2,4-dioxo-3,4-dihydropyrimidin-1(2H)-yl)-4-fluoro-3-hydroxy-4-methyltetrahydrofuran-2-yl)methoxy) (phenoxy) phosphorylamino) propanoate, is a nucleotide analogue HCV NS5B polymerase inhibitor that is used in the treatment of chronic hepatitis C genotypes 1,2,3 or 4.^[7-21]

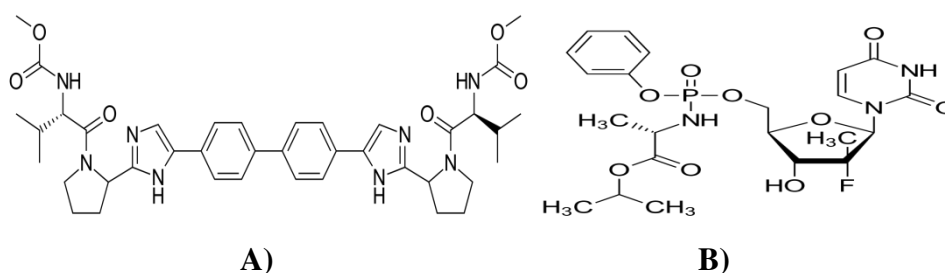


Fig.1: Chemical Structures of A) Daclatasvir B) Sofosbuvir.

The reported methods have some drawbacks in terms of sensitivity, ruggedness and robustness. This study describes a validated RP-HPLC method for the simultaneous quantitative detection of sofosbuvir and daclatasvir in its pure form and which is commercially available in tablet form. This method was more sensitive than the previously reported HPLC methods. The study was analytically validated according to the ICH guidelines.^[22] The purpose of this study was to develop simple, rapid, precise and accurate RP-HPLC method for the simultaneous estimation of daclatasvir and sofosbuvir in combined tablet dosage form.

MATERIALS AND METHODS

Instrumentation

Chromatographic separation was performed on a Agilent chromatographic system equipped with 1200 series isocratic pump; Rheodyne injector with 20 μ L fixed volume loop, variable wavelength programmable UV detector and the output signal was monitored and integrated by EZICHROME ELITE Chromatographic Software. Double beam UV-Visible spectrophotometer (Labindia-3120) was used to carry out spectral analysis and the data was recorded by UVWIN-5 software. Ultrasonicator (1.5L) was used for degasification of mobile phase and samples. Standard and sample drugs were weighed by using shimadzu electronic analytical balance (AX-220) and pH of the mobile phase was adjusted by using systronics digital pH meter.

Chemicals and solvents

All chemicals and reagents used were of HPLC grade. Pure standards of Daclatasvir and (DCV) Sofosbuvir (SFV) employed in the study were obtained as gift sample from Micro labs, Bangalore. The other reagents used were methanol and acetonitrile from Qualigens Ltd. Mumbai, India, triethylamine from Hi-media, Mumbai, India, and HPLC grade water from Merck chemicals, Mumbai, India.

Preparation of standard stock solution

Standard stock solution of Daclatasvir and Sofosbuvir (1mg/mL) were prepared by accurately weighing about 100mg pure drug and transferring in to 100mLvolumetric flask and dissolved in methanol.

The standard stock solution was further diluted with mobile phase to get calibration curve standard concentrations of 5, 10, 15, 20 and 25 μ g/mL of Daclatasvir and 2, 4, 6, 8 and 10 μ g/mL of Sofosbuvir.

Preparation of 0.1% triethylamine buffer (pH- 3.0)

Buffer solution was prepared by taking accurately a quantity of 0.1 mL of triethylamine dissolved in 100 mL HPLC grade water. The pH of the solution was adjusted to 3.0 with ortho- phosphoric acid and degassed for about 30 min in an ultra bath sonicator.

Preparation of mobile phase

The mobile phase used was acetonitrile, methanol and freshly prepared 0.1% triethylamine buffer solution (pH- 3.0) in the ratio of 25:35:40 (v/v/v) and the mobile phase was filtered through 0.45 μ membrane filter and sonicated before use.

Method Development

For developing the method, a systematic study of the effect of various factors was undertaken by varying on parameter errata time and keeping all other conditions constant. Method development consists of selecting the appropriate wavelength and choice of stationary and mobile phases. The following studies were conducted for this purpose.

Detection wave length

The spectrum of diluted solutions of the Daclatasvir in and Sofosbuvir and methanol was recorded. The absorption spectrum of Daclatasvirin and Sofosbuvir obtained by scanning the sample separately on UV spectrophotometer in UV region (200-400 nm) in spectrum mode showed that the drug has maximum absorbance at isobestic point 250 nm. Analysis was carried out by adjusting the UV detector of the HPLC system at 250 nm.

Choice of stationary phase

Preliminary development trials have performed with octadecyl columns with different types, configurations and from different manufacturers. Finally the expected separation and shapes of peak was succeeded analytical column Inertsil ODS-C₁₈ column (250x4.6 mm, 5 μ).

Selection of the mobile phase

Several systematic trials were performed to optimize the mobile phase. Different solvents like methanol, water and acetonitrile in different ratios and different pH values of the mobile phase ratio, by using different buffer solutions in order to get sharp peak and baseline separation of the components and without interference of the excipients. Satisfactory peak symmetry, resolved and free from tailing was obtained in mobile phase acetonitrile:methanol: 0.1% triethylamine buffer (pH-3.0); 25:35:40 (v/v/v) in isocratic condition.

Selection of the mobile phase flow rate

Flowrates of the mobile phase were changed from 0.5-1.2mL/min for optimum separation. A minimum flow rate as well as minimum run time gives the maximum saving on the usage of solvents. It was found from the experiments that 1.0mL/min flow rate was ideal for the

successful elution of the analyte.

After completion of several systematic trials to optimize the chromatographic conditions, a sensitive, precise and accurate RP-HPLC method was developed for the analysis of Daclatasvirin and Sofosbuvir in pharmaceutical dosage forms.

RESULTS AND DISCUSSION

The proposed method was validated as per ICH guidelines. The parameters studied for validation were system suitability, specificity, linearity, precision, accuracy (recovery), ruggedness and robustness, limit of detection and limit of quantification, filter validation and solution stability.^[39-40]

Specificity and Selectivity

The selectivity of an analytical method is its ability to measure accurately and specifically the analyte of interest in the presence of components that maybe expected to be present in the sample matrix. If an analytical procedure is able to separate and resolve the various components of a mixture and detect the analyte qualitatively the method is called selective. It has been observed that there were no peaks of diluents and placebo at main peaks. Hence, the chromatographic system used for the estimation of Daclatasvirin and Sofosbuvir was very selective and specific. Specificity studies indicating that the excipients did not interfere with the analysis. The standardsolution shown symmetric peak with retention times of 2.0 min for Daclatasvirin and 3.5 min for Sofosbuvir. The results were depicted in Fig. 2 to 4.

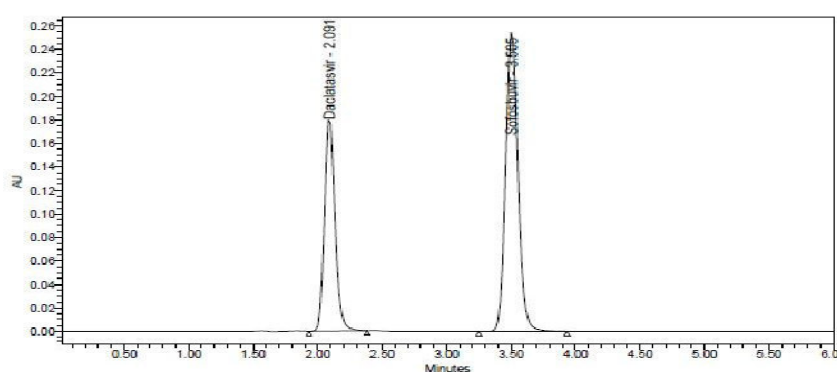


Fig.2: Chromatogramrepresentingspecificityofstandard solution.

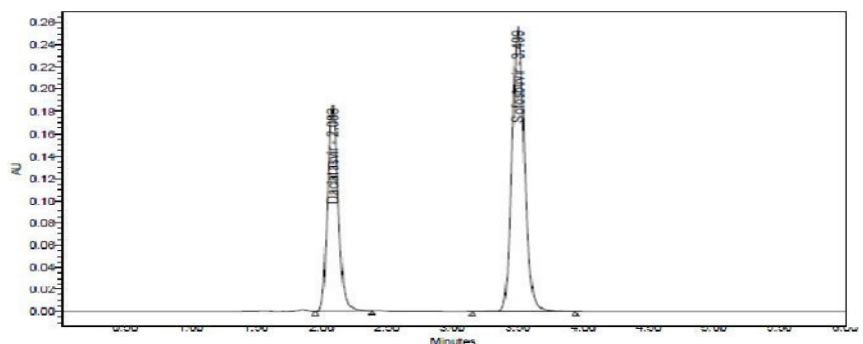


Fig.3: Chromatogram representing specificity of test sample solution.

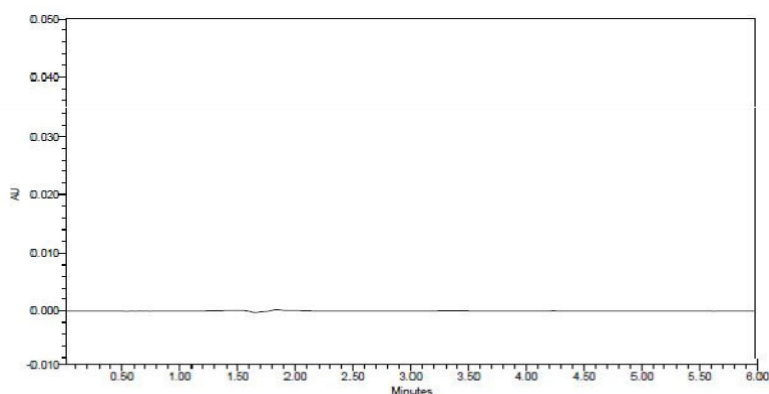


Fig.4: Typical chromatogram of the Placebo.

System suitability

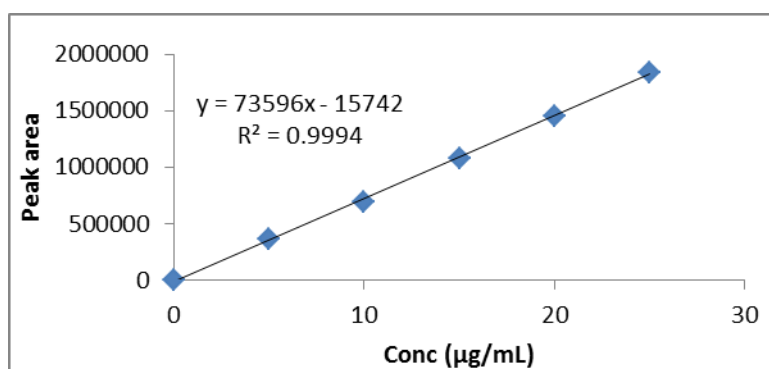
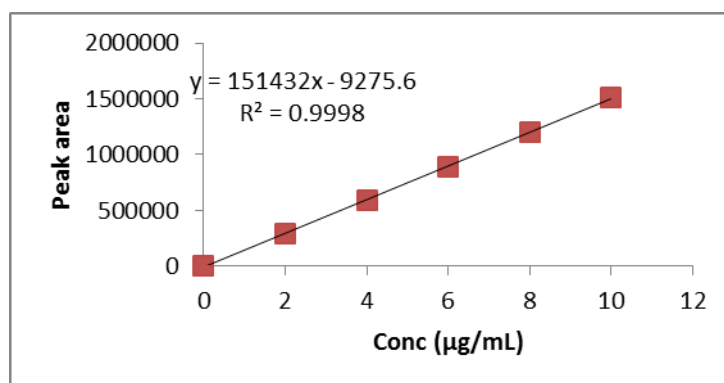
Standard solution (15 µg/mL of DCV and 6 µg/mL of SFV) was prepared as per the proposed method and injected into the HPLC system in five replicates and system suitability parameters were evaluated.

Linearity & Range

A series of standard concentrations were prepared from 50 % to 150 % of the target concentration of DCV and SFV. Linearity was assessed by performing single measurement at several analyte concentration varying quantities of stock standard solution diluted with the mobile phase to give a concentration of 5, 10, 15, 20 and 25 µg/mL of DCV and 2, 4, 6, 8 and 10 µg/mL of SFV. Injection was made at intervals of 10.0 min. Linearity of DCV was found to exist between 5-25 µg/mL and for SFV was 2-10 µg/mL. The chromatograms were recorded and linearity graph was plotted by using peak area of drug against respective concentrations to obtain the linearity range. The results were depicted in Table.1 and Fig.5 to 6.

Table 1.0: Linearity and range of DCV and SFV.

%Level	Concentration $\mu\text{g/mL}$	Areaof Daclatasvirin	Concentration $\mu\text{g/mL}$	Areaof Sofosbuvir
50%	5	358575	2	289124
75%	10	691556	4	591568
100%	15	1081521	6	890541
125%	20	1451468	8	1205846
150%	25	1842135	10	1510214
Concentration range	5-25 $\mu\text{g/mL}$		2-10 $\mu\text{g/mL}$	
Slope (m)	73596		151432	
Correlation coefficient (r^2)	0.9992		0.9997	

**Fig.5: Linearity of Daclatasvirin.****Fig.6: Linearity of Sofosbuvir.**

Precision

The intra-day and inter-day precision studies were carried out using a test sample assay method with six replicates on the same day and different days. The results were depicted in Table. 2 to 3.

Table 2: Intraday precision data for Daclatasvirin and Sofosbuvir.

Sample. No	Area of DCV	% Assay	Area of SFV	% Assay
1	1091521	98.88	894541	98.45
2	1091063	98.83	892265	98.20
3	1099852	99.63	893215	98.31
4	1082413	98.05	893426	98.33
5	1086315	98.40	891757	99.33
6	1099514	99.60	893475	98.34
Mean	1091780	98.90	893113	98.49
SD	6973.67	0.63	983.51	0.42
% RSD	0.64	0.64	0.11	0.43

Table 3: Interday precision data for Daclatasvirin and Sofosbuvir.

Sample. No	Area of DCV	% Assay	Area of SFV	% Assay
1	1098259	99.48	898126	98.85
2	1083695	98.17	893421	98.33
3	1084237	98.21	893825	98.37
4	1091595	98.88	891618	98.13
5	1093572	99.06	896481	98.67
6	1095285	99.21	897523	98.78
Mean	1091107	98.84	895166	98.52
SD	5950.73	0.54	2587.58	0.28
% RSD	0.55	0.55	0.29	0.29

Ruggedness

This is to prove the lack of influence of operational and environmental variables of the test results by using the method. Ruggedness is a measure of reproducibility of test results under the variation in conditions normally expected from system to system and from analyst to analyst. It was carried out by using a test sample assay method with six replicates using different analyst, column and HPLC system. The results were depicted in Table.4.

Table 4: Ruggedness of Daclatasvirin and Sofosbuvir.

Sr. No.	DCV (% Assay)			SFV (% Assay)		
	SET I	SET II	SET III	SET I	SET II	SET III
1	99.89	99.45	99.40	99.50	101.60	102.60
2	98.77	99.20	99.70	101.90	101.40	99.60
3	98.43	99.67	99.88	99.60	99.50	101.90
4	99.81	99.54	99.60	102.00	101.60	101.40
5	98.20	98.98	98.20	99.40	99.90	101.60
6	96.60	98.20	99.56	100.60	101.00	99.50
Average	98.62	99.17	99.39	100.50	100.83	101.10
SD	1.21	0.54	0.60	1.20	0.91	1.27

% RSD	1.23	0.54	0.61	1.20	0.91	1.25
Overall Average	99.06			100.81		
Overall % RSD	1.23			1.20		

SET – I : Variability due to HPLC system

SET – II : Variability due to HPLC column

SET – III : Variability due to Analyst

Accuracy (Recovery)

The accuracy of the method was determined by calculating recoveries of DCV and SFV by method of standard additions. Known amount of DCV and SFV were added to a pre quantified sample solution (containing DCV and SFV in 10 and 4 μ g/ mL proportion, respectively), and the amount of DCV and SFV were estimated by measuring the peak areas and by fitting these values to the straight-line equation of calibration curve. The results were depicted in Table. No- 5 to 6.

Table 5: Accuracy of Daclatasvirin.

Level of % recovery	Target Conc. (μ g/mL)	Amount of drug spiked (μ g/mL)	Nominal Conc. (μ g/mL)	Drug recovered (μ g/mL)	% Recovery	Mean	SD	% RSD
80	10	8	18.00	18.13	100.72	100.72	0.67	0.66
				18.01	100.06			
				18.25	101.39			
100	10	10	20.00	20.12	100.60	100.70	0.26	0.26
				20.20	101.00			
				20.10	100.50			
120	10	12	22.00	22.21	100.95	100.36	0.91	0.90
				22.18	100.82			
				21.85	99.32			

Table 6: Accuracy of Sofosbuvir.

Level of % recovery	Target Conc. (μ g/mL)	Amount of drug spiked (μ g/mL)	Nominal Conc. (μ g/mL)	Drug recovered (μ g/mL)	% Recovery	Mean	SD	% RSD
80	4	3.20	7.20	7.25	100.69	100.37	0.29	0.29
				7.21	100.14			
				7.22	100.28			
100	4	4.00	8.00	8.12	101.50	101.08	0.38	0.38
				8.08	101.00			
				8.06	100.75			
120	4	4.80	8.80	8.82	100.23	100.30	0.24	0.24
				8.85	100.57			
				8.81	100.11			

$$\% \text{ Recovery} = \frac{\text{Drug Recovered}}{\text{Nominal Concentration}} \times 100$$

Robustness

Robustness was performed by change in mobile phase ratio, mobile phase flow rate and wavelength of the detector. The test was carried out by small variation in the chromatographic conditions at a concentration equal to standard concentrations 15 µg/mL for DCV and 6µg/mL for SFV and % change was calculated. % change in the results was calculated. The results were depicted in Table. 7.0.

Table 7: Robustness of Daclatasvirin and Sofosbuvir.

S.No.	Parameter	Condition	DCV		SFV	
			Area (n=3)	% change	Area (n=3)	% change
1	Standard	Standard conditions	1078259	0.00	90541	0.00
2	Mobile Phase composition (±2%)	Acetonitrile : Methanol: 0.1%Triethylamine buffer (pH-3.0); 23:33:44 (v/v/v)	1089257	-1.02	90244	0.33
		Acetonitrile : Methanol: 0.1%Triethylamine buffer(pH-3.0); 27:37:36 (v/v/v)	1073285	0.46	90549	-0.01
3	Mobile phase pH (±0.2units)	2.8	1078576	-0.03	90243	0.33
		3.2	1072254	0.56	90939	-0.77
4	Wavelength (±2nm)	248	1088259	-1.49	90140	0.44
		252	1079257	-0.09	90596	-0.06
5	Flow rate (mL) ±0.2mL	1.2	1078296	0.09	90141	0.44
		0.8	1078651	-0.04	90595	-0.06

$$\% \text{ change} = \frac{\text{Peak area of standard} - \text{Peak area of test (parameter change)}}{\text{Peak area of standard}} \times 100$$

Limit of detection and Limit of quantification

The limit of detection (LOD) is defined as the lowest concentration of an analyte that can reliably be differentiated from background levels. Limit of quantification (LOQ) of an individual analytical procedure is the lowest amount of analyte that can be quantitatively determined with suitable precision and accuracy. LOD and LOQ were calculated using the following equation as per ICH guidelines. $\text{LOD} = 3.3 \times \sigma / S$; $\text{LOQ} = 10 \times \sigma / S$;

Where σ is the standard deviation of y-intercepts of regression lines and S is the slope of the calibration curve.

Solution Stability

Solution stability was assed using standard and test stock solutions. These stocks were prepared and stored at room temperature and refrigerated conditions (2-8°C) for 36 h and % differences was calculated. The results were depicted in Table. 8 to 11.

$$\% \text{ Difference} = \frac{\text{Fresh stock Peak area} - \text{Stability stock peak area}}{\text{Fresh stoock peak area}} \times 100$$

Table 8: Solution stability of Daclatasvirinat room temperature.

Time	Standard stock			Test stock		
	Fresh	Stability Stock	% Diff.	Fresh	Stability Stock	% Diff.
Initial	1078253	1078252	NA	1078252	1078243	NA
6h	1083695	1081634	0.190	1083634	1083695	-0.006
12h	1084234	1081256	0.275	1084256	1084214	0.004
20h	1081595	1081542	0.005	1081542	1081595	-0.005
26h	1073571	1083564	-0.931	1073564	1073501	0.006
30h	1075282	1065211	0.937	1075211	1075282	-0.007
36h	1078253	1079452	-0.111	1078252	1078053	0.018

Table 9: Solution stability of Sofosbuvir at room temperature.

Time	Standard stock			Test stock		
	Fresh	Stability Stock	% Diff.	Fresh	Stability Stock	% Diff.
Initial	890127	890167	NA	890127	890167	NA
6h	890511	890012	0.056	890422	890412	0.001
12h	890821	890356	0.052	890821	890856	-0.004
20h	890618	890696	-0.009	890618	890696	-0.009
26h	890482	890043	0.049	890482	890413	0.008
30h	890528	890589	-0.007	890528	890589	-0.007
36h	890127	890421	-0.033	890127	890421	-0.033

Table 10: Solution stability of Daclatasvirin at refrigerated temperature.

Time	Standard stock			Test stock		
	Fresh	Stability Stock	% Diff.	Fresh	Stability Stock	% Diff.
Initial	1078253	1078252	NA	1078252	1078253	NA
6h	1083695	1083634	0.006	1083634	1083695	-0.006
12h	1084234	1084256	-0.002	1084256	1084234	0.002
20h	1081595	1071542	0.929	1081542	1081595	-0.005
26h	1073571	1073564	0.001	1073564	1073571	-0.001

30h	1075282	1075211	0.007	1075211	1075282	-0.007
36h	1078253	1069452	0.816	1078252	1077253	0.093

Table 11: Solution stability of Sofosbuvir at refrigerated temperature.

Time	Standard stock			Test stock		
	Fresh	Stability Stock	% Diff.	Fresh	Stability Stock	% Diff.
Initial	890127	890167	NA	890127	890167	NA
6h	890422	890412	0.001	890422	890412	0.001
12h	890821	890056	0.086	890821	890856	-0.004
20h	890618	890696	-0.009	890618	890696	-0.009
26h	890482	891443	-0.108	890482	890443	0.004
30h	890528	891589	-0.119	890528	890589	-0.007
36h	890127	890421	-0.033	890127	895421	-0.595

Filter validation

A study was conducted to determine the effect of filter on the assay, dissolution and impurities. Test solution was prepared as per the test method. Some portion of the above solution was filtered through three different filters namely 0.45 µ PVDF filter, 0.45 µ PTFE and 0.45µ Nylon filter and some portion was centrifuged and injected into the HPLC system. The % difference values between centrifuged and filtered sample were calculated. The results were depicted in Table. 12.

Table 12: Filter interference results for Daclatasvirin and Sofosbuvir.

DCV				
Filtration Method	Centrifuged	Nylon	PTFE	PVDF
Area (Inj. 1)	1078253	1073564	1073571	1073564
Area (Inj. 2)	1083695	1075211	1075282	1075211
Avg. Area	1080974	1074388	1074427	1074388
% Difference		0.609	-0.004	0.004
SFV				
Filtration Method	Centrifuged	Nylon	PTFE	PVDF
Area (Inj. 1)	890482	890127	890167	890443
Area (Inj. 2)	890528	890422	890412	890589
Avg. Area	890505	890275	890290	890516
% Difference		0.026	-0.002	-0.025

$$\% \text{ Difference} = \frac{\text{Centrifuge Peak area} - \text{Filter peak area}}{\text{Centrifuge peak area}} \times 100$$

Analysis of Marketed Formulation

Preparation test solution

A total of 20 tablets were accurately weighed and powdered in a mortar. An amount equivalent to one tablet (Containing 400 mg of SFV and 60 mg of DCV) was transferred to 25 mL volumetric flask, 10 mL of methanol was added and content of the flask were ultrasonicated for 10 min. The solution was filtered through whatmann filter paper No. 41. The sample solution thus prepared was diluted with mobile phase to get the solution containing SFV and DCV in 15 & 6 µg/mL proportion, respectively. The test solution was injected in to HPLC and % assay was calculated.

CONCLUSION

A new, reversed-phase HPLC method has been developed for simultaneous analysis of Daclatasvir and Sofosbuvir in a tablet formulation. It was shown that, the method was linear, accurate, reproducible, repeatable, precise, selective and specific proving the reliability of the method. The run time is relatively short (6.0 min), which enables rapid determination of many samples in routine and quality control analysis of tablet formulations. Hence, the proposed method was successfully applied to analyze preparation containing Daclatasvir and Sofosbuvir.

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AN ECO-FRIENDLY HPLC METHOD FOR THE SIMULTANEOUS ESTIMATION OF ABACAVIR, DOLUTEGRAVIR AND LAMIVUDINE IN ACTIVE PHARMACEUTICAL INGREDIENTS AND MARKETING TABLET FORMULATION

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Keywords:

Abacavir, Dolutegravir and Lamivudine, Bulk drug, Formulation, Eco-friendly, HPLC

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ABSTRACT: Selective and novel method has been optimized to evaluate Abacavir, Dolutegravir, and Lamivudine in bulk and formulation by HPLC. The principle analytes were eluted with the conditions of the mobile phase having the Ethanol: Ethyl acetate (80:20, % v/v) using the Lichrosphere RP C8 column (Phenomenex, USA (250 x 4.6 mm, 5 μ) analytical column with the 1.0 ml/min flow rate and 10 μ l sample volume at 260 nm in UV detector. The retention times of Abacavir, Dolutegravir, and Lamivudine were 2.31min, 3.120 min, and 4.59min with a total run time of 6 min. The curve indicates correlation coefficient (r^2) was superior by having a value nearer to 1.000 with a linear range of 40 μ g/ml-130.0 μ g/ml for Abacavir, Dolutegravir, and Lamivudine. The correlation coefficient (r^2) 0.9971 for Abacavir, 0.9979 for Dolutegravir and 0.9947 for Lamivudine were found. The LOD and LOQ for the Abacavir, Dolutegravir, and Lamivudine were found at 1.40 μ g/ml, 3.01 μ g/ml, 5.84 μ g/ml, and 4.25 μ g/ml, 9.12 μ g/ml and 17.71 μ g/ml. The developed method was applied for the bulk and formulation.

INTRODUCTION: Abacavir, which is chemically called 1S,cis-4-6- cyclopropyl-2-amino-9H-purin-9-yl-2-cyclopentene-1-methanol sulphate (abacavir), is a C7- cyclic purine that is transformed to 9-dihydro-2 residues sulfurate and then to 9-amino-7-(dichloro)adenosine. The nano-particle transforms to the activated neurotransmitter carbovir triphosphate if the liver absorbs it. Carbovir triphosphate is an analogue that represents deoxyguanosine-5'-triphosphate (dGTP). Carbovir triphosphate is an antiviral medication used to block HIV-1 reverse transcriptase (RT).

The drug is also a powerful inhibitor of RT and, therefore a macromolecule that gets integrated into the viral DNA. The chemical compound Dolutegravir (DTG) 1 which is chemically (4R, 12aS) - 9 - {[(2, 4difluorophenyl) methyl] carbamoyl} - 4 - methyl-6, 8-dioxo-3, 4, 6, 8, 12, 12a-hexahydro-2 Hpyrido[1',2':4, 5] pyrazino [2,1-b] [1,3] oxazin-7-olate, might disrupt the retroviral DNA integration steps. This is seen as a benefit because within this chemical compound; there is a mechanism that can block retroviral DNA integration, which is essential to HIV infection.

Lamivudine (3TC) 1 is a compound that is chemically [2R, cis]4-amino-1-(2hydroxymethyl-1,3-oxathiolan-5-yl)-(1H)-pyrimidin-2-one (*i.e.* a synthetic nucleoside analogue). Like several proteins, the HIV-1 RT is phosphorylated to the active 5'-triphosphate metabolite, lamivudine triphosphate (L-TP).

<p>QUICK RESPONSE CODE</p> 	<p>DOI: 10.13040/IJPSR.0975-8232.13(9).1000-07</p> <hr/> <p>This article can be accessed online on www.ijpsr.com</p> <hr/> <p>DOI link: http://dx.doi.org/10.13040/IJPSR.0975-8232.13(9).1000-07</p>
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The key mode of action of L-TP is the rapid inhibition of HIV-1 RT and the subsequent termination of viral DNA chains¹⁻⁴. Based on the literature survey, there was no Eco-friendly analytical method for this formulation, i.e., Abacavir, Dolutegravir, and Lamivudine. Several methods were developed for Abacavir, Dolutegravir, and Lamivudine with combinations⁵⁻

²⁵. For the Abacavir, Dolutegravir, and Lamivudine combination, there was a lack of an eco-friendly analytical method for identifying and quantifying bulk and formulation. And there was no sensitive Eco-friendly analytical method having the 40 µg/ml detectability to quantify the product traces of the manufacturing area when the product change is over.

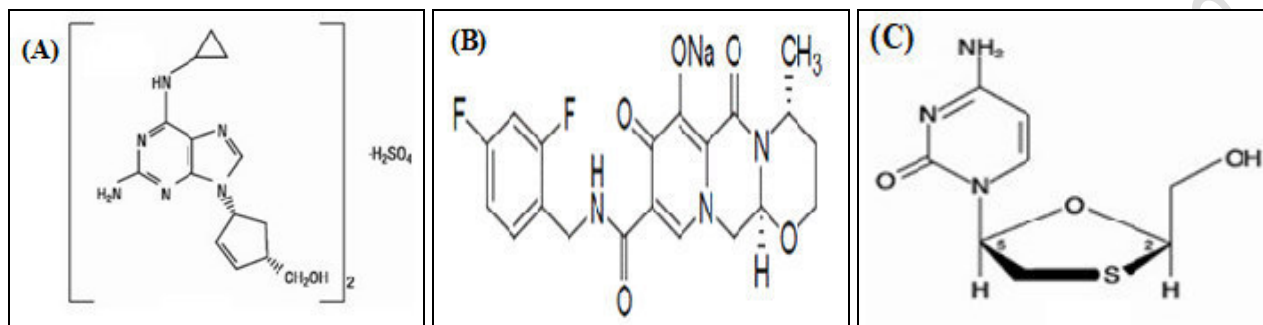


FIG. 1: CHEMICAL STRUCTURES OF ABACAVIR (A), DOLUTEGRAVIR (B), LAMIVUDINE (C)

MATERIALS AND METHODS: Abacavir, Dolutegravir, and Lamivudine Fig. 1, high purity Ethyl acetate (J. T. Baker, Phillipsburg, NJ, USA), Ethanol (HPLC grade, Sigma Aldrich).

Preparation of Standard Solution: Abacavir, Dolutegravir, and Lamivudine standards stock solution prepared by taking 10 mg in 10 ml volumetric flask then adding 10 ml ethanol and sonicated for 3 min. Then makeup to 10ml with the ethanol.

Preparation of Mobile Phase: Added 500 ml of ethanol to the 500 ml of Ethyl acetate, degassed to prepare 1000 ml of the mobile phase.

Optimization of Chromatographic Conditions: After series of trials, the chromatographic conditions were accomplished with the Ethanol:

Ethyl acetate (80:20, % v/v) by utilizing the stationary phase Lichrosphere RP C8 column (Phenomenex, USA (250 x 4.6 mm, 5µ) Spherisorb C₁₈, 5 µm, 4.6 mm x150 mm to obtain the best peak shape. The Abacavir, Dolutegravir, and Lamivudine separation was good at 260nm with a column temperature 25°C and sample compartment temperature 10° C with the flow 1.0 ml/min with the sample volume 10µl.

Assay Sample Preparation: One tablet has Abacavir 60 mg, Dolutegravir 30 mg, and Lamivudine 60 mg into 1000 ml volumetric flask and dissolved in the diluent and make up to the 100 ml. This preparation is considered a stock solution. From the stock solution, take 1ml to 10 ml in a volumetric flask and make up to the mark with the diluents and filter.

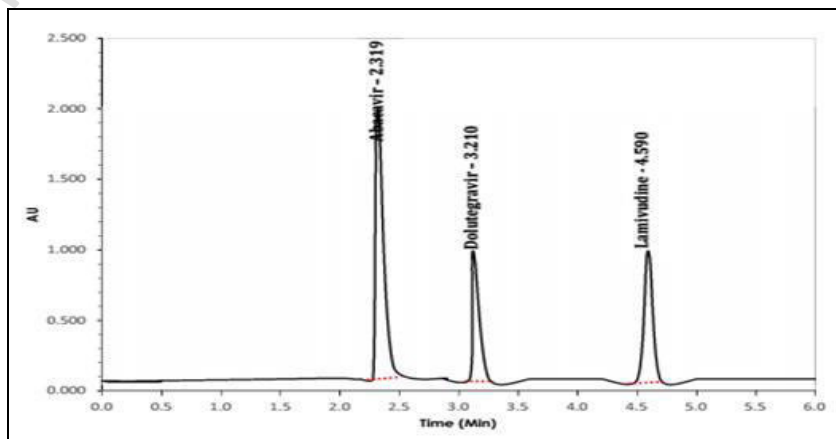


FIG. 2: TEST SAMPLE CHROMATOGRAM

Validation of Analytical Methods: Validation was performed for the developed method with the stringent limit to prove the efficiency of this method²⁶.

System Suitability: To verify the system produces the consistent results with the optimized method injected the standard six times with the criteria of % RSD for retention time and area NMT 2.0%, theoretical plates NLT 3000 plates, tailing factor NMT 1.5, and resolution NLT 4.

TABLE 1: SYSTEM SUITABILITY PARAMETERS

Parameter	Compound	Result
Retention Time	Abacavir	2.31 min
	Dolutegravir	3.21 min
	Lamivudine	4.59 min
Peak Area	Abacavir	294621
	Dolutegravir	867991
	Lamivudine	512422
Theoretical plates	Abacavir	5120
	Dolutegravir	4200
	Lamivudine	12013
Tailing factor	Abacavir	0.72
	Dolutegravir	0.64
	Lamivudine	0.32
Resolution	Abacavir	-
	Dolutegravir	5.61
	Lamivudine	6.53
% Rsd	Abacavir	0.53
	Dolutegravir	0.17
	Lamivudine	0.23

Selectivity: To verify the method validation in terms of the selectivity and exactness, injected triplicate preparations of 100 % concentration, *i.e.*,

TABLE 2: PRECISION AND ACCURACY OF DATA

Intraday precision						
	Abacavir		Dolutegravir		Lamivudine	
Mean	293930	97.48	861297	97.40	502985	99.80
SD	1222.49	0.74	8167.69	1.20	8934.62	1.80
% RSD	0.42	0.76	0.95	1.23	1.78	1.80
Intermediate precision						
	Abacavir		Dolutegravir		Lamivudine	
Mean	294904	96.61	865243	96.20	507801.17	98
SD	2154.36	0.74	2056.04	1.22	5110.41	1.02
% RSD	0.73	0.76	0.24	1.27	1.01	1.04

Accuracy and Recovery: To verify the method's accuracy, triplicate preparations were prepared at 80% and 100%, and 120% levels of 100 %

TABLE 3: RECOVERY DATA

Abacavir			Dolutegravir			Lamivudine		
Mean	SD	%RSD	Mean	SD	%RSD	Mean	SD	%RSD
97.71	1.11	1.14	97.87	1.42	1.45	98.36	1.10	1.12
98.59	0.96	0.97	96.59	1.30	1.35	97.24	1.06	1.09
97.27	1.78	1.83	96.60	1.42	1.47	96.12	1.60	1.66

100 µg/ml of Abacavir, Dolutegravir, and Lamivudine.

Then injected one blank to prove the method did not have the carryover issue. The specificity's limit is that it should pass the system suitability criteria, and there should not be an RT shift for all three preparations.

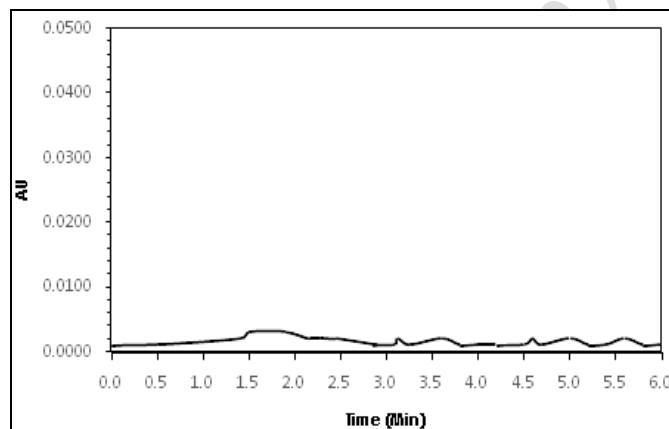


FIG. 3: BLANK CHROMATOGRAM

Precision: After passing the specificity and system suitability criteria, the method was verified for the system precision and method precision with the limit of % RSD for the retention time and area NMT 2%.

The intermediate precision was verified the next day with another column by following the limit as % RSD for the retention time, and the area should be NMT 2%.

concentration by spiking the standard into the diluent. Calculated the recovery with the acceptance criteria of 95-105%.

Linearity: The method linearity was verified with the six concentrations of 100 % concentration as 40 µg/ml, 60 µg/ml, 80 µg/ml, 100 µg/ml, 120 µg/ml, and 130 µg/ml for the Abacavir, Dolutegravir, and Lamivudine with the acceptance criteria of the regression coefficient (R^2) NLT 0.99.

To verify the method efficiency when the minor changes happened in the optimized method parameters like mobile phase composition, flow rate, and wavelength parameters were performed with the criteria, it should pass the system suitability criteria.

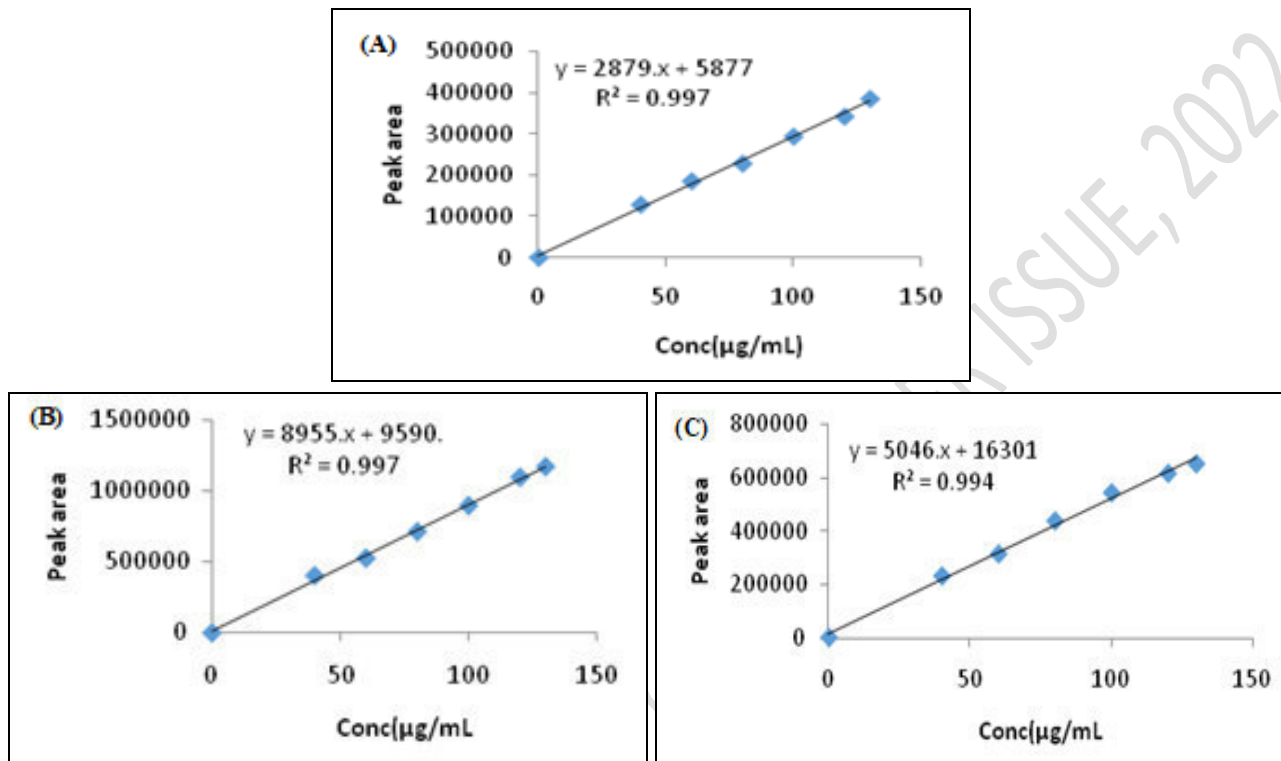


FIG. 4: LINEARITY DATA OF ABACAVIR (A), DOLUTEGRAVIR (B), LAMIVUDINE (C) ROBUSTNESS

TABLE 4: ROBUSTNESS EVALUATION OF CHROMATOGRAPHIC METHOD

Compound	Altered conditions	Condition	System suitability parameters							
			Area	% Change	RT	% Change	N	% Change	Tailing factor	% Change
Abacavir	Mobile Phase composition (±2%)	A = 80%	294621	0.38	2.310	-0.43	5120	-0.04	0.710	-1.41
		a = 70%	293511		2.320		5122		0.720	
	Wavelength(±2nm)	A = 260 nm	289512	-1.76	2.310	0.87	5110	-0.20	0.730	1.37
Dolutegravir	Mobile Phase composition (±2%)	a = 262 nm	294611		2.290		5120		0.720	
		A = 1.0 mL/min	294621	0.34	2.300	-0.87	5210	-0.38	0.710	-1.41
		a = 1.2 mL/min	293620		2.320		5230		0.720	
Lamivudine	Mobile Phase composition (±2%)	A = 80%	856814	0.11	2.300	-0.87	5140	-0.21	0.640	1.56
		a = 70%	855914		2.320		5151		0.630	
	Wavelength (±2nm)	A = 260 nm	854814	-0.23	2.300	-0.87	5260	-0.19	0.610	-1.64
	Flow rate ±0.2mL	a = 262 nm	856814		2.320		5270		0.620	
		A = 1.0 mL/min	853814	-0.35	2.330	0.43	5180	-0.19	0.642	-0.16
		a = 1.2 mL/min	856814		2.320		5190		0.643	
	Mobile Phase composition (±2%)	A = 80%	512422	0.19	2.300	-0.43	5300	-0.19	0.320	-0.31
		a = 70%	511432		2.310		5310		0.321	
	Wavelength (±2nm)	A = 260 nm	520442	0.57	2.330	0.43	5220	-0.19	0.323	0.93
	Flow rate ±0.2mL	a = 262 nm	517472		2.320		5230		0.320	
		A = 1.0 mL/min	515481	-0.19	2.300	-0.43	5240	-0.19	0.311	-1.29
		a = 1.2 mL/min	516482		2.310		5250		0.315	

A= Average values obtained at nominal concentration; a = Average values obtained at altered conditions.

Lower Level of Quantification: By considering the 10% concentration of the target concentration, injected the sample into the system with the acceptance criteria S/N ratio NLT 10. The LOQ concentration was injected with the different concentration preparation to identify the detectability with the acceptance criteria 3:1 and minimum detectability five times out of six injections from the same concentration.

Lower Level of Quantification Precision: LOQ precision verified with the limit NMT 2.0% for the RT and area.

Assessment of Stability of Standard: The prepared standards were verified up to 72 h for stability at room temperature and refrigerated conditions.

Filter Compatibility: To evaluate the impact of PVDF and Nylon filters on the assay results, the samples were analyzed after passing through the filters.

TABLE 5: FILTER COMPATIBILITY AND ASSAY OF TEST FORMULATION

Filter type	Label claimed (mg/Tab)		
PVDF	Abacavir	Dolutegravir	Lamivudine
	600	50	300
	Conc. found (mg/Tab)		
	594.26	49.32	296.18
	% Assay		
	99.04	98.64	98.73
Filter type	Label claimed (mg/Tab)		
Nylon	Abacavir	Dolutegravir	Lamivudine
	600	50	300
	Conc. found (mg/Tab)		
	593.94	48.75	298.82
	% Assay		
	98.99	97.50	99.61

RESULTS: There was a clear separation and good resolution, and without any carryover was achieved with this method, as shown in **Fig. 1** and **2**. The system suitability acceptance criteria were also satisfactory, as shown in **Table 1**. For the system precision parameter, the %RSD of RT and area for the Abacavir, Dolutegravir, and Lamivudine achieved 0.53%, 0.17, and 0.23% as shown in **Table 3** against the limit NMT 2.0%. The linearity parameter was quantified by peak area vs. concentration methodology. Different concentrations from 40 µg/ml to 130 µg/ml standard solutions for Abacavir, Dolutegravir, and

Lamivudine were prepared and injected into the system. The calculated regression coefficient for Abacavir, Dolutegravir, and Lamivudine is nearer to 1.000, as shown in **Fig. 4**.

For the method precision parameter, the % RSD of area for the Abacavir, Dolutegravir, and Lamivudine achieved 0.73%, 0.24, and 1.01 % against the limit NMT 2.0%. The method was verified for the ruggedness as intraday and interday precision. For the intermediate precision parameter, the %RSD of area for the Abacavir, Dolutegravir, and Lamivudine achieved in day-1 as 0.76%, 1.23%, and 1.80% on the next day (Day-2) 0.76%, 1.27% and 1.04% against the limit NMT 2.0% as shown in **Table 2**.

The recovery for the 80%, 100%, and 120% was more than 95% against the acceptance criteria of 95-105% as shown in **Table 3**. To evaluate the method's capability of producing precise results with the minor variations of flow, mobile phase composition, and wavelength variations as robustness was performed. The results were shown in **Table 4**. The results proved that the method was stable to produce consistent results with the minor variation of the method parameters.

The LOQ and LOD were identified by injecting the lower concentration of 40 µg/ml with the S/N ratio criteria. The LOQ for the Abacavir, Dolutegravir and Lamivudine was 1.40 µg/ml, 3.01 µg/ml and 5.84 µg/ml. The LOD for the Abacavir, Dolutegravir and Lamivudine was 4.25 µg/ml, 9.12 µg/ml and 17.71 µg/ml. Based on the stability results, the standards were stable up to 72 h at room temperature and refrigerated conditions. The compatibility of the filters was verified with the PVDF and Nylon filters. The assay of the Abacavir, Dolutegravir, and Lamivudine was more accurate (99.04% for Abacavir, 98.64% for Dolutegravir, and 98.73% for Lamivudine) with the PVDF filter compared to Nylon filter (98.99% for Abacavir, 97.50% for Dolutegravir and 99.61% for Lamivudine) as shown in **Table 5**.

DISCUSSION: During method optimization, organic solvents were initially used as mobile phases in different compositions. But three compounds were not detected. Then, an organic solvent such as ethanol and ethyl acetate were used

in different ratios with the Lichrosphere RP C8 column (Phenomenex, USA (250 x 4.6 mm, 5 μ). Finally, the method was found to be optimized with the conditions of mobile phase (ethanol and ethyl acetate (80:20 % v/v), wavelength 260 nm, flow rate of 1.0 ml/min, column temperature of 25°C, sample compartment temperature of 10°C, a sample volume of 10 μ l. With this method, both actives *i.e.* Abacavir, Dolutegravir, and Lamivudine eluted at 2.31 min, 3.120 min, and 4.59 with good resolution and symmetry.

After the method optimization, the method was validated as per ICH guidelines. As per the results obtained in the method validation, there was no interference of the blank and carryover problem even at the LOQ level quantification. Both LOQ and LOD of this method were verified practically in the instrument with S/N ratio criteria. The results were found satisfactory. Based on the recovery results, it proves that the method has the capability of high extraction efficiency (NLT 90%). The method was successfully applied to the assay of dosage forms to verify filter capability. The assay results show satisfactory and free from the interference of nylon and PVDF filters.

CONCLUSION: Based on the results obtained, the developed method was very sensitive, accurate, linear, and economical. Due to the short time of the chromatographic program, more samples can be analyzed within the short period, which will be helpful in the industry at the time of multiple products manufacturing continuously. The method met all the predefined acceptance criteria. The bulk and formulation samples can be analyzed in various dosage forms containing Abacavir, Dolutegravir, and Lamivudine.

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Reviewer's recommendations:

1. Specify designation and current full address of corresponding author.
2. Check for spelling, grammar and punctuation error(s).

Simultaneous Estimation of Daclatasvir and Sofosbuvir in Tablet Dosage form by Reverse Phase High-Performance Liquid Chromatography

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Abstract

A simple, rapid reversed-phase high performance liquid chromatographic method had been developed and validated for estimation of Daclatasvir and Sofosbuvir in tablet dosage form. The estimation was carried out on Inertsil ODS-C₁₈ column (250 x 4.6 mm, 5 μ) column with a mixture of Acetonitrile: Methanol: 0.1% Triethylamine buffer (pH-3.0) 25:35:40 (v/v/v) as mobile phase. UV detection was performed at 250 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 2.09 and 3.50 min for Daclatasvir and Sofosbuvir respectively and total run time was 6.0min at a flow rate of 1.0 mL/ min. The calibration curve was linear over the concentration range of 5.0-25.0 μ g/ mL for Daclatasvir and 2.0-10.0 μ g/ mL for Sofosbuvir. The LOD and LOQ values were found to be 0.313 and 0.948 μ g/ mL for Daclatasvir and 0.021 and 0.065 μ g/mL for Sofosbuvir, respectively. The high percentage of recovery and low percentage coefficient of variance confirm the suitability of the method for the simultaneous estimation of Daclatasvir and Sofosbuvir in tablet dosage form.

Keywords: Sofosbuvir, Daclatasvir; RP- HPLC; Validation, Chromatography

INTRODUCTION

Hepatitis C is a comprehensive liver disease produced by the hepatitis C virus (HCV) and can increase liver cirrhosis, liver failure, liver cancer and liver transplantation. The standard treatment for HCV is pegylated-interferon (Peg-IFN) and ribavirin (RBV) whoever these agents caused side effects such as bacterial infections, anemia, hematological toxicity, and neutropenia and anorectal symptoms.

Telaprevir and boceprevir were the first generation direct-acting protease inhibitors that developed and approved for the treatment of genotype I chronic hepatitis C. However, they have to be co-administered with interferon and ribavirin therefore they were associated with their common side effects so their effectiveness were limited [1-2].

Second-generation direct-acting antiviral drugs were developed and aimed to have a high pangenotypic activity with fewer undesirable side effects. These drugs include daclatasvir and sofosbuvir. Both medicines have effective antiviral activity and genotypic coverage [3-5].

Daclatasvir, Methyl [(2S)-1-{(2S)-2-[4-(4'-{2-[(2S)-1-{(2S)-2-[(methoxycarbonyl) amino]-3-methylbutanoyl]-2-pyrrolidinyl]-1H-imidazol-4-yl]-4-biphenyl)-1H-imidazol-2-yl]-1-pyrrolidinyl}-3-methyl-1-oxo-2-butanyl] carbamate, is a nucleotide analogue NS5A polymerase inhibitor [6].

Sofosbuvir, (S)-Isopropyl 2-((S)-(((2R,3R,4R,5R)-5-(2,4-dioxo-3,4-dihydropyrimidin-1(2H)-yl)-4-fluoro-3-hydroxy-4-methyltetrahydrofuran-2-yl)methoxy) (phenoxy) phosphorylamino) propanoate, is a nucleotide analogue HCV NS5B polymerase inhibitor that is used in the treatment of chronic hepatitis C genotypes 1,2,3 or 4 [21]. The sofosbuvir and daclatasvir combination is associated with a high rate of SVR4 in difficult-to-treat patients

infected with genotype 1 or 4. Combination with ribavirin increases the SVR rate in cirrhotic and treatment experienced patients with no additive effect of extension of treatment from 12 to 24 weeks. Since patient compliance is an important point in the treatment so taking the two drugs in one tablet will be a better choice. On another hand, the combined therapy is economically reduced the cost of the treatment and this will give a chance for many companies to formulate the three drugs in one tablet sooner. Additionally, the co-administered drugs might affect each other and there is no sufficient information about drug-drug interaction and thus the establishment of separation method is of great importance [31].

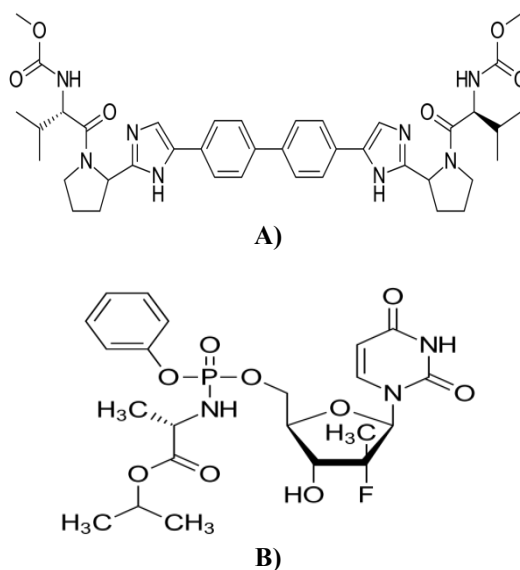


Fig.1: Chemical Structures of A) Daclatasvir B) Sofosbuvir

Literature survey reveals that there are few reported HPLC [6-11] and UV [12], UHPLC [13] and LC-MS/MS [14-19] for sofosbuvir and HPLC methods [21-27], UV [28-30] for daclatasvir individually and simultaneous estimation with different drugs like ledipasvir [31-35], velpatasvir [36-37] and simeprevir [38].

The reported methods have some drawbacks in terms of sensitivity, ruggedness and robustness. This study describes a validated RP-HPLC method for the simultaneous quantitative detection of sofosbuvir and daclatasvir in its pure form and which is commercially available in tablet form. This method was more sensitive than the previously reported HPLC methods. The study was analytically validated according to the ICH guidelines [39-40]. The purpose of this study was to develop simple, rapid, precise and accurate RP-HPLC method for the simultaneous estimation of daclatasvir and sofosbuvir in combined tablet dosage form.

MATERIALS AND METHODS

Instrumentation

Chromatographic separation was performed on a Agilent chromatographic system equipped with 1200 series isocratic pump; Rheodyne injector with 20 μ L fixed volume loop, variable wavelength programmable UV detector and the output signal was monitored and integrated by EZICHROME ELITE Chromatographic Software. Double beam UV-Visible spectrophotometer (Labindia-3120) was used to carry out spectral analysis and the data was recorded by UVWIN-5 software. Ultrasonicator (1.5L) was used for degasification of mobile phase and samples. Standard and sample drugs were weighed by using shimadzu electronic analytical balance (AX-220) and pH of the mobile phase was adjusted by using systronics digital pH meter.

Chemicals and solvents

All chemicals and reagents used were of HPLC grade. Pure standards of Daclatasvir and (DCV) Sofosbuvir (SFV) employed in the study were obtained as gift sample from Micro labs, Bangalore. The other reagents used were methanol and acetonitrile from qualigens ltd. Mumbai, India, triethylamine from Hi-media, Mumbai, India, and HPLC grade water from Merck chemicals, Mumbai, India.

Preparation of standard stock solution

Standard stock solution of Daclatasvir and Sofosbuvir (1mg/mL) were prepared by accurately weighing about 100 mg pure drug and transferring in to 100 mL volumetric flask and dissolved in methanol.

The standard stock solution was further diluted with mobile phase to get calibration curve standard concentrations of 5, 10, 15, 20 and 25 μ g/mL of Daclatasvir and 2, 4, 6, 8 and 10 μ g/mL of Sofosbuvir.

Preparation of 0.1% triethylamine buffer (pH - 3.0)

Buffer solution was prepared by taking accurately a quantity of 0.1 mL of triethylamine dissolved in 100 mL HPLC grade water. The pH of the solution was adjusted to 3.0 with ortho- phosphoric acid and degassed for about 30 min in a ultra bath sonicator.

Preparation of mobile phase

The mobile phase used was acetonitrile, methanol and freshly prepared 0.1% triethylamine buffer solution (pH-3.0) in the ratio of 25:35:40 (v/v/v) and the mobile phase was filtered through 0.45 μ membrane filter and sonicated before use.

Method Development

For developing the method, a systematic study of the effect of various factors were undertaken by varying one parameter at a time and keeping all other conditions constant. Method development consists of selecting the appropriate wave length and choice of stationary and mobile phases. The following studies were conducted for this purpose.

Detection wavelength

The spectrum of diluted solutions of the Daclatasvirin and Sofosbuvir and methanol was recorded. The absorption spectrum of Daclatasvirin and Sofosbuvir obtained by scanning the sample separately on UV spectrophotometer in UV region (200-400 nm) in spectrum mode showed that the drug has maximum absorbance at isobestic point 250 nm. Analysis was carried out by adjusting the UV detector of the HPLC system at 250 nm.

Choice of stationary phase

Preliminary development trials have performed with octadecyl columns with different types, configurations and from different manufacturers. Finally the expected separation and shapes of peak was succeeded analytical column Inertsil ODS-C₁₈ column (250 x 4.6 mm, 5 μ).

Selection of the mobile phase

Several systematic trials were performed to optimize the mobile phase. Different solvents like methanol, water and acetonitrile in different ratios and different pH values of the mobile phase ratios, by using different buffer solutions in order to get sharp peak and base line separation of the components and without interference of the excipients. Satisfactory peak symmetry, resolved and free from tailing was obtained in mobile phase acetonitrile: methanol: 0.1% triethylamine buffer (pH-3.0); 25:35:40 (v/v/v) in isocratic condition.

Selection of the mobile phase flow rate

Flow rates of the mobile phase were changed from 0.5- 1.2 mL/min for optimum separation. A minimum flow rate as well as minimum run time gives the maximum saving on the usage of solvents. It was found from the experiments that 1.0 mL/min flow rate was ideal for the successful elution of the analyte.

After completion of several systematic trials to optimize the chromatographic conditions, a sensitive, precise and accurate RP-HPLC method was developed for the analysis of Daclatasvirin and Sofosbuvir in pharmaceutical dosage forms.

Method Validation

The proposed method was validated as per ICH guidelines. The parameters studied for validation were system suitability, specificity, linearity, precision, accuracy (recovery), ruggedness and robustness, limit of detection and limit of quantification, filter validation and solution stability [39-40].

Specificity and Selectivity

The selectivity of an analytical method is its ability to measure accurately and specifically the analyte of interest in the presence of components that may be expected to be present in the sample matrix. If an analytical procedure is able to separate and resolve the various components of a mixture and detect the analyte qualitatively the method is called selective. It has been observed that there were no peaks of diluents and placebo at main peaks. Hence, the chromatographic system used for the estimation of Daclatasvirin and Sofosbuvir was very selective and specific. Specificity studies indicating that the excipients did not interfere with the analysis. The standard solution shown symmetric peak with retention times of 2.0 ± 0.05 min for Daclatasvirin and 3.5 ± 0.05 min for Sofosbuvir. The results were depicted in Fig. 2 to 4.

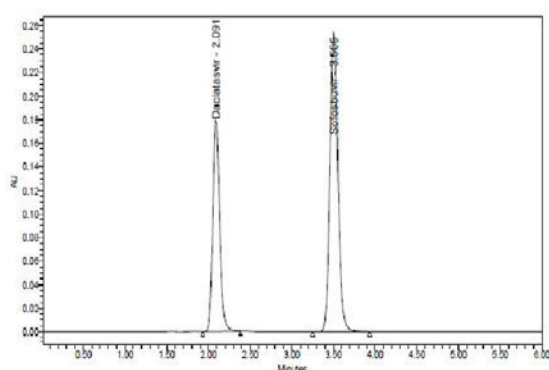


Fig.2: Chromatogram representing specificity of standard solution

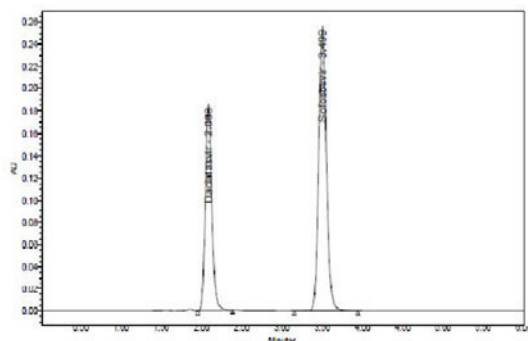


Fig.3: Chromatogram representing specificity of test sample solution

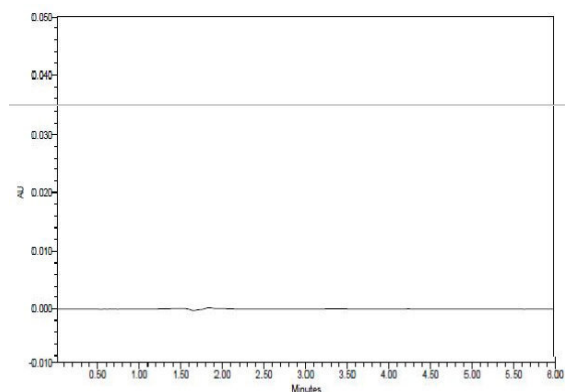


Fig.4: Typical chromatogram of the Placebo

System suitability

Standard solution (15 $\mu\text{g/mL}$ of DCV and 6 $\mu\text{g/mL}$ of SFV) was prepared as per the proposed method and injected into the HPLC system in five replicates and system suitability parameters were evaluated.

Linearity & Range

A series of standard concentrations were prepared from 50 % to 150 % of the target concentration of DCV and SFV. Linearity was assessed by performing single measurement at several analyte concentration varying quantities of stock standard solution diluted with the mobile phase to give a concentration of 5, 10, 15, 20 and 25 $\mu\text{g/mL}$ of DCV and 2, 4, 6, 8 and 10 $\mu\text{g/mL}$ of SFV. Injection was made at intervals of 10.0 min. Linearity of DCV was found to exist between 5-25 $\mu\text{g/mL}$ and for SFV was 2-10 $\mu\text{g/mL}$. The chromatograms were recorded and linearity graph was plotted by using peak area of drug against respective concentrations to obtain the linearity range. The results were depicted in Table.1 and Fig.5 to 6.

Table.1.0: Linearity and range of DCV and SFV

%Level	Concentration $\mu\text{g/mL}$	Area of Daclatasvirin	Concentration $\mu\text{g/mL}$	Area of Sofosbuvir
50%	5	358575	2	289124
75%	10	691556	4	591568
100%	15	1081521	6	890541
125%	20	1451468	8	1205846
150%	25	1842135	10	1510214
Concentration range	5-25 $\mu\text{g/mL}$		2-10 $\mu\text{g/mL}$	
Slope (m)	73596		151432	
Correlation coefficient (r^2)	0.9992		0.9997	

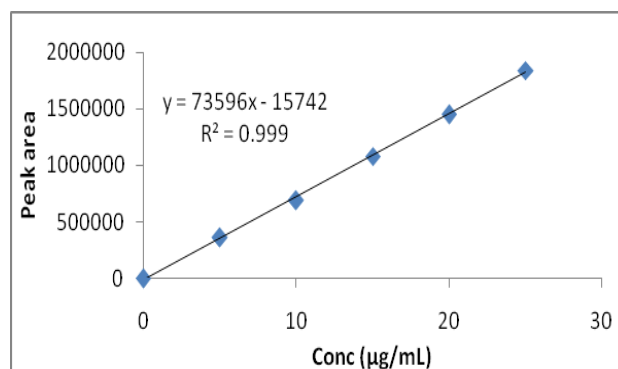


Fig.5: Linearity of Daclatasvirin

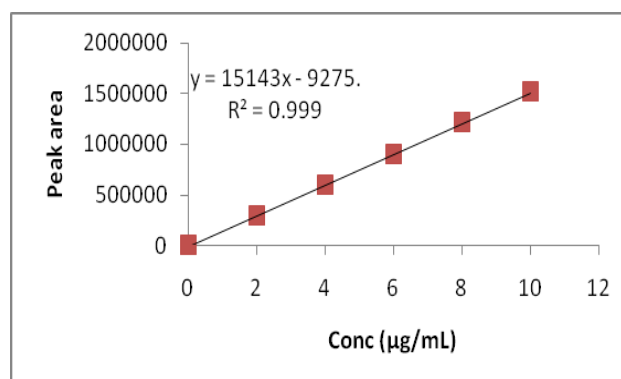


Fig.6: Linearity of Sofosbuvir

Precision

The intra-day and inter-day precision studies were carried out using a test sample assay method with six replicates on the same day and different days. The results were depicted in Table. 2 to 3.

Ruggedness

This is to prove the lack of influence of operational and environmental variables of the test results by using the method. Ruggedness is a measure of reproducibility of test results under the variation in conditions normally expected from system to system and from analyst to analyst. It was carried out by using a test sample assay method with six replicates using different analyst, column and HPLC system. The results were depicted in Table.4.

Table. 2: Intraday precision data for Daclatasvirin and Sofosbuvir

Sample. No	Area of DCV	%Assay	Area of SFV	%Assay
1	1091521	98.88	894541	98.45
2	1091063	98.83	892265	98.20
3	1099852	99.63	893215	98.31
4	1082413	98.05	893426	98.33
5	1086315	98.40	891757	99.33
6	1099514	99.60	893475	98.34
Mean	1091780	98.90	893113	98.49
SD	6973.67	0.63	983.51	0.42
% RSD	0.64	0.64	0.11	0.43

Table.3: Interday precision data for Daclatasvirin and Sofosbuvir

Sample. No	Area of DCV	%Assay	Area of SFV	%Assay
1	1098259	99.48	898126	98.85
2	1083695	98.17	893421	98.33
3	1084237	98.21	893825	98.37
4	1091595	98.88	891618	98.13
5	1093572	99.06	896481	98.67
6	1095285	99.21	897523	98.78
Mean	1091107	98.84	895166	98.52
SD	5950.73	0.54	2587.58	0.28
% RSD	0.55	0.55	0.29	0.29

Accuracy (Recovery)

The accuracy of the method was determined by calculating recoveries of DCV and SFV by method of standard additions. Known amount of DCV and SFV were added to a pre quantified sample solution (containing DCV and SFV in 10 and 4 µg/ mL proportion, respectively), and the amount of DCV and SFV were estimated by measuring the peak areas and by fitting these values to the straight-line equation of calibration curve. The results were depicted in Table. No- 5 to 6.

$$\% \text{ Recovery} = \frac{\text{Drug Recovered}}{\text{Nominal Concentration}} \times 100$$

Table.4: Ruggedness of Daclatasvirin and Sofosbuvir

Sr. No.	DCV (%Assay)			SFV (%Assay)		
	SET I	SET II	SET III	SET I	SET II	SET III
1	99.89	99.45	99.40	99.50	101.60	102.60
2	98.77	99.20	99.70	101.90	101.40	99.60
3	98.43	99.67	99.88	99.60	99.50	101.90
4	99.81	99.54	99.60	102.00	101.60	101.40
5	98.20	98.98	98.20	99.40	99.90	101.60
6	96.60	98.20	99.56	100.60	101.00	99.50
Average	98.62	99.17	99.39	100.50	100.83	101.10
SD	1.21	0.54	0.60	1.20	0.91	1.27
% RSD	1.23	0.54	0.61	1.20	0.91	1.25
Overall Average	99.06			100.81		
Overall % RSD	1.23			1.20		

SET – I : Variability due to HPLC system

SET – II : Variability due to HPLC column

SET – III : Variability due to Analyst

Table.5: Accuracy of Daclatasvirin

Level of % recovery	Target Conc. (µg/mL)	Amount of drug spiked (µg/mL)	Nominal Conc. (µg/mL)	Drug recovered (µg/mL)	% Recovery	Mean	SD	% RSD
80	10	8	18.00	18.13	100.72	100.72	0.67	0.66
				18.01	100.06			
				18.25	101.39			
100	10	10	20.00	20.12	100.60	100.70	0.26	0.26
				20.20	101.00			
				20.10	100.50			
120	10	12	22.00	22.21	100.95	100.36	0.91	0.90
				22.18	100.82			
				21.85	99.32			

Table.6: Accuracy of Sofosbuvir

Level of % recovery	Target Conc. (µg/mL)	Amount of drug spiked (µg/mL)	Nominal Conc. (µg/mL)	Drug recovered (µg/mL)	% Recovery	Mean	SD	% RSD
80	4	3.20	7.20	7.25	100.69	100.37	0.29	0.29
				7.21	100.14			
				7.22	100.28			
100	4	4.00	8.00	8.12	101.50	101.08	0.38	0.38
				8.08	101.00			
				8.06	100.75			
120	4	4.80	8.80	8.82	100.23	100.30	0.24	0.24
				8.85	100.57			
				8.81	100.11			

Table. 7: Robustness of Daclatasvirin and Sofosbuvir

S.No.	Parameter	Condition	DCV		SFV	
			Area (n=3)	% change	Area (n=3)	% change
1	Standard	Standard conditions	1078259	0.00	90541	0.00
2	Mobile Phase composition (±2%)	Acetonitrile : Methanol: 0.1%Triethylamine buffer (pH-3.0); 23:33:44 (v/v/v)	1089257	-1.02	90244	0.33
		Acetonitrile : Methanol: 0.1%Triethylamine buffer(pH-3.0); 27:37:36 (v/v/v)	1073285	0.46	90549	-0.01
3	Mobile phase pH (±0.2units)	2.8	1078576	-0.03	90243	0.33
		3.2	1072254	0.56	90939	-0.77
4	Wavelength (±2nm)	248	1088259	-1.49	90140	0.44
		252	1079257	-0.09	90596	-0.06
5	Flow rate (mL) ±0.2mL	1.2	1078296	0.09	90141	0.44
		0.8	1078651	-0.04	90595	-0.06

Table.8. Solution stability of Daclatasvirin at room temperature

Time	Standard stock			Test stock		
	Fresh	Stability Stock	% Diff.	Fresh	Stability Stock	% Diff.
Initial	1078253	1078252	NA	1078252	1078243	NA
6h	1083695	1081634	0.190	1083634	1083695	-0.006
12h	1084234	1081256	0.275	1084256	1084214	0.004
20h	1081595	1081542	0.005	1081542	1081595	-0.005
26h	1073571	1083564	-0.931	1073564	1073501	0.006
30h	1075282	1065211	0.937	1075211	1075282	-0.007
36h	1078253	1079452	-0.111	1078252	1078053	0.018

Robustness

Robustness was performed by change in mobile phase ratio, mobile phase flow rate and wavelength of the detector. The test was carried out by small variation in the chromatographic conditions at a concentration equal to standard concentrations 15 µg/mL for DCV and 6µg/mL for SFV and % change was calculated. %change in the results was calculated. The results were depicted in Table. 7.0.

$$\% \text{ change} = \frac{\text{Peak area of standard} - \text{Peak area of test (parameter change)}}{\text{Peak area of standard}} \times 100$$

Limit of detection and Limit of quantification

The limit of detection (LOD) is defined as the lowest concentration of an analyte that can reliably be differentiated from background levels. Limit of

quantification (LOQ) of an individual analytical procedure is the lowest amount of analyte that can be quantitatively determined with suitable precision and accuracy. LOD and LOQ were calculated using the following equation as per ICH guidelines. $\text{LOD} = 3.3 \times \sigma / S$; $\text{LOQ} = 10 \times \sigma / S$; Where σ is the standard deviation of y-intercepts of regression lines and S is the slope of the calibration curve.

Solution Stability

Solution stability was assed using standard and test stock solutions. These stocks were prepared and stored at room temperature and refrigerated conditions (2-8°C) for 36 h and % differences was calculated. The results were depicted in Table. 8 to 11.

$$\% \text{ Difference} = \frac{\text{Fresh stock Peak area} - \text{Stability stock peak area}}{\text{Fresh stock peak area}} \times 100$$

Table. 9. Solution stability of Sofosbuvir at room temperature

Time	Standard stock			Test stock		
	Fresh	Stability Stock	% Diff.	Fresh	Stability Stock	% Diff.
Initial	890127	890167	NA	890127	890167	NA
6h	890511	890012	0.056	890422	890412	0.001
12h	890821	890356	0.052	890821	890856	-0.004
20h	890618	890696	-0.009	890618	890696	-0.009
26h	890482	890043	0.049	890482	890413	0.008
30h	890528	890589	-0.007	890528	890589	-0.007
36h	890127	890421	-0.033	890127	890421	-0.033

Table. 10. Solution stability of Daclatasvirin at refrigerated temperature

Time	Standard stock			Test stock		
	Fresh	Stability Stock	% Diff.	Fresh	Stability Stock	% Diff.
Initial	1078253	1078252	NA	1078252	1078253	NA
6h	1083695	1083634	0.006	1083634	1083695	-0.006
12h	1084234	1084256	-0.002	1084256	1084234	0.002
20h	1081595	1071542	0.929	1081542	1081595	-0.005
26h	1073571	1073564	0.001	1073564	1073571	-0.001
30h	1075282	1075211	0.007	1075211	1075282	-0.007
36h	1078253	1069452	0.816	1078252	1077253	0.093

Table. 11. Solution stability of Sofosbuvir at refrigerated temperature

Time	Standard stock			Test stock		
	Fresh	Stability Stock	% Diff.	Fresh	Stability Stock	% Diff.
Initial	890127	890167	NA	890127	890167	NA
6h	890422	890412	0.001	890422	890412	0.001
12h	890821	890056	0.086	890821	890856	-0.004
20h	890618	890696	-0.009	890618	890696	-0.009
26h	890482	891443	-0.108	890482	890443	0.004
30h	890528	891589	-0.119	890528	890589	-0.007
36h	890127	890421	-0.033	890127	895421	-0.595

Table. 12. Filter interference results for Daclatasvirin and Sofosbuvir

DCV				
Filtration Method	Centrifuged	Nylon	PTFE	PVDF
Area (Inj. 1)	1078253	1073564	1073571	1073564
Area (Inj. 2)	1083695	1075211	1075282	1075211
Avg. Area	1080974	1074388	1074427	1074388
% Difference		0.609	-0.004	0.004
SFV				
Filtration Method	Centrifuged	Nylon	PTFE	PVDF
Area (Inj. 1)	890482	890127	890167	890443
Area (Inj. 2)	890528	890422	890412	890589
Avg. Area	890505	890275	890290	890516
% Difference		0.026	-0.002	-0.025

Filter validation

A study was conducted to determine the effect of filter on the assay, dissolution and impurities. Test solution was prepared as per the test method. Some portion of the above solution was filtered through three different filters namely 0.45 µ PVDF filter, 0.45 µ PTFE and 0.45µ Nylon filter and some portion was centrifuged and injected into the HPLC system. The % difference values between centrifuged and filtered sample were calculated. The results were depicted in Table. 12.

$$\% \text{ Difference} = \frac{\text{Centrifuge Peak area} - \text{Filter peak area}}{\text{Centrifuge peak area}} \times 100$$

Analysis of Marketed Formulation**Preparation test solution**

A total of 20 tablets were accurately weighed and powdered in a mortar. An amount equivalent to one tablet (Containing 400 mg of SFV and 60 mg of DCV) was transferred to 25 mL volumetric flask, 10 mL of methanol was added and content of the flask were ultra sonicated for 10 min. The solution was filtered through whatmann filter

paper No. 41. The sample solution thus prepared was diluted with mobile phase to get the solution containing SFV and DCV in 15 & 6 µg/mL proportion, respectively. The test solution was injected in to HPLC and % assay was calculated.

RESULTS AND DISCUSSION

In this RP-HPLC method, the conditions were optimized to obtain an adequate separation of eluted compounds. Initially, various mobile phase compositions were tried, to separate analytes. The mobile phase and flow rate selection was based on peak parameters (height, tailing, theoretical plates, capacity or symmetry factor), run time and resolution. The system with acetonitrile: methanol: 0.1% triethylamine buffer (pH-3.0); 25:35:40 (v/v/v) at isocratic flow rate of 1.0 mL/min was found to be robust method.

The developed method was validated as per the ICH guidelines for the quantification of Daclatasvir and Sofosbuvir in pharmaceutical formulations.

A suitability test was applied to various system suitability parameters and the results obtained were within acceptable limits of tailing factor ≤ 2.0 and theoretical plates >2000 .

The calibration curve was constructed with series of concentration in the range of 5-25 µg/mL and 2-10 µg/mL for Daclatasvir and Sofosbuvir. The correlation co-efficient of Daclatasvir and Sofosbuvir was found to be > 0.998 . This concluded that the method was linear throughout the range selected.

Specificity was studied for the quantification of excipients in the tablet dosage form of Daclatasvir and Sofosbuvir. From the results it was indicated that none of excipients were interfere at analytes retention time. Hence the developed method was specific.

The precision of the method was measured in terms of repeatability, which was determined by sufficient number of aliquots of a homogenous sample with in the day (intraday) and next consequent three days for inter day precision.

For each cases % RSD was calculated and results were the acceptable limits. The low values of RSD indicate that the method was precise.

The % recovery for each case was calculated and was found to be 100.36 to 100.72 % for Daclatasvir and 100.30 to 101.08 % for Sofosbuvir and found to be results were within acceptance limits. Hence the developed method is accurate throughout the selected range.

Robustness test was carried out by small variation in the chromatographic conditions and % change was calculated. The % change in the results was calculated and it was found robust as % change was below 2.0 %.

A signal-to-noise ratio 2:1 is generally considered acceptable for estimating the detection limit. LOD is found to be 0.313µg/mL for Daclatasvir and 0.021µg/mL for Sofosbuvir and LOQ is found to be 0.948µg/mL for Daclatasvir and 0.065µg/mL for Sofosbuvir.

Sample and standard solution are stable at 5°C for 36 hrs

as the % difference in the area was found to be less than 2.0 %. Filter interference was done on three types of 0.45µ filters (Nylon, PVDF, PTFE), and the % difference was found to be below 2.0 % for sample solutions and standard solutions calculated against centrifuged samples and standard.

The validated method was applied for the assay of commercial tablets of Daclatasvir and Sofosbuvir (HEPCINAT-PLUS Tablets: 400mg of Sofosbuvir and 60 mg of Daclatasvir). Peak area of the detector response was used to calculate % assay. The % assay was found to be 99.57 % for Daclatasvir and 99.38 % for Sofosbuvir. The results presented good agreement with the labelled content.

Thus the method developed in the present investigation is simple, sensitive, accurate, rugged, robust, rapid and precise. The absence of additional peaks in the chromatogram indicated that there is no interference of the common excipients used in the tablets. Hence, the developed method can be successfully applied for the estimation of Daclatasvir and Sofosbuvir in tablet dosage forms by RP-HPLC.

CONCLUSION

A new, reversed-phase HPLC method has been developed for simultaneous analysis of Daclatasvir and Sofosbuvir in a tablet formulation. It was shown that, the method was linear, accurate, reproducible, repeatable, precise, selective and specific proving the reliability of the method. The run time is relatively short (6.0 min), which enables rapid determination of many samples in routine and quality control analysis of tablet formulations. Hence, the proposed method was successfully applied to analyze preparation containing Daclatasvir and Sofosbuvir.

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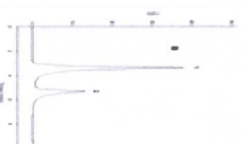
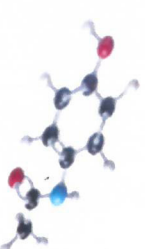
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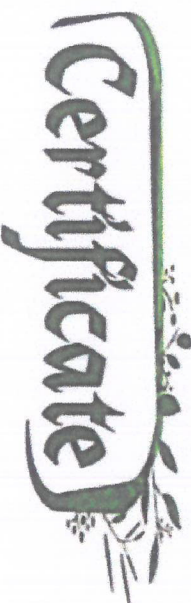


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PARTICIPATION CERTIFICATE

Mr. G. Nagaraju.

Title Simultaneous Estimation of Abacavir, Dolutegravir and Lamivudine By RP-HPLC
for his/her active participation and "Poster / Oral" Presentation during

7TH INDO-WEST INDIES INTERNATIONAL CONFERENCE

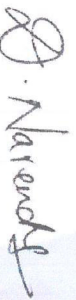
"Global Aspects of Drug Discovery, Drug Design & Pharmaceutical Technology"

organized by APP Tamilnadu State Branch and APP West Indies International Branch

in collaboration with APP DrugDesign MedChem Division at VJ's College of Pharmacy, Rajahmundry

East Godavari, Andhra Pradesh, in commemoration of "International Youth Day 2022".

Given on 17th day of August 2022.



DR. D. NARENDRA

Convener & Principal

VJ's College of Pharmacy, Rajahmundry
East Godavari, Andhra Pradesh



DR. SUNITA DAHIYA

General Secretary APP

School of Pharmacy, University of Puerto Rico
San Juan, Puerto Rico, USA



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Awards this

Certificate of Appreciation

This is to certify that Mr. G. Nagaraju for imparting his
Knowledge and Ideas as an exotic Speaker titled Analytical Validation Techniques by RP-HPCL in a One Week
Student Enhancement Programme on **Role of Pharmacy Teachers : A Holistic Approach to Create an End Product of
Education be a free Creative Man** from 12-09.2020 to 18.09.2020. Organized by Dept of Pharmaceutical Analysis in Association
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Dept. of Pharmaceutical Analysis
Vignan Pharmacy College

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STUDENTS' FORUM

11th NATIONAL IPA STUDENTS' CONGRESS - 2020

28-29, February 2020 (Friday - Saturday)

VENUE: VIGNAN INSTITUTE OF PHARMACEUTICAL TECHNOLOGY

Theme: Work Force for Future Needs



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CERTIFICATE OF PARTICIPATION

Mr/Ms.
This is to certify that

of *Chalapathi Institute of Pharmaceutical Sciences, Guntur.*

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Certificate of Participation

This Certificate is Awarded to Dr/Mr./Mrs./Ms. Gr. Nagaraju for successful completion of training in the area of Quantitative Structure Active Relationship, on July 15, 2019 organized by Department of Pharmaceutical Analysis, Chalapathi Institute of Pharmaceutical Sciences, Guntur in association with vLife Sciences Technologies, Pune and Alumni Association of CLPT.

Sri. Y. V. Anjaneyulu
President,
Chalapathi Educational Society
Lam, Guntur

Prof. K. N. Rajini Kanth
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