DEVELOPMENT OF VALIDATED UV SPECTROSCOPIC, HPTLC AND RP-HPLC METHODS FOR THE SIMULTANEOUS ESTIMATION OF LEDIPASVIR AND SOFOSBUVIR IN PURE AND FIXED DOSE COMBINATION

A Dissertation submitted to

THE TAMIL NADU Dr. M.G.R. MEDICAL UNIVERSITY,

CHENNAI – 600 032

In partial fulfilment of the requirements for the award of the Degree of

MASTER OF PHARMACY

IN

BRANCH–V- PHARMACEUTICAL ANALYSIS

Submitted by

S.SATHESHKUMAR
REGISTRATION No. 261530104

Under the guidance of

Dr. A. SUGANTHI, M.Pharm., Ph.D.,
Department of Pharmaceutical Analysis

COLLEGE OF PHARMACY
SRI RAMAKRISHNA INSTITUTE OF PARAMEDICAL SCIENCES
Coimbatore - 641 044.

OCTOBER 2017

CERTIFICATE

This is to certify that the dissertation entitled **"Development of Validated UV Spectroscopic, HPTLC and RP-HPLC Methods for the Simultaneous Estimation of Ledipasvir and Sofosbuvir in Pure and Fixed Dose Combination"** being submitted to The Tamil Nadu Dr.M.G.R Medical University, Chennai was carried out by **S.SATHESHKUMAR** in the **Department of Pharmaceutical Analysis,** College of Pharmacy, Sri Ramakrishna Institute of Paramedical Sciences, Coimbatore, under my direct supervision and guidance.

Dr. A. SUGANTHI, M.Pharm., Ph.D.,
Associate Professor,
Department of Pharmaceutical Analysis,
College of Pharmacy, SRIPMS,
Coimbatore-641 044.

Place: Coimbatore

Date:

CERTIFICATE

This is to certify that the dissertation entitled **"Development of Validated UV Spectroscopic, HPTLC and RP-HPLC Methods for the Simultaneous Estimation of Ledipasvir and Sofosbuvir in Pure and Fixed Dose Combination"** being submitted to The Tamil Nadu Dr.M.G.R Medical University, Chennai was carried out by **S.SATHESHKUMAR** in the **Department of Pharmaceutical Analysis,** College of Pharmacy, Sri Ramakrishna Institute of Paramedical Sciences, Coimbatore, under the supervision and guidance of **Dr. A. Suganthi, M.Pharm., Ph.D.,** Associate Professor, Department of Pharmaceutical Analysis, College of Pharmacy, Sri Ramakrishna Institute of Paramedical Sciences, Coimbatore.

Dr. T. K. RAVI, M. Pharm., Ph.D., FAGE.,
Principal & HOD,
Department of Pharmaceutical Analysis,
College of Pharmacy, SRIPMS,
Coimbatore-641 044.

Place: Coimbatore

Date:

ACKNOWLEDGEMENT

It's my honour to thank everyone personally who have stood by my side for the completion of my project work and without whose support I would have found it difficult to finish the work.

First and foremost I express my heartfelt gratitude to my lovable respected guide for her guidance, constant support in all aspects **Dr.A.Suganthi, M.Pharm., Ph.D.,** Associate Professor, Department of Pharmaceutical Analysis for her remarkable guidance, valuable suggestions and also sparing her time for me whenever needed throughout the work.

I also express gratitude to our beloved Principal and Head of the Department of Pharmaceutical Analysis **Dr.T.K.Ravi, M.Pharm., Ph.D., FAGE.,** for providing every need from time to time to complete the research work.

I extol my gratitude and respectful regards to our Managing Trustee, **Thiru.R. Vijayakumar** and the Management of SRIPMS for providing complete facilities required for the work.

My solemn thanks to my dear teachers, **Dr. M.Gandhimathi, M.Pharm., Ph.D., Dr.Susheel John Varghese, M.Pharm., Ph.D., Dr. Jayaprakasam, M.Pharm., Ph.D., Mrs.Sangeetha, M.Pharm (Ph.D).,** Department of Pharmaceutical Analysis and **Dr. K. Asok Kumar, M. Pharm., Ph.D., Professor & Head,** Department of Pharmacology for their timely help during the course of the work.

I sincerely thank to **Mr.Sorna Kumar.N** and **Dr. Jayaprakasam** providing drug for my project work.

A special thanks to **Mrs.Kalaivani** and **Mrs.Dhanalakshmi** for unstinted support at the time of need.

My heartfelt thanks to my dear **Naveen, Haritha, Pavithra, Suthakaran, Hariharshan, Nadha kumar, Rittu, Naseem, Sneha** who have helped me during my project work, their valuable support and advice at all the time.

I also thank to my seniors **Dhivya, Veerapandiyan, Arthi, Sangeetha, Arya, Aju, Liji, Sumithra, Aravind** and **Gopi.**

Above all I dedicate myself before the constant love and encouragement given to me by my beloved grandmother and Parents **K.Subamal, J.Rani, E.Sasi** and **S.Jansirani,** and my lovable caring sweet uncle **K.Jaishankar** who have been the backbone of all my achievements .

Above all, I bow with reverence before the gracious presence and boundless blessings of **"The Almighty"** who is the source of all wisdom and knowledge for the successful completion of this thesis work.

CONTENTS

ABBREVIATIONS

LEDI	-	Ledipasvir
SOFO	-	Sofosbuvir
Hrs	-	Hours
HPLC	-	High Performance Liquid Chromatography
HPTLC	-	High Performance Thin Layer Chromatography
ICH	-	International conference on harmonization
LOD	-	Limit of detection
LOQ	-	Limit of quantification
min	-	Minute
RP-HPLC	-	Reverse phase high performance liquid chromatography
RSD	-	Relative standard deviation
UV	-	Ultra violet
A_S	-	Asymmetric factor
gm	-	Gram
M	-	Molar
Mg	-	Milligram
mL	-	Millilitre
mM	-	Millimolar
mm	-	Millimeter
ng	-	Nanogram
R_f	-	Retardation factor
Rs	-	Resolution
Rt	-	Retention time
Tf	-	Tailing factor
µg	-	Microgram
µL	-	Microlitre
λ_{max}	-	Wavelength of maximum absorbance

INTRODUCTION [1-9]

Pharmaceutical analysis may be defined as the application of analytical procedures used to determine the purity, safety and quality of drugs and chemicals. Most manufacturing industries rely upon both qualitative and quantitative chemical analysis to ensure that the raw materials used meet certain specifications, and also to check the quality of the final product. The examination of raw materials is carried out to ensure that there are no unusual substances present which might be deleterious to the manufacturing process or appear as a harmful impurity in the final product. The final manufactured product is subjected to quality control to ensure that its essential components are present within a pre-determined range of composition, whilst impurities do not exceed certain specified limits.

Reasons for developing newer analytical methods for drugs:

- Marketed drug may not be official in pharmacopoeias.
- Literature search may not contain complete analytical procedure for such drugs.
- No proper method may be available for drug in the form of formulation due to interference caused by pharmaceutical excipients.
- Procedures for the estimation of drug in biological fluids may not be available.
- Analytical techniques may not be available for the drugs in combination with other drugs.
- Expenses may be more for reagents and solvents used in existing procedures.
- There may be difficulties in extraction and separation process and these may not be sensitive and time consuming.

Analytical Techniques

The instrument techniques employed for the drug analysis can be categorized into following techniques such as:

- Spectrophotometric techniques
- Electrochemical techniques
- Chromatographic techniques
- Hyphenated techniques
- Miscellaneous techniques

UV-VISIBLE SPECTROSCOPY

The technique of UV-Vis spectrophotometry is one of the most frequently employed in pharmaceutical analysis. It involves the measurement of amount of ultraviolet (190-380 nm) or visible (380-800 nm) radiation absorbed by a substance in solution. Absorption of light in both the ultraviolet and visible regions of the electromagnetic spectrum occurs when the energy of the light matches that required to induce in the molecule an electronic transition and its associated vibrational and rotational transitions.

Many molecules absorb ultraviolet or visible light. The absorbance of a solution increases as the attenuation of beam increases. Absorbance is directly proportional to the absorptivity, *a,* path length, *b*, and the concentration, *c*, of the absorbing species. *Beer's Law* states that

$$A = abc$$

Optimum conditions for spectrophotometric measurement

In developing an analytical method based on UV visible spectrophotometry most appropriate sample and instrumental conditions are required. The accuracy and precision of spectrophotometric measurements depend on the intensity of stray light and the choice of sample conditions(solvents, concentration and path length) and instrumental parameters(wavelength, slit width and scan speed).

Selection of solvent

The choice of solvent is governed by the solubility of absorbing substance and by the absorption of the solvent at the analytical wavelength. The solubility of the substance in polar and non-polar solvents can often be predicted from a consideration of its chemical structure.

Selection of analytical wavelength

In spectrophotometer the wavelength of maximum absorbance is selected by wavelength scan, the wavelength at which the maximum absorbance was attained when scanned in the range of 200-400 nm was selected as detection wavelength for the method.

SIMULTANEOUS EQUATION METHOD

If a sample containing two absorbing drugs (X and Y) each of which absorbs at the λ_{max} of the other , it may be possible to determine both drugs by simultaneous equation method(vierodt's method.

$C_x = A_2ay_1 - A_1ay_2 / ax_2ay_1 - ax_1 ay_2$

$C_y = A_2ax_2 - A1ax_1 / ax_2ay_1 - ax_1 ay_2$

Where,

C_x and C_y = Concentration of both drug respectively

A_1 and A_2 = Absorbance's of the unknown sample at λ1 and λ2 respectively

ax_1 and ax_2= Absorptivity's of the unknown sample (x) at λ1 and λ2 respectively

ay_1 and ay_2 = Absorptivity's of the unknown sample (y) at λ1 and λ2 respectively

The criteria are satisfied only when the λ_{max} of the two components are reasonably dissimilar and additional criterion is that the two components do not interact chemically.

DERIVATIVE SPECTROPHOTOMETRY

Derivative spectrophotometry is mainly used in the process of conversion of a simple spectrum in to its first, second or higher order derivative spectrum. The transformations that occur in the derivative spectra are understood by reference to a Gaussian band which represents an ideal absorption band.

The second derivative (D^2) spectrum is a plot of the $d^2A/d\lambda^2$ vs. λ. It is characterized by two satellite maxima and an inverted band of which the minimum corresponds to the λ_{max} of the fundamental band.

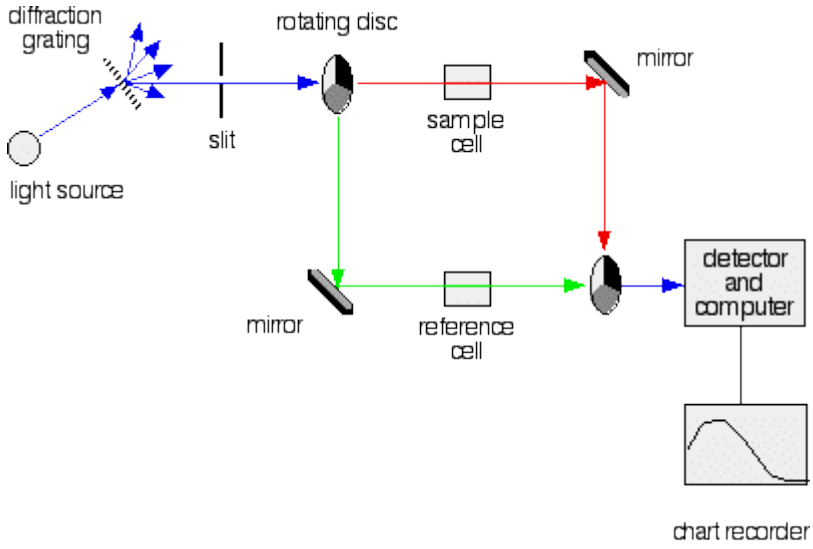
These spectral transformations confer two principal advantages on derivative spectrophotometry.

1. Derivative spectrum shows better resolution of overlapping bands than the fundamental spectrum and may permit the accurate determination of the λ_{max} of the individual bands,
2. Derivative spectrophotometry discriminates in favour of substances of narrow spectral bandwidth against broad bandwidth substances.

The substances of narrow spectral bandwidth display larger derivative amplitudes than those of broad bandwidth substances.

These advantages of derivative spectrophotometry, enhanced resolution and bandwidth discrimination, permit the selective determination of certain absorbing substances in samples, and most importantly, to reduce the effects of interference from scattering, matrix, or other absorbing compounds in quantitative analysis.

INSTRUMENTATION OF UV-VISIBLE SPECTROSCOPY



CHROMATOGRAPHY

Chromatography is defined as a method of separating a mixture of components into individual components through equilibrium distribution between two phases. The technique of chromatography is based on the differences in the rate at which the components of a mixture move through a porous medium (called stationary phase) under the influence of some solvent or gas (called moving phase).

The chromatographic method of separation, in general, involves the following steps:

- Adsorption
- Separation of the adsorbed substances by the mobile phase.
- Recovery of the separated substances by a continuous flow of the mobile phase; the method being called elution.
- Qualitative and quantitative analysis of the eluted substances.

HPLC and HPTLC comes under the classification of liquid chromatography and principle involved in the separation of components is adsorption.

HIGH PERFORMANCE THIN LAYER CHROMATOGRPHY

HPTLC is a versatile separation powerful, reliable and efficient method of quantitative and qualitative analysis, HPTLC can simultaneously handle several samples even of divergent nature and composition supporting several analysts at a given time. HPTLC is the most simple separation technique today available to the analyst. It is considered as a time machine that can speed up your work and allows you to do many things at a time usually not possible with other analytical techniques.

The basic difference between conventional TLC and HPTLC is particle and pore size. In HPTLC high performance grade of silica in which the particle are small about 5µm and very uniform in size are used. The high performance silica gel gives more efficient and reproducible separation than conventional grade of silica. The plates are smaller and the development time is shorter.

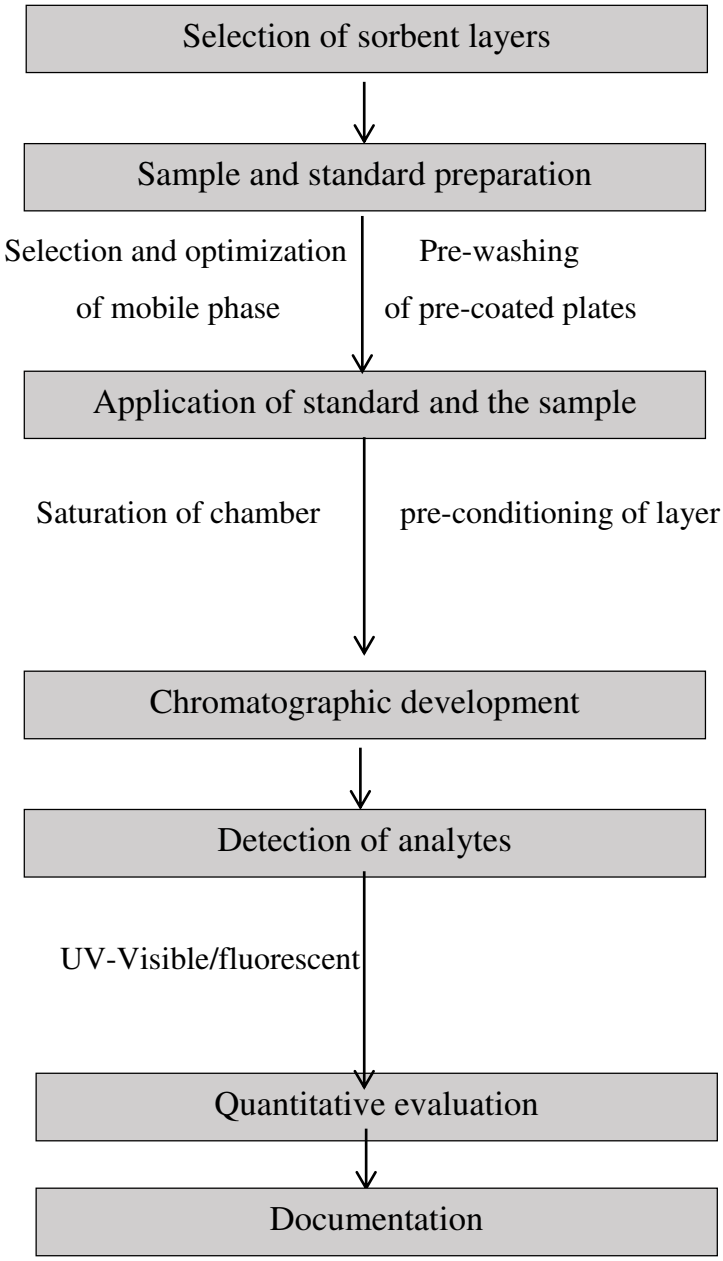
Principle

HPTLC is one of the most widely used methods for both quantitative and qualitative analysis. The principle of separation is adsorption. The mobile phase flows through because of capillary action. The components are separated based on the affinity of the components towards the stationary phase.

Advantages of HPTLC:

- Visual chromatogram and simplicity
- Multiple sample handling
- Quantification of crude drugs
- Simultaneous analysis of samples
- Small quantity of mobile is required
- Automatic sample application

Various steps involved in HPTLC chromatography



There are different HPTLC plates available:

- ❖ Hand-made plates(self-prepared layers)
- ❖ Pre-coated plates
- ❖ Polyester sheets
- ❖ Glass support
- ❖ Aluminium sheets

Plate size

Pre-coated HPTLC plates in size of 20×20 with aluminium or polyester support are usually procured mainly for economic reasons. These plates can be cut into size and shape to suit a particular analysis.

Sample preparation

Proper sample preparation is an important pre-requisite for successful chromatographic separation. The sample preparation procedure is to dissolve the dosage form with complete recovery of intact compounds of interest and minimum of matrix with a suitable concentration of analytes for direct application on the HPTLC plate.

Choice of solvent for the sample

- ▪ It should dissolve the analytes.
- ▪ It should be reasonably volatile.
- ▪ It should have low viscosity.
- ▪ It should wet the sorbent layer.

Sample application

This is the most critical step for obtaining good resolution for quantification by HPTLC. Automatic application devices are recommended for quantitative analysis. The advantages of application of sample as band are even distribution of the sample, better resolution and greater accuracy. Application in small increments has disadvantage.

Chamber saturation:

Chamber saturation has pronounced influence on the separation profile. When the plate is introduced into an unsaturated chamber, during the course of development, the solvent evaporates from the plate mainly at the solvent front. Therefore larger quantity of the solvent shall be required for a given distance, hence resulting in increase in R_f values. If the tank is saturated prior to development, solvent vapours soon get uniformly distributed throughout the chamber.

Chromatographic development

The precoated plate then placed in the saturated chamber containing mobile phase and allowed to run the desired running distance and then kept out for drying.

Detection:

After completion of the development process the plate is removed from the chamber and dried to remove the mobile phase completely. The zone can be visualized by various physical and chemical methods. There is apparently no difficulty in detecting coloured substances or colourless substances absorbing in shortwave ultraviolet region. The substances which do not have such properties have to be transferred into detectable substances by means of using chromatographic reagents. Detection under UV light is the first choice and non-destructive in most cases and is commonly employed for densitometric scanning.

HIGH PERFORMANCE LIQUID CHROMATOGRAPH

A variety of methods are available for analysing pharmaceutical compounds; however HPLC is currently the method of choice for the analysis of these compounds. HPLC offers high performance over ambient pressure or low pressure liquid chromatography.

High performance liquid chromatography is used in analytical development to quantitate the active pharmaceutical ingredient (API) and to evaluate impurity and degradation product profiles of drugs substances and drug products. Additional uses of HPLC include the determination of content uniformity of dosage forms, monitoring of dissolution profiles, determination of antioxidant and microbial preservative content and support of cleaning validations. Separation of these types require only a monitoring of one, or a limited number of predefined compounds. A significantly larger challenge is presented in the composite assays of drug substances and drug products where the goal is to quantitate API and relevant impurities and degradation products in a single chromatographic run.

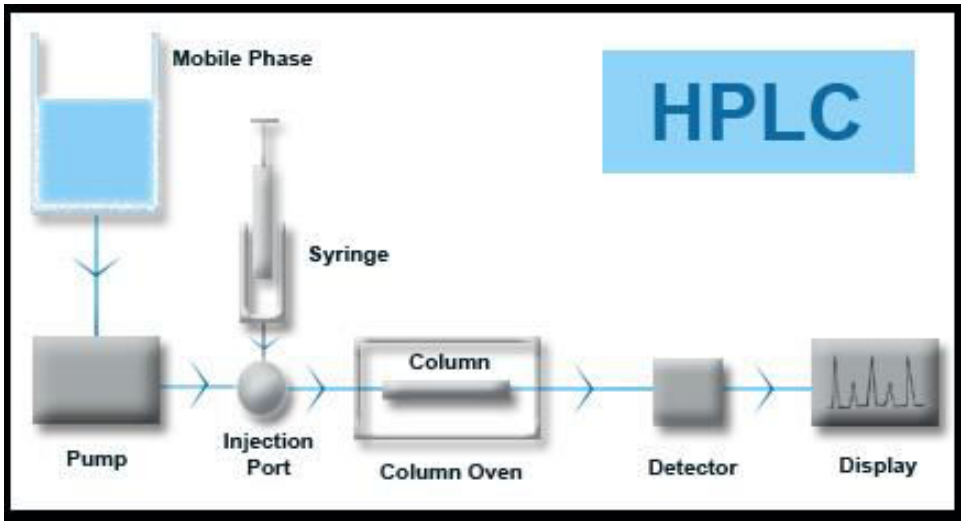
The development of any new or improved method usually tailors existing approaches and instrumentation to the current analyte, as well as to the final needs or requirements of the method. Method development usually requires selecting the requirements and deciding on what type of instrumentation to utilize at the development stage, decisions regarding

Choice of column,

Mobile phase,

Detector(s) and

Method of quantitation must be addressed.



Goals for new or improved HPLC method might include the following:

1. Qualitative identification; the specific analyte(s) of interest, providing some structural information to confirm general behaviour(i.e. retention time, pH)
2. Quantitative determination; at trace level when necessary (i.e. accurate, precise and reproducible in any laboratory setting when performed according to established procedures).
3. Ease of use, ability to be automated, high sample throughout and rapid sample turn around time.
4. Sample preparation that minimizes time, effort, materials and volume of sample consumed.

The following criteria are to be met for developing method:

i. **For drug substances**

Methods should separate the API, synthetic process impurities, and drug substances and degradation products. Methods should be able to detect impurities and degradation products present at level greater than 0.05% relative to the API. Impurities and degradation products present at the levels greater than 0.1% should be identified and specifications should be placed on limits.

ii. For drug products

Methods should separate the API, drug products degradation products from excipients. Drug products methods are not required to monitor synthetic process impurities, unless they are also drug product degradation products. Methods should be able to detect degradation products present at level greater than 0.1% relative to API. Degradation products present at level greater than 0.2% should be identified and specifications should be placed on limits.

Types

Most commonly used methods in chromatography are as follows:

- *Normal–phase chromatography:*

Normal phase chromatography depends upon the interaction of stationary phase's polar surface with polar parts of the sample molecules.

- *Reversed-phase chromatography:*

The mechanism is retention by interaction of stationary phase's non-polar hydrocarbon chain with non-polar parts of the sample molecules.

- *Reversed phase ion-pair chromatography:*

The technique is based on ionic sample molecules that are ionically bound to an ion-pair reagent. The ion-pair reagent contains an unpolar part suitable for interaction with the unpolar hydrocarbon chain of the stationary phase.

- *Ion-exchange chromatography:*

Ion-exchange chromatography is due to the retention by reversible ionic bonds to charged groups on the stationary phase.

System suitability parameters

Parameters concerning column specification and performance are needed for use in method development and they are as follows:

- **Plate number (N):**

The column plate number (N) is an important characteristic of a column. N defines the ability of the column to produce sharp, narrow peaks for achieving good resolution of band pairs with small values.

$$N = \frac{13500\ L\ (cm)}{dp\ (\mu m)}$$

Where, L = Column length

T_R = Band retention time

dp = Diameter of the particle

- **Peak asymmetry and peak tailing:**

Peak shape is important in method development. Peaks with poor symmetry can result in:

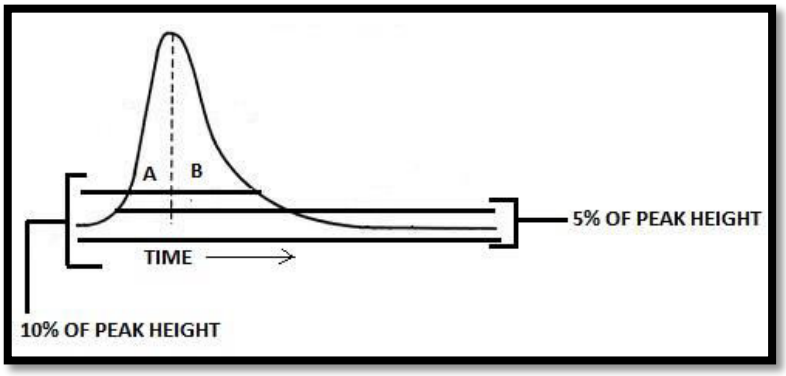
* Inaccurate plate number and resolution measurement
* Imprecise quantitation
* Degraded resolution and undetected minor bands in the peak tail
* Poor retention reproducibility

A useful and practical measurement of peak shape is the peak asymmetry factor, A_s. Peak asymmetry is measured at 10% of full peak height. Good columns produce peaks with A_s values of 0.95 to 1.1.

Another useful way to define peak shape is the tailing factor and is calculated at 5% of full peak height.

Peak asymmetry factor = B/A

Peak tailing factor = A+B/2A



Where, A = Distance between the perpendicular connecting the baseline to the peak maximum and the earliest eluting portion of the curve

B = Distance between the perpendicular connecting the baseline to the peak maximum and the latest eluting portion of the curve

- **Retention:**

The retention of a drug with a given packing material and eluent can be expressed as retention time or retention volume, but both of this are dependent on flow rate, column length and column diameter. The retention is best described as a column capacity ratio (k), which is independent of these factors. The column capacity ratio of a compound (A) is defined as

$$k_A = \frac{V_A - V_o}{V_o} = \frac{t_A - t_o}{t_o}$$

Where, V_A = Elution volume of A

V_0 = Elution volume of a non-retained compound (void volume)

- **Resolution:**

Measure of quality of separation of adjacent bands and it is calculated from width and retention time of two peaks or separation of two peaks.

$$R_s = \frac{2(t_2 - t_1)}{W_1 + W_2}$$

Where, t_1 and t_2 = Retention time of first and second adjacent bands for the latest and the earliest eluting peak

W_1 and W_2 = Baseline bandwidth

$R_S \geq 1$ = Components completely separated

$R_S \leq 1$ = Component overlap

- **Capacity Factor (k'):**

Capacity factor is the measure of position of a sample peak in the chromatogram. It is a parameter which specifies the extent of the delay of a substance to be separated. It depends upon stationary phase, mobile phase, temperature and quality of column packing.

For good chromatographic performance with isocratic separation, k' value should be in the range of 1-10. If k' is less than 1; the bands are inadequately separated from unretained material eluting at t_0 and if k' is more than 10; separation takes too long and bands broadened.

$$k'_1 = t_{R1} - t_0 / t_0 \quad ,$$

$$k'_2 = t_{R2} - t_0 / t_0$$

Where, $t_{R=}$ Retention time

t_0 = Void volume

- **Separation Factor (α):**

Separation factor is also known as selectivity. It is the measure of peak spacing and is expressed as

$$\alpha = \frac{K'_2}{K'_1}$$

Where, subscripts refer to the order of elution.

α is always ≥ 1

FORCED DEGRADATION STUDIES

Chemical stability of pharmaceutical molecules is a matter of great concern as it affects the safety and efficacy of the drug product. The FDA and ICH guidance's state the requirement of stability testing data to understand how the quality of a drug substance and drug product changes with time under the influence of various environmental factors. Knowledge of the stability of molecule helps in selecting proper formulation and package as well as providing proper storage conditions and shelf life, which is essential for regulatory documentation. Forced degradation is a process that involves degradation of drug products and drug substances at conditions more severe than accelerated conditions and thus generates degradation products that can be studied to determine the stability of the molecule. The ICH guideline states that stress testing is intended to identify the likely degradation products which further helps in determination of the intrinsic stability of the molecule and establishing degradation pathways, and to validate the stability indicating procedures used.

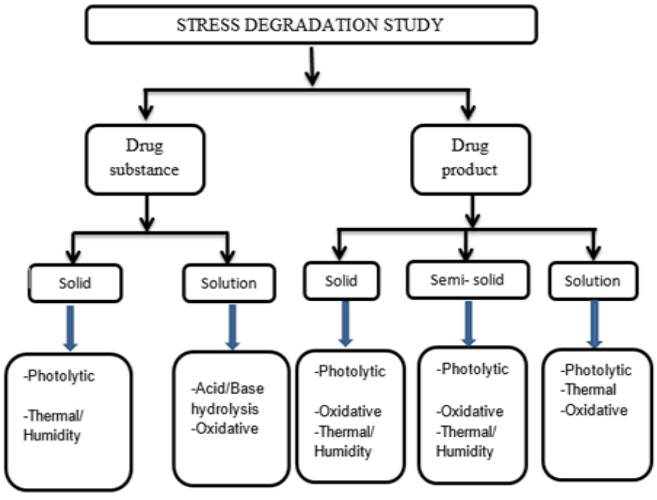
Forced degradation studies provide knowledge about possible degradation pathways and degradation products of the active ingredients and help to elucidate the structure of the degradants. Degradation products generated from forced degradation studies are potential degradation products that may or may not be formed under relevant storage conditions but they assist in the developing stability indicating method. It is better to start degradation studies earlier in the drug development process to have sufficient time to gain more information about the stability of the molecule. This information will in turn help improve the formulation manufacturing process and determine the storage conditions. The aim of any strategy used for forced degradation is to produce the desired amount of degradation i.e., 5 – 20%.

OBJECTIVES OF FORCED DEGRADATION STUDIES

Forced degradation studies are carried out to achieve following purposes:

- ❖ To establish degradation pathways of drug substances and drug products.
- ❖ To differentiate degradation products that is related to drug products from those that are generated from non-drug product in a formulation.
- ❖ To elucidate the structure of degradation products.
- ❖ To determine the intrinsic stability of a drug substance in a formulation.
- ❖ To reveal the degradation mechanisms such as hydrolysis, oxidation, thermolysis or photolysis of the drug substance and drug product.
- ❖ To establish stability indicating nature of a developed method.
- ❖ To understand the chemical properties of drug molecules.
- ❖ To generate more stable formulations.
- ❖ To produce a degradation profile similar to that of what would be observed in a formal stability study under ICH conditions.
- ❖ To solve stability related problems.

The ICH guidelines entitled "stability testing for new drug substance and products" (Q1A) requires that stress testing is carried out to elucidate the inherent stability characteristics of temperature, humidity, where appropriate, oxidative photolysis and susceptibility to hydrolysis.



ANALYTICAL METHOD VALIDATION

The work presented is validated by ICH guidelines Q2 (R1). The validation parameters being linearity, precision, accuracy, limit of detection, limit of quantification and robustness.

Specificity:

Specificity is the ability of the method to accurately measure the analyte response in the presence of all the potential sample components. The response of the analyte in test mixtures containing the analyte and all the potential sample components is compared with the response of a solution containing only the analyte.

Linearity:

The ability of the procedure to obtain results of the test directly proportional to the concentration of the analyte present in the sample. This will result in a linear relationship. This relationship is evaluated by statistical methods. To obtain linearity a minimum of 5 concentrations are needed. The parameters to be calculated are coefficient correlation, y intercept and slope.

Range:

The range of an analytical method is the concentration over which acceptable accuracy, linearity, and precision are obtained in practice; the range is determined using data from the linearity and accuracy studies.

Accuracy:

The accuracy of an analytical procedure expresses the closeness of agreement between the value which is accepted either as a conventional true value or an accepted reference value and the value found.

Precision:

The precision of an analytical method is the amount of scatter in the results obtained from multiple analyses of a homogenous sample. It is determined at three levels.

a) Reproducibility

When the subject method is carried out by different analysts of different laboratories using different experimental conditions using the samples from same homogenous batch.

b) Intermediate precision

It is determined by relating the results of a method within the same laboratory but different days, analysts, equipments and reagents.

Limit of Detection (LOD)

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.

$$DL=3.3\sigma/S$$

Limit of Quantitation (LOQ)

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. The quantitation limit is a parameter of quantitative assays for low levels of compounds in sample matrices, and is used particularly for the determination of impurities and/or degradation products.

$$DQ=10\sigma/S$$

Robustness

The robustness of a method is its ability to remain unaffected by small changes in parameters such as percentage organic concentration and pH of mobile phase, concentration of buffer, temperature, injection volume and flow rate.

LITERATURE REVIEW [10-15]

- Vikas p. *et al*. (2016) developed a validated RP-HPLC method for the determination of sofosbuvir in pure form. Separation of Sof was successfully achieved on a Hisil C_{18} (4.6 x 250mm, 5 µm) Waters or equivalent in an isocratic mode utilizing Phosphate Buffer (4.0 pH): Methanol (50:50%v/v) at a flow rate of 0.8 mL /min and eluate was monitored at 262 nm, with a retention time of 1.01 minutes. The method was validated and the response was found to be linear in the concentration range of 5 µg/mL to 30µg/mL.

- Devilal.j. *et al. (2016)* described a RP- HPLC method for the determination of ledipasvir in bulk drug form. The procedure involved use of isocratic elution where the stationary phase was a ODS column (250 mm, 4.6 mm, 5 µm), mobile phase 0.05% triflouroacetic acid in methanol and 0.05% triflouroacetic acid in acetonitrile (55:45). pH of the chromatographic system was maintained at 3.0, flow rate 1 ml/minute, eluent was monitored by PDA detector wavelength at 270 nm. Retention time was found to be 2.749 minutes, regression analysis shown the value of correlation coefficient 0.999. Value for limit of detection (LOD) was 1.064µgm/ml and limit of quantification (LOQ) was 3.224 µg/ml. Linearity range was designed 15µg/ml to 300µg/ml for Ledipasvir. Accuracy study revealed percentage recovery 99.81%-100.10%.

- Chenwei.P. *et al. (2015)* established a UPLC–MS/MS method for the Simultaneous determination of ledipasvir, sofosbuvir and its metabolite in rat plasma and its application to a pharmacokinetic study. The analytes and the internal standard (diazepam) were separated on an Acquity UPLC BEH C_{18} chromatography column (2.1 mm × 50 mm, 1.7 m) 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.

- Nebsen.M. *et al. (2016)* has developed a LC–MS/MS method for Characterization of Forced Degradation Products of Sofosbuvir. However, it was stable under thermal and neutral hydrolysis stress conditions. Chromatographic separation of the drug from its degradation products was performed on Inertsil ODS-3 C_{18} (250mm × 4.6mm i.d., 5 μm) column using a methanol:water 70:30 (v/v).

- Elkady.F. *et al. (2016)* presented *a* LC-MS/MS Method for the Simultaneous Extraction and Determination of Sofosbuvir and Ledipasvir in Human Plasma. The chromatographic separation of the three analytes was achieved within 2.8 min by an isocratic mobile phase consisting of 10 mM ammonium acetate, (pH 4.0) by acetic acid–acetonitrile–0.1% methanolic formic acid (12 + 25 + 63, v/v/v) flowing through a C_{18} Zorbax eclipse plus column (5 μm, 100 × 4.6 mm; Agilent). Linearity range was found to be 0.5 to 2500 and 5 to 2100 ng/mL for sofosbuvir and ledipasvir, respectively.

- Swain.D. *et al.(2017)* analysed a LC-QTOF-MS/MS and NMR methods for the forced degradation behaviour of ledipasvir. Identification of major degradation products using Ledipasvir degraded in hydrolytic (acid, alkaline and neutral) and oxidative stress conditions. The drug was found to be stable in thermal and photolytic conditions. The degradation products obtained were well separated using an HPLC C_{18} stationary phase (150 x 4.6 mm, 5 μ) and mobile phase composed of formic acid/acetonitrile in gradient elution mode. All the degradation products were characterized using tandem mass spectrometry with a time-of-flight analyser.

AIM AND OBJECTIVE [16-18]

Hepatitis C is an infectious disease caused by the hepatitis C virus (HCV) that primarily affects the liver. During the initial infection people often have no symptoms. Occasionally a fever, dark urine, abdominal pain and yellow tinged skin occurs. The virus persists in the liver 75%- 85%.

There is no vaccine currently against hepatitis C (however research in this area is ongoing). Antiviral medicines such as ledipasvir and sofosbuvir (cimivir-L tablet) can cure more than 95% of persons with hepatitis C, which is lifesaving drug. An estimated 143 million people (2%) worldwide are infected with hepatitis C as of 2015. It occurs most commonly in Africa and central and East Asia. Each year 3,99,000 people died for hepatitis C.

Literature survey revealed that no Spectrophotometric and HPTLC methods for the simultaneous estimation of Ledipasvir and Sofosbuvir. This combination tablet approved by FDA (10, Oct, 2014) which is a lifesaving drug. Which is not official in any of the pharmacopoeias and hence requires much more investigation.

Hence an attempt has been made to develop new, simple and economic and validated methods for the determination of Ledipasvir and Sofosbuvir in bulk and formulation

Hence, the objectives of the present work is to develop a validated

- UV-spectroscopic methods for the simultaneous estimation of ledipasvir and sofosbuvir in bulk and formulation.
- HPTLC method for the simultaneous determination of ledipasvir and sofosbuvir in bulk and formulation.
- RP-HPLC method for the simultaneous determination of ledipasvir and sofosbuvir in bulk and formulation.

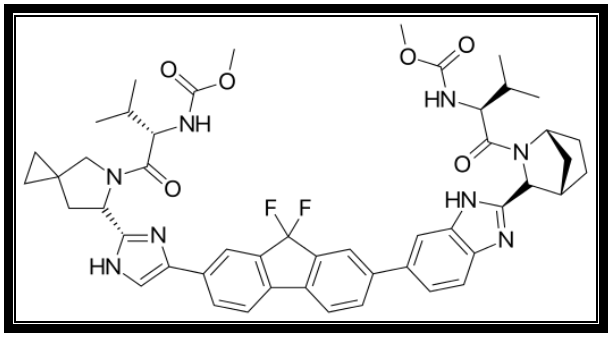
DRUG PROFILE [19, 20]

Name : Ledipasvir

IUPAC Name : methyl N-[(2S)-1-[(6S)-6-[5-[9,9-difluoro-7-[2-[(1S,2S,4R)-3-[(2S)-2-(methoxycarbonylamino)-3-methylbutanoyl]-3-azabicyclo[2.2.1]heptan-2-yl]-3H-benzimidazol-5-yl]fluoren-2-yl]-1H-imidazol-2-yl]-5-azaspiro[2.4]heptan-5-yl]-3-methyl-1-oxobutan-2-yl]carbamate

Empirical Formula : $C_{49}H_{54}F_2N_8O_6$

Structure Formula :



Molecular Weight : 889.018 g/mol

Description : Slightly yellow solid

Solubility : Freely soluble in methanol.

Bioavailability : 76%

Storage : 25°C

Category : Antiviral agent (Hepatitis C virus)

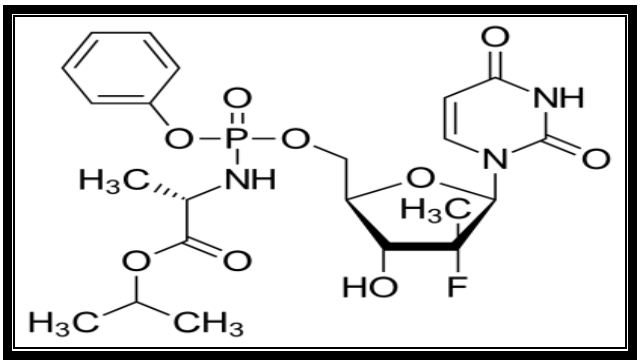
Dose : 90mg

Name : Sofosbuvir

IUPAC Name : propan-2-yl (2S)-2-[[[(2R,3R,4R,5R)-5-(2,4-dioxopyrimidin-1-yl)-4-fluoro-3-hydroxy-4-methyloxolan-2-yl] methoxy-phenoxyphosphoryl] amino]propanoate

Empirical Formula : $C_{22}H_{29}FN_3O_9P$

Structure Formula :



Molecular Weight : 529.458 g/mol

Description : White to off-white solid

Solubility : Freely soluble in methanol.

Bioavailability : 92%

Storage : 30ºC

Category : Antiviral agent (Hepatitis C virus)

Dose : 400mg

BRANDS AVAILABLE

Brand names	Strength(mg)	Manufacturer
Harvoni	Ledipasvir 90/sofosbuvir 400	Mylan
Hepcinat	Ledipasvir 90/sofosbuvir 400	Natco
Hepcvir-L	Ledipasvir 90/sofosbuvir 400	Cipla
Ledviclear	Ledipasvir 90/sofosbuvir 400	Abbott
Resof-L	Ledipasvir 90/sofosbuvir 400	Dr.Reddy
Cimivir-L*	**Ledipasvir 90/sofosbuvir 400**	**Biocon**
Sofocure-L	Ledipasvir 90/sofosbuvir 400	Emcure
Sofab-LP	Ledipasvir 90/sofosbuvir 400	Ranbaxy
Ledihep	Ledipasvir 90/sofosbuvir 400	Zydus Cadila
Ledifos	Ledipasvir 90/sofosbuvir 400	Hetero
Virpas	Ledipasvir 90/sofosbuvir 400	Strides Shasun

*Formulation used in present work

MATERIALS AND INSTRUMENTS

Active Pharmaceutical Ingredient (API)

Ledipasvir and sofosbuvir were procured from sequent Labs Pvt. Ltd., Mangalore, India.

Formulation

Cimivir-L tablet, Ledipasvir 90 / sofosbuvir 400 from Biocon Pharmaceuticals, Bangalore.

Chemicals and solvents used

- ✓ Water- HPLC grade
- ✓ Methanol HPLC grade, AR grade
- ✓ Acetonitrile - HPLC grade
- ✓ Formic acid- AR grade
- ✓ Toluene- LR grade
- ✓ Chloroform- LR grade
- ✓ Sodium hydroxide- LR grade
- ✓ Hydrochloric acid- AR grade
- ✓ Hydrogen peroxide 6 %-LR grade
- ✓ Hydrogen peroxide 30 %-LR grade
- ✓ Ammonium acetate
- ✓ Triethyl amine
- ✓ Ammonia 25%
- ✓ Ethyl acetate
- ✓ Hexane
- ✓ Orthophosphoric acid

All the above chemicals and solvents were supplied by S.D. Fine chemicals Ltd., India, Sigma-aldrich Chemicals Pvt. Ltd., Maharashtra, India and Ranbaxy chemicals Ltd., New Delhi, India.

MATERIALS USED

- ✓ Pre-coated silica gel $60F_{254}$ on aluminium sheets were procured from Merck, Germany.
- ✓ Hibar, C_{18} column (250mm×4.0mm, 5µm) column was obtained from Merck Pvt. Ltd., Mumbai.

INSTRUMENTS USED

1. Shimadzu digital electronics balance
2. Systronics Pvt. Limited, India, pH meter
3. Jasco V-630 UV/ Vis- spectrophotometer
4. Camag HPTLC system (with TLC Scanner-3, Win CATS software and Linomat 5 as application device)
5. Shimadzu HPLC prominence *i* LC-*2030* liquid chromatography system with UV-VISIBLE detector and auto sampler injector. Chromatogram were recorded and integrated on PC installed with lab solutions chromatographic sofotware.
6. Shimadzu HPLC system with SPD-M10 A VP system PDA with 20µl fixed volume manual injector and LC-MS solution software.

DEVELOPMENT OF VALIDATED UV-SPECTROSCOPIC METHODS FOR THE SIMULTANEOUS ESTIMATION OF LEDIPASVIR AND SOFOSBUVIR IN BULK AND FORMULATION

Ledipasvir and sofosbuvir is having π electron in its structure and hence absorbs electromagnetic radiation. UV- spectroscopic methods are simple and suitable to determine ledipasvir and sofosbuviri in pharmaceutical formulation.

SELECTION OF PARAMETERS FOR METHOD DEVELOPMENT:

1. SELECTION OF SOLVENT

The solubility of ledipasvir and sofosbuvir were checked in various solvents like methanol and acetone, etc. The one which showed good spectrum and good stability was selected as solvent of choice for the determination of ledipasvir and sofosbuvir.

2. PREPARATION OF STOCK SOLUTION

Ten milligram of pure drug ledipasvir and sofosbuvir were weighed, transferred and made up to 10 ml with methanol in a standard flask previously rinsed with methanol to get concentration of 1000 µg/ml. From the stock 1ml was diluted to 10 ml, to get a concentration of 100µg/ml. Further, the working standard solutions were prepared from 100µg/ml. The prepared stock solution was used for further studies.

3. SELECTION OF WAVELENGTH

After selection of suitable solvent for ledipasvir and sofosbuvir and preparation of stock solution, a solution containing 20µg/ml of ledipasvir and sofosbuvir was prepared and scanned in the range of 200 to 400 nm against methanol as blank. This was known as normal spectrum. Further this was derivatised to obtain second order derivative spectrum.

VALIDATION

LINEARITY AND RANGE

From the stock solution containing 100µg/ml of ledipasvir about 1 to 4ml and sofosbuvir 1 to 7ml were transferred into 10ml volumetric flasks and the working standards were prepared by making up to the volume using methanol to get concentration of ledipasvir about 10-40µg/ml and sofosbuvir about 10-70µg/ml. The absorbances were noted for all the standard solutions of ledipasvir and sofosbuvir at the selected wavelength. Standards were scanned in the wavelength range of 200 nm – 400 nm. The linear graph was plotted between concentrations (µg/ml) versus absorbance. Then it is derivatised to second order derivative and the amplitudes (mm) were measured at ledipasvir 333nm and sofosbuvir 261nm. The respective calibration graphs were prepared by plotting concentration against corresponding amplitude.

PRECISION

Precision of the method was determined by repeating the assay six times. Repeatability was carried out using 20µg/ml for both drugs normal and second order derivative methods. To study intraday precision, it was repeated 3 times on the same day using 20 and 30 µg/ml for both drugs and % RSD was calculated. Similarly the interday precision was performed by repeating the above procedure on different days and average of % RSD was calculated. Precision studies were carried out for both the methods.

RECOVERY STUDIES

To study the accuracy of the proposed method, standard addition procedure was adopted. To a pre - analysed sample solutions a known quantity of standard ledipasvir and sofosbuvir were added at 50%, 100% and 150% levels. Recovery was calculated from the ratio of difference between the amount of drug found after the addition of standard drug and amount of analyte present in preanalysed formulation to that of the standard ledipasvir and sofosbuvir added to the formulation.

LOD AND LOQ:

From the limit of detection and limit of quantification the sensitivity of the method was determined. The LOD and LOQ were calculated using the equation,

$LOD = 3.3 \times \sigma/S$

$LOQ = 10 \times \sigma/S$

Where, σ is the standard deviation of y intercept of regression line, & S is slope of calibration curve.

STABILITY STUDIES:

When the prepared solution is exposed to atmosphere, the analytes are likely to decompose; hence it is necessary to conduct stability studies. Stability of the analyte in methanol was studied, at room temperature for 8 hours and in refrigeration for 4 days and absorbance was compared with the absorbance of freshly prepared solution.

APPLICATION OF THE PROPOSED METHODS FOR ANALYSIS OF FORMULATION:

Cimivir-L was assayed by Zero and second order UV spectrophotometric methods. Twenty tablets were weighed and finely powdered. The powder mass equivalent to 5mg of ledipasvir and sofosbuvir were accurately weighed and transferred to 50ml standard flask containing 10ml of methanol and made up to volume with the same and sonicated for dissolution for five minutes. The solution was filtered using whatman(no.1) filter paper. Suitable dilutions were made with methanol to get the concentrations of 10µg/ml for ledipasvir and 44µg/ml for sofosbuvir. Normal and second order derivative were recorded against methanol as blank. The content of cimivir-L was calculated using respective calibration graph.

DEVELOPMENT AND VALIDATION OF HPTLC METHOD FOR THE SIMULTANEOUS DETERMINATION OF LEDIPASVIR AND SOFOSBUVIR IN BULK AND FORMULATION

The experimental work consists of optimization of chromatographic conditions development and validation of HPTLC method of ledipasvir and sofosbuvir.

1. Selection of plate

A pre-coated silica gel G_{60} F_{254} coated on aluminum sheet was selected for the study.

2. Selection of solvent

Drug should be soluble in the solvent used.

Drug should show stability in the solvent used.

Solvent should be volatile.

3. Selection of wavelength

The sensitivity of HPTLC method depends upon the proper selection of wavelength for UV detection. An ideal wavelength is that the overlay that give maximum absorbance and good response for the drug to be selected at the lower concentration is to be selected. The UV spectrum of ledipasvir and sofosbuvir were scanned 200-400nm in HPTLC scanner and the isobestic point was selected for the further study.

4. Optimization of mobile phase system

Initially different solvent systems were tried and A solvent system with compact spots and good separation has been selected for the study.

5. Selection of ratio of mobile phase system

Different ratios hexane : ethyl acetate : methanol (3:5:2, 8:2:2, 5:3:2, v/v/v etc) were tried along with few drops of ammoniato get dense compact spots with good resolution**.**

6. Optimization of chamber saturation

After selection of mobile phase ratio the twin trough chamber was saturated for 15 minutes to 25 minutes using mobile phase system fix the chamber saturation time.

7. Preparation of stock solutions

The mixed stock solution of ledipasvir (100µg/ml) and sofosbuvir (440µg/ml) was prepared using methanol in 10ml volumetric flask.

VALIDATION OF THE METHOD:

The developed method was validated as per ICH guidelines. The validation of the method was carried out in terms of linearity, accuracy, precision, repeatability, LOD and LOQ.

LINEARITY AND RANGE:

The linear response for the mixture (ledipasvir and sofosbuvir) was assessed by spotting different volume of mixed stock solution from 1 to 7 µL on TLC plate.

ACCURACY:

The accuracy study was conducted at 50, 100 and 150% level for the pre-analysed samples by standard addition method.

PRECISION:

Precision of the method was studied by

- Intra-day precision
- Inter-day precision
- Repeatability precision

Repeatability of sample measurement

Repeatability of sample application

Intra-day precision:

Intra-day precision was studied by carrying out the analysis of the standard drug of two different concentrations for three times on the same day and %RSD was calculated.

Inter-day precision:

Inter-day precision was studied by carrying out the analysis of the standard drug of two different concentrations for three different days over a period of one week and %RSD was calculated.

REPEATABILITY:

Repeatability of sample measurement:

Repeatability of measurement was determined by spotting 300ng/spot of ledipasvir and 1320ng/spot of sofosbuvir drug solution on a pre-coated TLC plate and developed the plate was scanned six times and %RSD was calculated.

Repeatability of sample application:

Repeatability of sample application was carried by spotting 6 times of 2µl of drug solution on pre-coated TLC plate followed by development of plate and %RSD was calculated.

LIMIT OF DETECTION (LOD) AND LIMIT OF QUANTIFICATION (LOQ):

The lowest concentration detected (LOD) and lowest concentration quantified (LOQ) were estimated from the set of five calibration curves used to determine method linearity.

LOD = $3.3\times\sigma/S$ and LOQ = $10\times\sigma/S$

Where, σ = the standard deviation of y intercepts of regression lines

S = the slope of the calibration curve

ROBUSTNESS:

The effect of deliberate variations on method parameters like the composition of the mobile phase saturation time, development distance spot scanning time interval, wavelength scan time from spotting to chromatography and mobile phase volume was evaluated.

SPECIFICITY

The peak purity of ledipasvir and sofosbuvir was assessed by comparing its respective spectra at the three different levels, that is, peak start (S), peak apex (M) and peak end (E) positions of the spot.

STABILITY STUDIES:

When the developed chromatographic plate is exposed to atmosphere, the analytes are likely to decomposed. It is necessary to study the stability of drug on plate. It was studied by scanning the plate at different time interval and peak areas were compared with the peak area of freshly scanned plate. The developed plate was found to be stable for about 24 hours.

ANALYSIS OF FORMULATION

To determine the amount of ledipasvir and sofosbuvir in tablet dosage form (label claim 90& 400mg per tablet respectively) 20 tablets were weighed, their average weight was determined, and they were finely powdered. An accurately weighed powder sample equivalent to 9mg of ledipasvir or 40mg of sofosbuvir was transferred into a 100ml volumetric flask then added 10ml methanol, followed by sonication for 10 min and further dilution was made up to the mark with methanol. The resulting solution was filtered through whatman filter paper (No.1) and two microliters of the filtered solution (180ng/spot of ledipasvir & 800ng/spot of sofosbuvir) was applied on the TLC plate followed by development and scanning as per optimized chromatographic conditions.

STRESS STUDIES

Forced degradation studies for Ledipasvir and Sofosbuvir

For conducting the forced degradation studies, four samples were taken viz., the blank solution stored under normal conditions, the blank subjected to stress in the similar manner as the drug solution, zero time analyte solution which was stored under normal and the sample subjected to stress treatment. The study was conducted separately for ledipasvir and sofosbuvir individually and also for combination.

Acid Hydrolysis:

Hundred milligram of ledipasvir and sofosbuvir was weighed accurately and transferred into 25ml standard flasks separately. To this 10 ml of 0.1M hydrochloric acid was added and made up to 25ml with methanol. This solution was refluxed for 5 hours at 80˚C. Further 2.5 ml was made up to 10ml (1000µg/ml) for ledipasvir and sofosbuvir individually and also for mixture.

Alkaline Hydrolysis:

Hundred milligram of ledipasvir and sofosbuvir was weighed accurately and transferred into 25ml standard flasks separately. To this 10 ml of 0.1M sodium hydroxide was added and made up to 25ml with methanol. This solution was refluxed for 5 hours at 80˚C. Further 2.5 ml was made up to 10ml (1000µg/ml) for ledipasvir and sofosbuvir individually and also for mixture.

Neutral hydrolysis:

Hundred milligram of ledipasvir and sofosbuvir was weighed accurately and transferred into 25ml standard flasks separately. To this 10 ml of water was added separately and made up to 25 ml methanol. This solution was refluxed for 5 hours at 80˚C. Further 2.5 ml was made up to 10ml (1000µg/ml) for ledipasvir and sofosbuvir individually and also for mixture.

Oxidative Degradation:

Hundred milligram of ledipasvir and sofosbuvir was weighed accurately and transferred into 25ml standard flasks separately. To this 10 ml of 6% hydrogen peroxide was added separately and made up to 25ml with methanol. Further 1 ml was withdrawn 1 hour once for about 5 hours and Further 2.5 ml was made up to 10ml (1000µg/ml) for ledipasvir and sofosbuvir individually and also for mixture.

Thermal degradation:

Hundred milligram of ledipasvir and sofosbuvir was weighed and transferred to a petri dish separately. It was then placed in hot air oven at 80ºC for 5 hours then dissolved and made up to 25 ml with methanol. Further 2.5 ml was taken and made up to 10ml (1000µg/ml) for ledipasvir and sofosbuvir individually and also for mixture.

Photolytic degradation:

Hundred milligram of ledipasvir and sofosbuvir was weighed and transferred to a petri dish. It was then exposed to sunlight for about 5 hours. The drug solution was prepared using methanol. Further 2.5 ml was taken and made up to 10ml (1000µg/ml) for ledipasvir and sofosbuvir individually and also for mixture.

DEVELOPMENT AND VALIDATION OF RP-HPLC METHOD FOR THE SIMULTANEOUS DETERMINATION OF LEDIPASVIR AND SOFOSBUVIR IN BULK AND FORMULATION

1. Chromatographic method for separation:

The drug Ledipasvir and Sofosbuvir is polar in nature, RP-HPLC method was selected as separation technique.

2. Selection of solvent:

The drugs are soluble in acetonitrile and also showed good stability. Hence methanol was selected as the solvent.

3. Selection of Detection wavelength:

Good analytical separation can be obtained only by careful selection of wavelength for the detection. This choice requires knowledge of the UV spectrum of the sample component. A UV spectrum of ledipasvir and sofosbuvir was recorded in acetonitrile and the overlay have shown acceptable absorbance at 254nm for both drugs and it was selected for the study.

OPTIMIZATION OF CHROMATOGRAPHIC CONDITIONS

Selection of mobile phase:

Here different mobile phase with different ratios were tried like water: methanol, water: acetonitrile and orthophosphoric acid buffer and acetonitrile.

Selection of strength of acetic acid:

Different strengths of formic acid such as 0.5%, 1% and 1.5% were tried. Good peak characteristics was observed for strength of 1% orthophosphoric acid and hence selected.

.

Selection of pH of orthophosphoric acid:

The pH which gave the good peak characteristic was selected for the further study.

Selection of mobile phase ratio:

Different ratio of 1% orthophosphoric acid: acetonitrile tried from which a ratio of **30:70% v/v** gave good resolution and satisfactory peak shapes.

Selection of flow rate:

Keeping all other parameters of mobile phase system constant, the chromatograms were recorded different flow rates like 0.7, 0.8, 0.9, 1 and 1.1 ml/min were tried. A flow rate which gave good symmetrical peaks and good resolution.

Chromatographic development procedure:

The HPLC system was stabilized for 60min, by passing mobile phase and detector was set as 254nm and flow rate of 0.8ml/min was maintained to get a steady baseline. One blank followed by six replicate of a single standard solution were injected to check the system suitability.

VALIDATION OF THE METHOD

The developed HPLC method was validated according to ICH guidelines in terms of linearity, accuracy, precision, specificity, robustness and LOD and LOQ.

LINEARITY AND RANGE:

The mixed standard stock solution (100µg/ml of ledipasvir and 100µg/ml of sofosbuvir) was further diluted to get ledipasvir and sofosbuvir concentration in the range of 1-10µg/ml and 1-10µg/ml respectively. Linearity of the method was studied by injecting ten concentration of the drug prepared in the mobile phase in triplicate into the LC system keeping the injection volume constant, The peak areas were plotted against the corresponding concentration to obtain the calibration graphs.

ACCURACY:

Accuracy of the method was carried out by applying the method to drug sample (ledipasvir and sofosbuvir combination tablet) to which known amount of ledipasvir and sofosbuvir standard powder corresponding to 50, 100 and 150% of label claim had been added (standard addition method) mixed and the powder was extracted and analyzed by running chromatogram in optimized mobile phase.

PRECISION:

The precision of the method was verified by intraday, interday and repeatability precision studies.

- Intra-day precision
- Inter-day precision
- Repeatability

Intra-day precision:

Intraday precision was determined by injecting standard solutions in between linearity range (4, 8µg/ml for ledipasvir and 4, 8µg/ml for sofosbuvir) were injected three times on the same day and % RSD was calculated.

Inter-day precision:

Inter-day precision was determined by injecting standard solutions in between linearity range (4, 8µg/ml for ledipasvir and 4, 8µg/ml for sofosbuvir) were injected for three days and % RSD was calculated.

Repeatability:

Repeatability studies were performed by analysis of different concentration 4µg/ml ledipasvir and 4µg/ml of sofosbuvir six times on the same day and %RSD was calculated.

LIMIT OF DETECTION (LOD) AND LIMIT OF QUANTITATION (LOQ):

The lowest concentration detected (LOD) and lowest concentration quantified (LOQ) were estimated from the set of five calibration curves used to determine method linearity.

$$LOD = 3.3 \times \sigma / S$$

$$LOQ = 10 \times \sigma / S$$

Where, σ = the standard deviation of y intercepts of regression lines

S = the slope of the calibration curve

SPECIFICITY:

The specificity of the method towards the drug was established through study of resolution factor of the drug peak from the nearest resolving peak. The

peak purity of ledipasvir and sofosbuvir were determined by comparing the spectrum at three different regions of the spot i.e. peak start (S), peak apex (M) and peak end (E). Effect of excipients of formulation was studied for whether it interfered with the assay.

ROBUSTNESS:

To evaluate robustness of a HPLC method, few parameters were deliberately varied. The parameters included variation of flow rate, percentage of acetonitrile in the mobile phase and solvents. The response factors like retention time, resolution, asymmetric factor for these changed conditions were noted.

STABILITY OF SOLUTION:

The standard solution of ledipasvir and sofosbuvir kept under room temperature. It was injected periodically. Stability was studied by looking for any change in retention time, resolution and peak shape, when compared to chromatogram of freshly prepared solution. The solution was stable 24hrs under room temperature.

SYSTEM SUITABILITY STUDIES:

The system suitability parameters like peak area, tailing factor, theoretical plate count, and resolution and retention time were calculated from the standard chromatograms.

ANALYSIS OF A MARKETED FORMULATION:

To determine the amount of ledipasvir and sofosbuvir in tablet dosage form (label claim 90& 400mg per tablet respectively) 20 tablets were weighed, their average weight was determined, and they were finely powdered. An accurately weighed powder sample equivalent to 9mg of ledipasvir and 40mg of sofosbuvir was transferred into a 100ml volumetric flask then added 10ml acetonitrile, followed by sonication for 10 min and further dilution up to the mark with

acetonitrile. The resulting solution was filtered through whatman filter paper (No.1) and using syringe 0.45µm. The above stock solution was further diluted to get sample solution of 0.9µg/ml of ledipasvir and 4µg/ml of sofosbuvir respectively. A 20µl volume of sample solution was injected into HPLC, six times, under the conditions described above. The peak areas were measured at 254nm.

STRESS STUDIES

Forced degradation studies for Ledipasvir and sofosbuvir:

For conducting the forced degradation studies, four samples were taken viz., the blank solution stored under normal conditions, the blank subjected to stress in the similar manner as the drug solution, zero time analyte solution which was stored under normal and the sample subjected to stress treatment. The study was conducted separately for ledipasvir and sofosbuvir individually and also for combination.

Acid Hydrolysis:

Hundred milligram of ledipasvir and sofosbuvir was weighed accurately and transferred into 25ml standard flasks separately. To this 10 ml of 0.1M hydrochloric acid was added and made up to 25ml with acetonitrile. These solution was refluxed for 5 hours at 80˚C. Further 0.25 ml was made up to 100ml (10µg/ml) for ledipasvir and sofosbuvir individually and also for mixture.

Alkaline Hydrolysis:

Hundred milligram of ledipasvir and sofosbuvir was weighed accurately and transferred into 25ml standard flasks separetely. To this 10 ml of 0.1M sodium hydroxide was added and made up to 25ml with acetonitrile. This solution was refluxed for 4 hours at 80˚C. Further 0.25 ml was made up to 100ml (10µg/ml) for ledipasvir and sofosbuvir individually and also for mixture.

Neutral hydrolysis:

Hundred milligram of ledipasvir and sofosbuvir was weighed accurately and transferred into 25ml standard flasks separetely. To this 10 ml of water was added separately and made up to 25 ml acetonitrile. This solution was refluxed for 4 hours at 80˚C. Further 0.25 ml was made up to 100ml (10µg/ml) for ledipasvir and sofosbuvir individually and also for mixture.

Oxidative Degradation:

Hundred milligram of ledipasvir and sofosbuvir was weighed accurately and transferred into 25ml standard flasks separately. To this 10 ml of 6% hydrogen peroxide was added separately and made up to 25ml with acetonitrile. Further 1 ml was withdrawn 1 hour once for about 5 hours and Further 0.25 ml was made up to 100ml (10µg/ml) for ledipasvir and sofosbuvir individually and also for mixture.

Thermal degradation:

Hundred milligram of ledipasvirand sofosbuvir was weighed and transferred to a petri dish separately. It was then placed in hot air oven at 80ºC for 5 hours then dissolved and made up to 25 ml with acetonitrile. Further 0.25 ml was made up to 100ml (10µg/ml) for ledipasvir and sofosbuvir individually and also for mixture.

Photolytic degradation:

Hundred milligram of ledipasvir and sofosbuvir was weighed and transferred to a petri dish separately. It was then exposed to sunlight for about 5 hours. The drug solution was prepared using acetonitrile. Further 0.25 ml was made up to 100ml (10µg/ml) for ledipasvir and sofosbuvir individually and also for mixture.

DEVELOPMENT OF VALIDATED UV-SPECTROSCOPIC METHODS FOR THE ESTIMATION OF LEDIPASVIR AND SOFOSBUVIR IN BULK AND FORMULATION

Ledipasvir and sofosbuvir possess chromophoric groups like benzene extended to carbonyl (=c=o) and alkene (=c=c) functional groups. Amide (-NH) and hydroxyl(-OH) groups as auxochrome which contribute into strong UV absorption of the molecule. This character is used for the determination of ledipasvir and sofosbuvir by UV spectroscopic methods.

Ideal solvent is the one which completely solublize the drug and should maintain good stability of the drug in solution. Initially solvents like acetone and methanol were tried, among these spectrum of ledipasvir and sofosbuvir was not good and smooth but, methanol gave a good absorption spectrum with acceptable stability and sensitivity. Hence methanol was selected as solvent of choice for further studies. The overlay spectra is shown in the Figure 1a and 1b.

Figure 1a: Overlay spectra of solvent selection of Ledipasvir

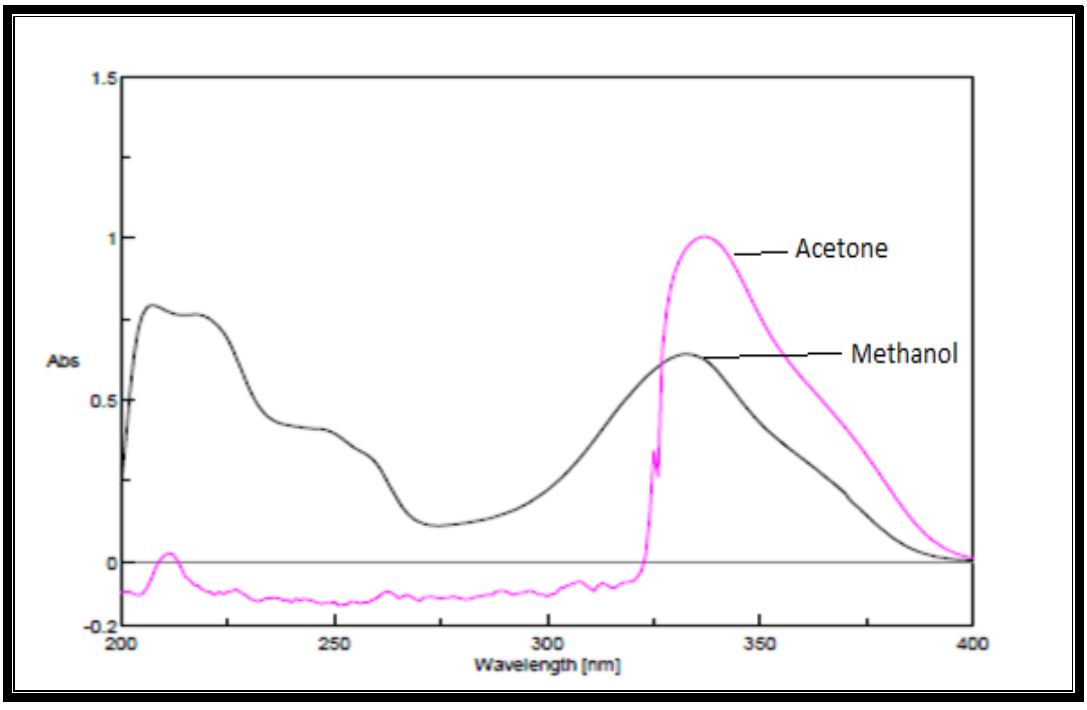
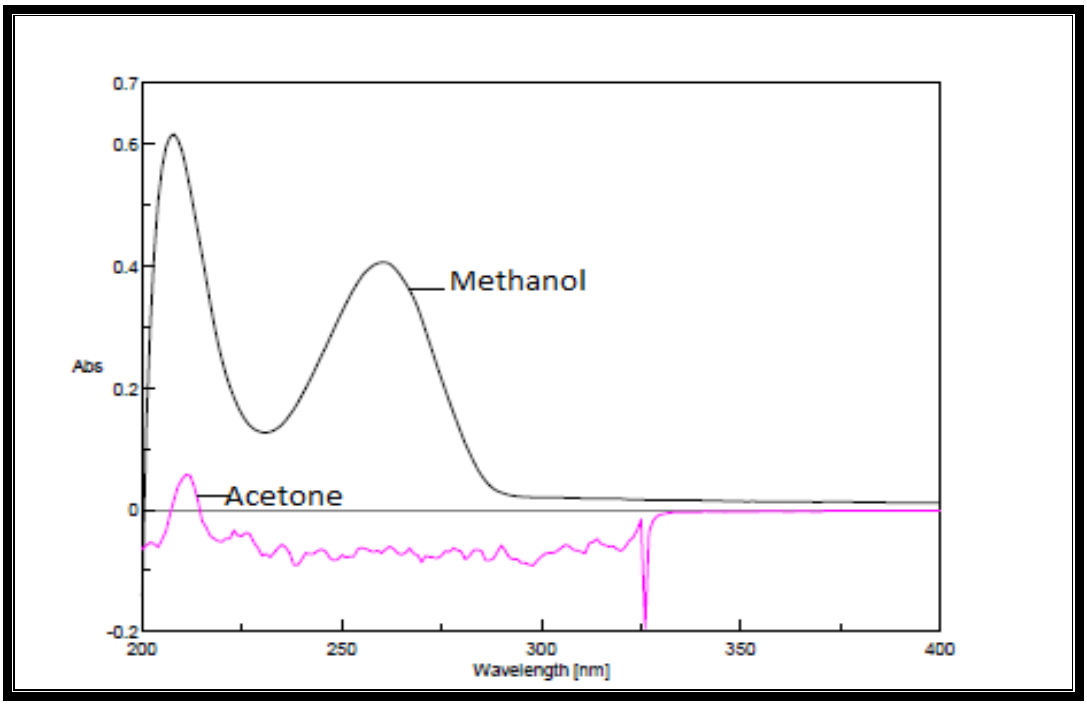


Figure 1b: Overlay spectra of solvent selection of Sofosbuvir



An ideal wavelength is the one that gives good response with maximum absorbance for the both drug to be detected. The normal UV spectrum of ledipasvir 333nm and sofosbuvir 261nm were selected as detection wavelength because the linear response was very good with maximum acceptable absorbance, but wavelength less than 215nm is not commonly used due to solvent interference. But the derivatised normal also spectrum showed the λ_{max} at 333nm for ledipasvir and 261nm for sofosbuvir which was used for further studies.

The normal spectrum and the derivative spectrum of both drugs for 20µg/ml are shown in the figure 2a & 2b and 3a & 3b, respectively.

Figure 2a: Normal spectrum of Ledipasvir (20µg/ml)

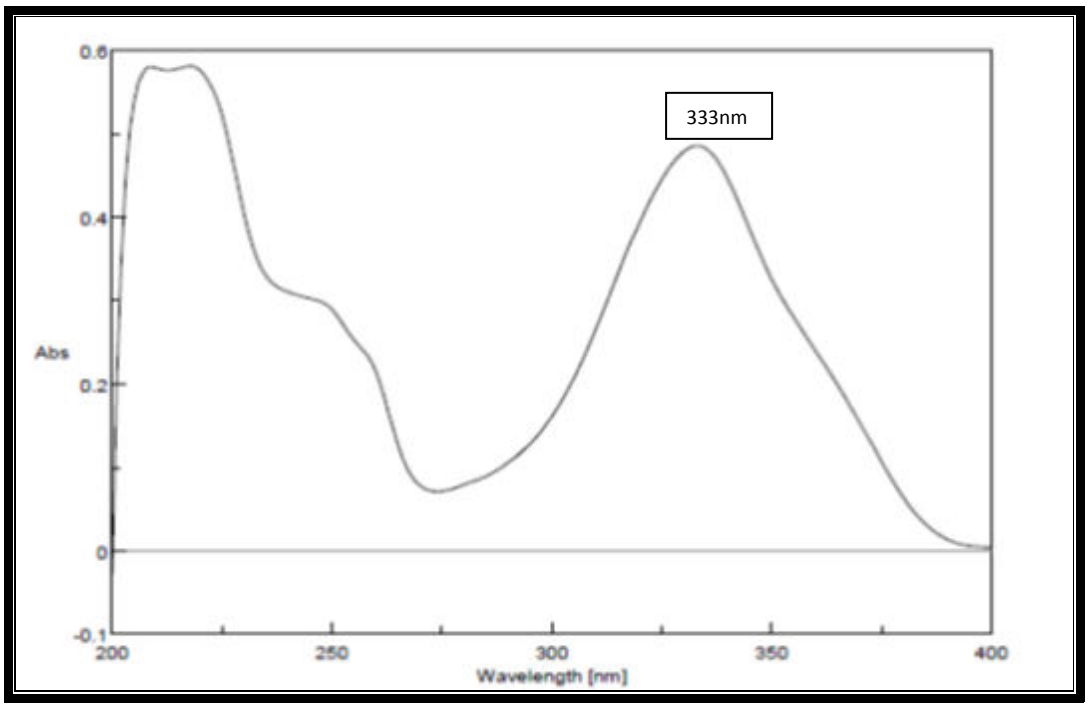


Figure 2b: Second order derivative spectrum of Ledipasvir (20µg/ml)

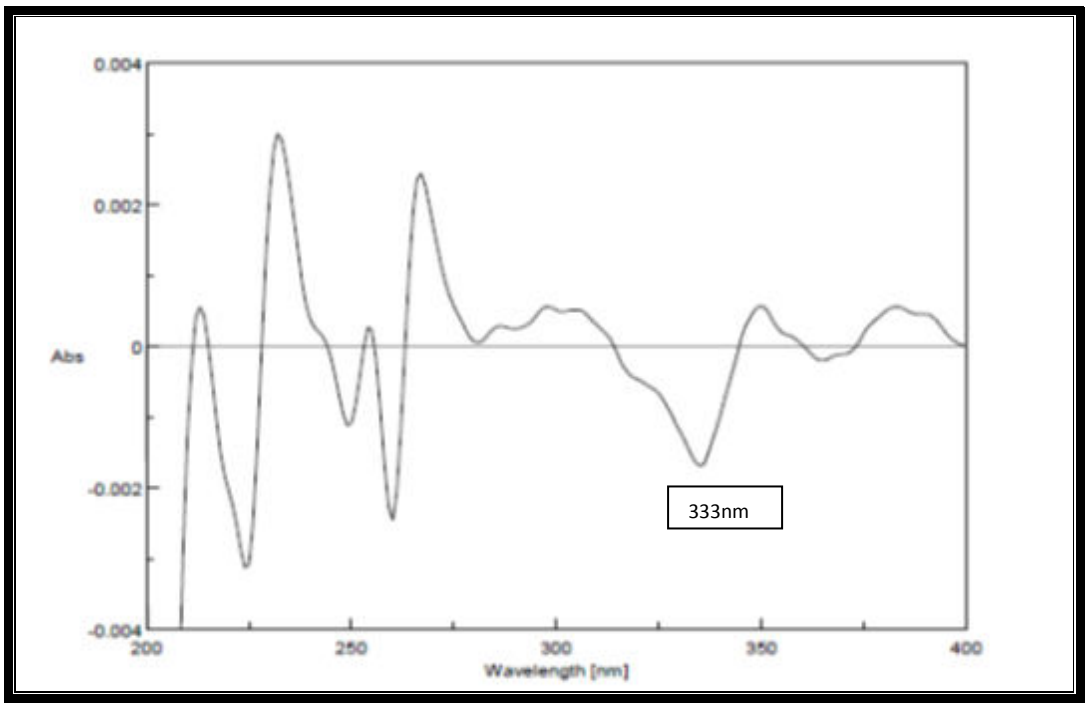


Figure 3a: Normal spectrum of Sofosbuvir (20µg/ml)

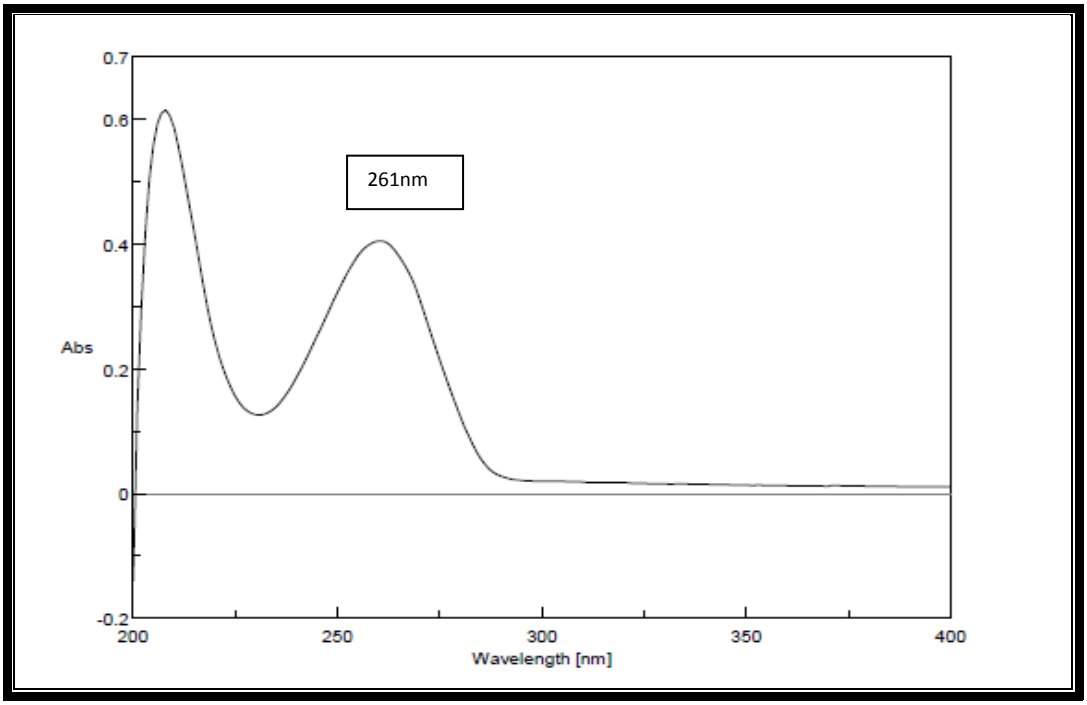
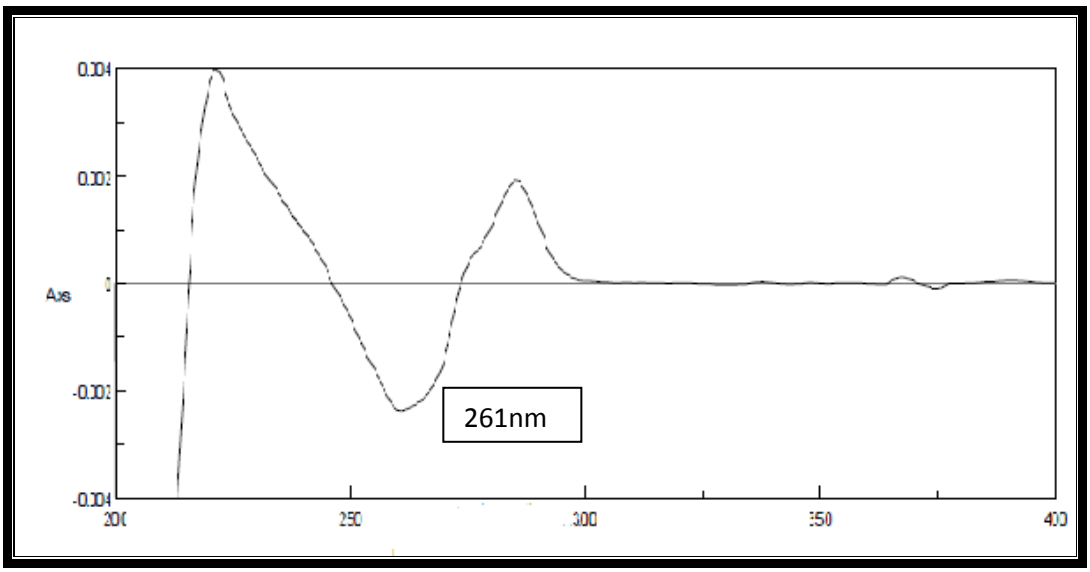


Figure 3b: Second order derivative spectrum of Sofosbuvir (20µg/ml)



VALIDATION PARAMETERS

Linearity and range:

The linear regression analysis showed good correlation coefficient and good linear relationship over concentration range of ledipasvir 10– 40µg/ml and sofosbuvir 10- 70 µg/ml, Overlay of normal spectra were shown in figure 4a & 4b and calibration graph was shown in figure 5a & 5b and the values are tabulated in table 1a & 1b, respectively.

Figure 4a: Overlay of normal spectrum of Ledipasvir

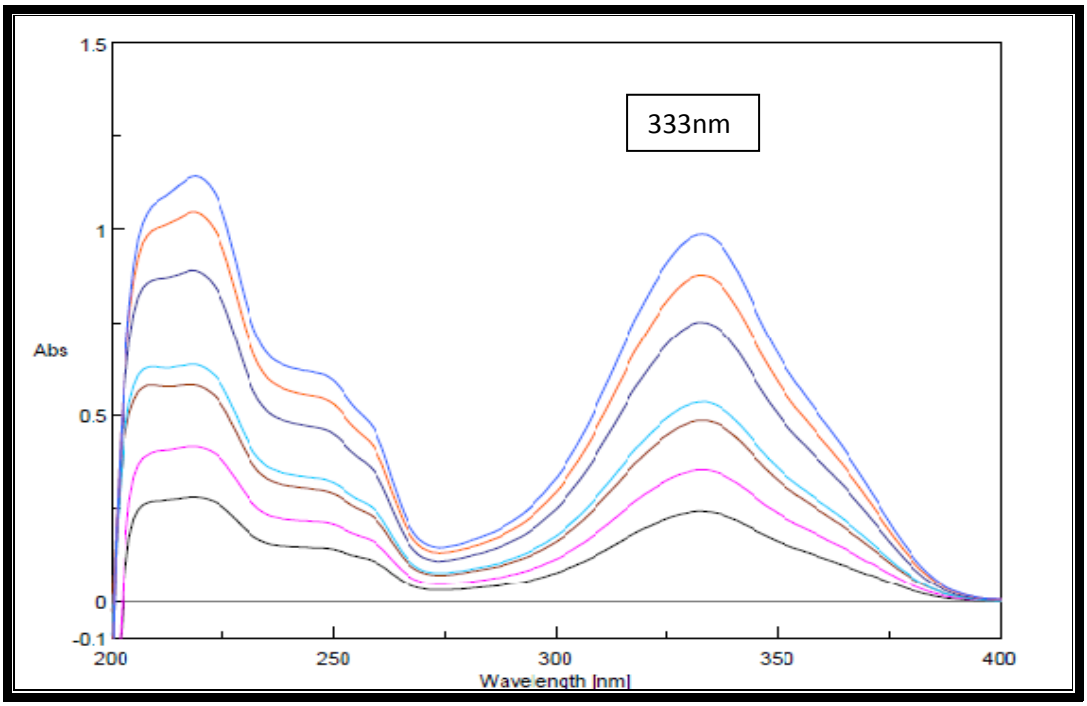


Figure 4b: Overlay of normal spectrum of Sofosbuvir

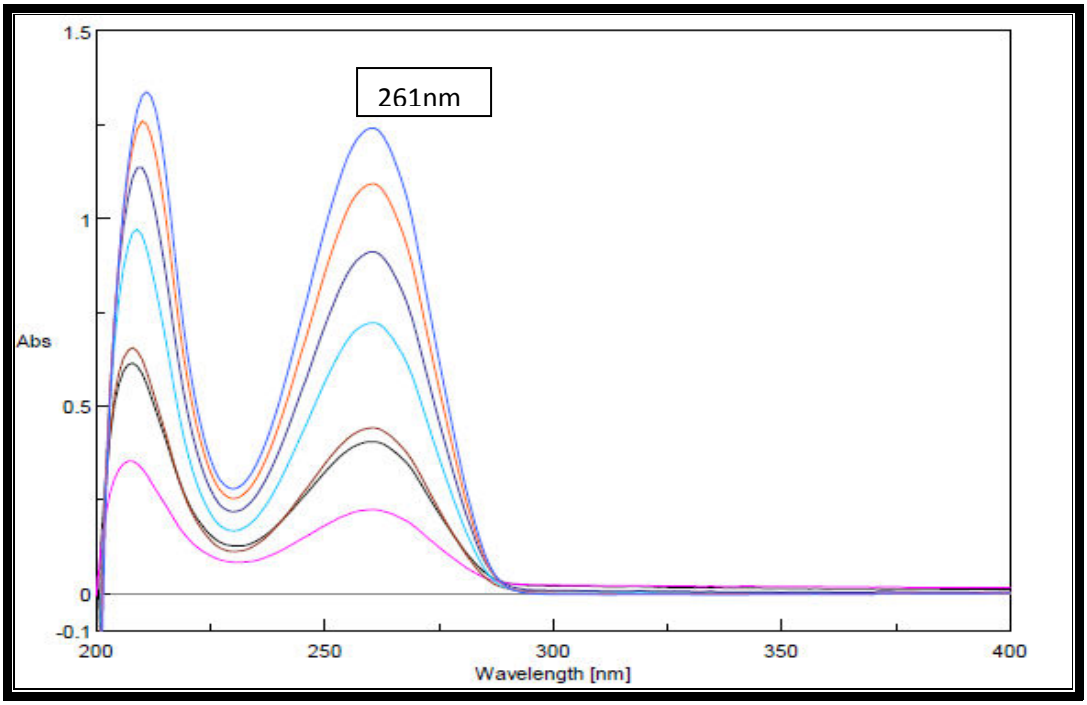


Table 1a: Calibration data for normal spectrum of Ledipasvir

S.No	Concentration (µg/ml)	Absorbance
1.	10	0.2422
2.	15	0.3533
3.	20	0.4853
4.	25	0.5346
5.	30	0.7491
6.	35	0.8756
7.	40	0.9859

Figure 5a: Linear graph of Ledipasvir (10-40µg/ml)

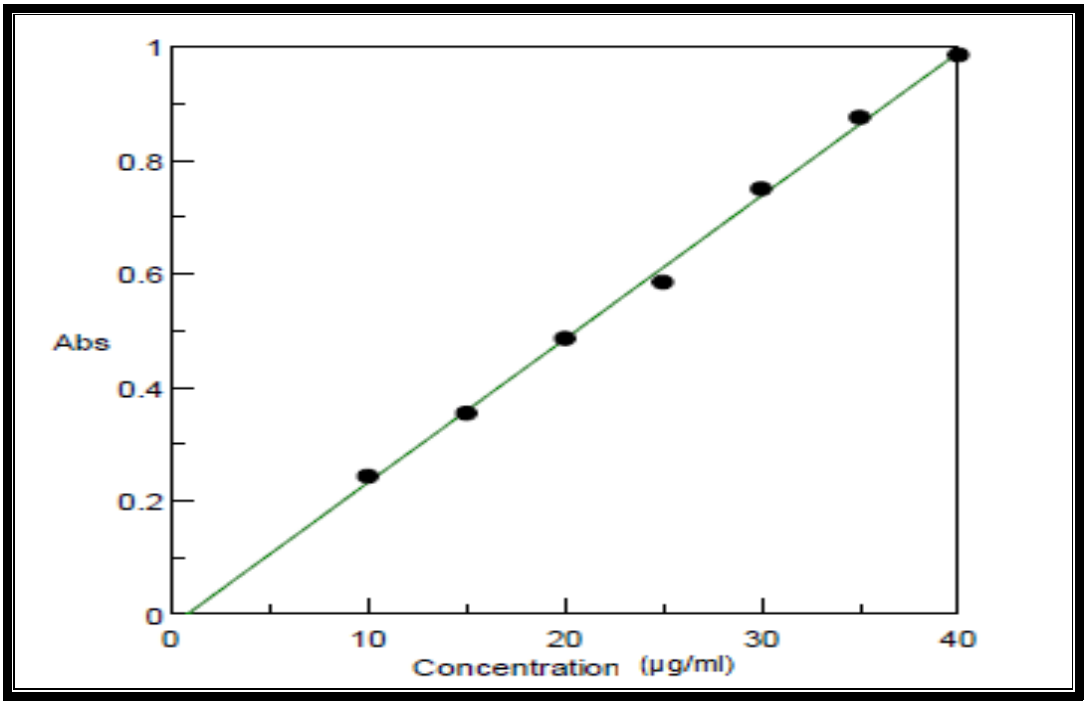
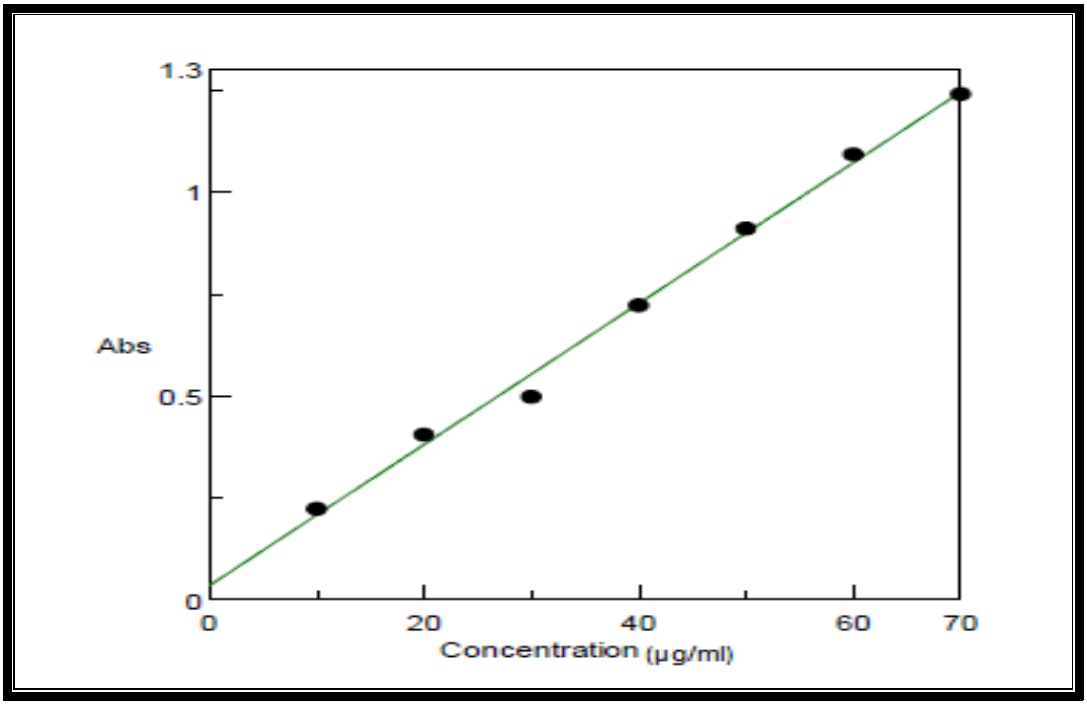


Table 1b: Calibration data for normal spectrum of sofosbuvir

S.No	Concentration (µg/ml)	Absorbance
1.	10	0.2222
2.	20	0.3985
3.	30	0.4404
4.	40	0.7211
5.	50	0.9096
6.	60	1.0913
7.	70	1.2395

Figure 5b: Linear graph of Sofosbuuvir (10-70µg/ml)



Similarly, the normal spectrum was converted to second order derivative, and the corresponding overlay and linear graph was shown in the figure 6a & 6b and 7a & 7b and data are shown in table 2a & 2b.

Figure 6a: Second order derivative spectrum of Ledipasvir

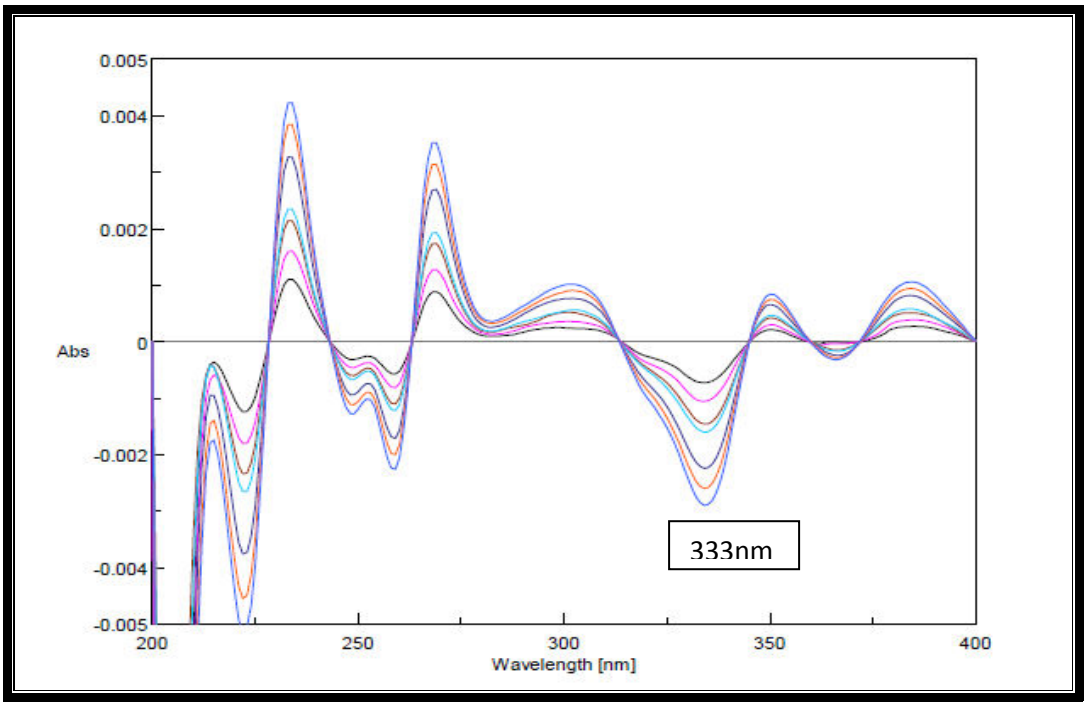


Figure 6b: Second order derivative spectrum of Sofosbuvir

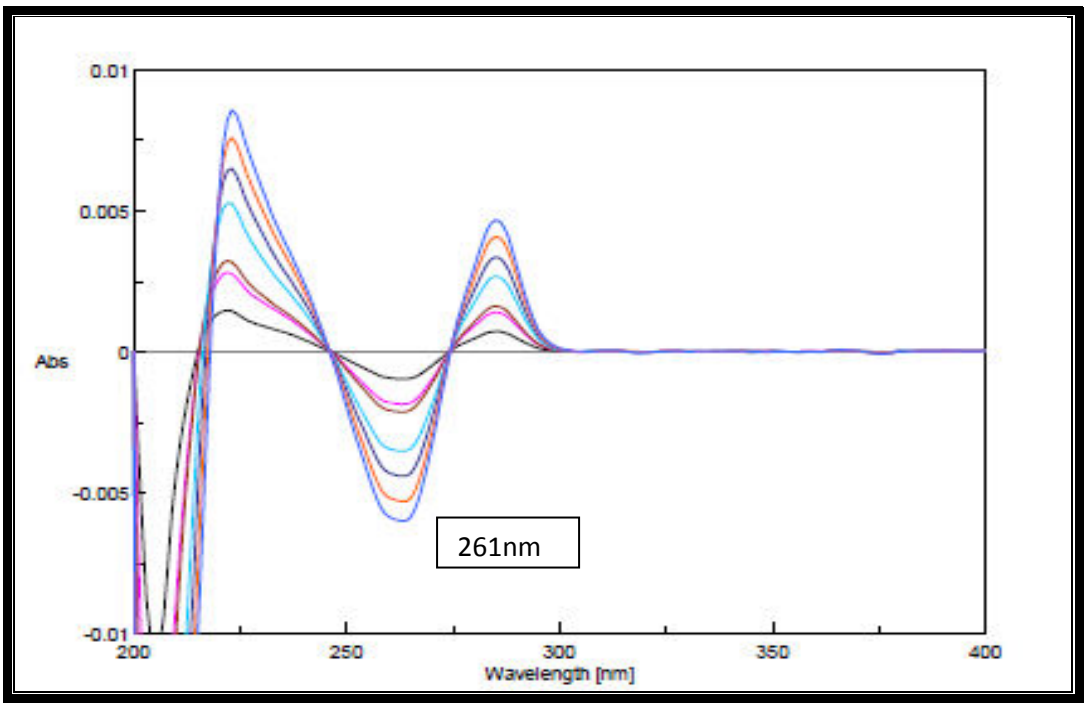


Table 2a: Calibration data for Second order derivative of Ledipasvir(10-40 µg/ml)

S.No	Concentration(µg/ml)	Amplitude(mm)
1.	10	8
2.	15	13
3.	20	16
4.	25	19
5.	30	24
6.	35	28
7.	40	32

Figure 7a: Linear graph for second order derivative of Ledipasvir

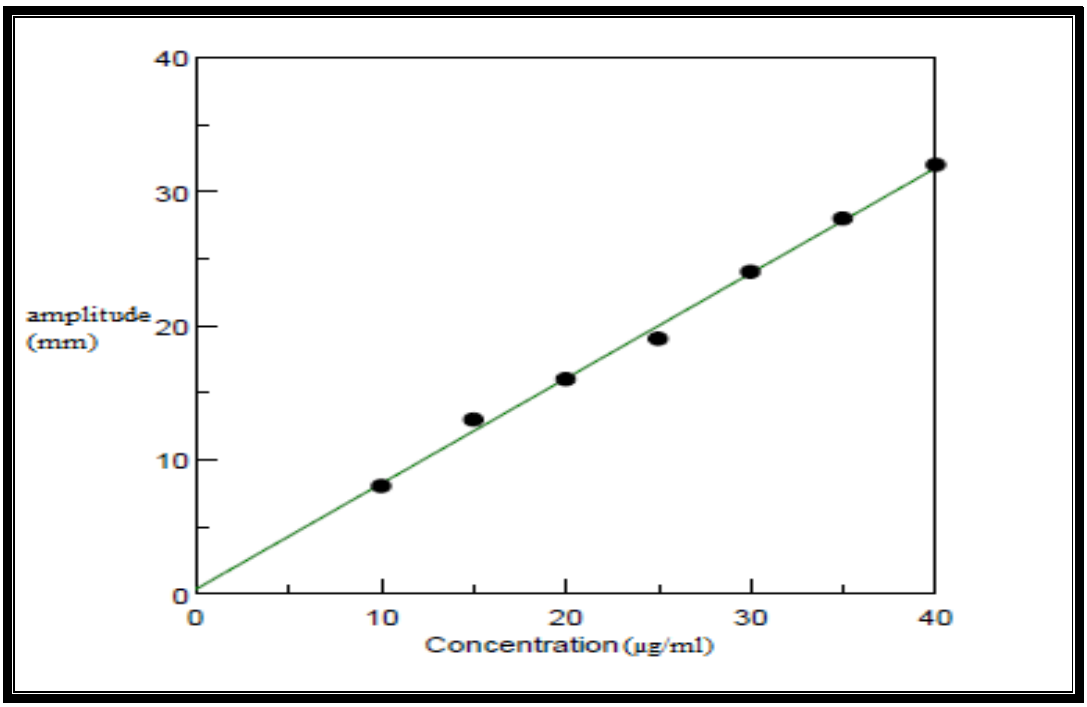
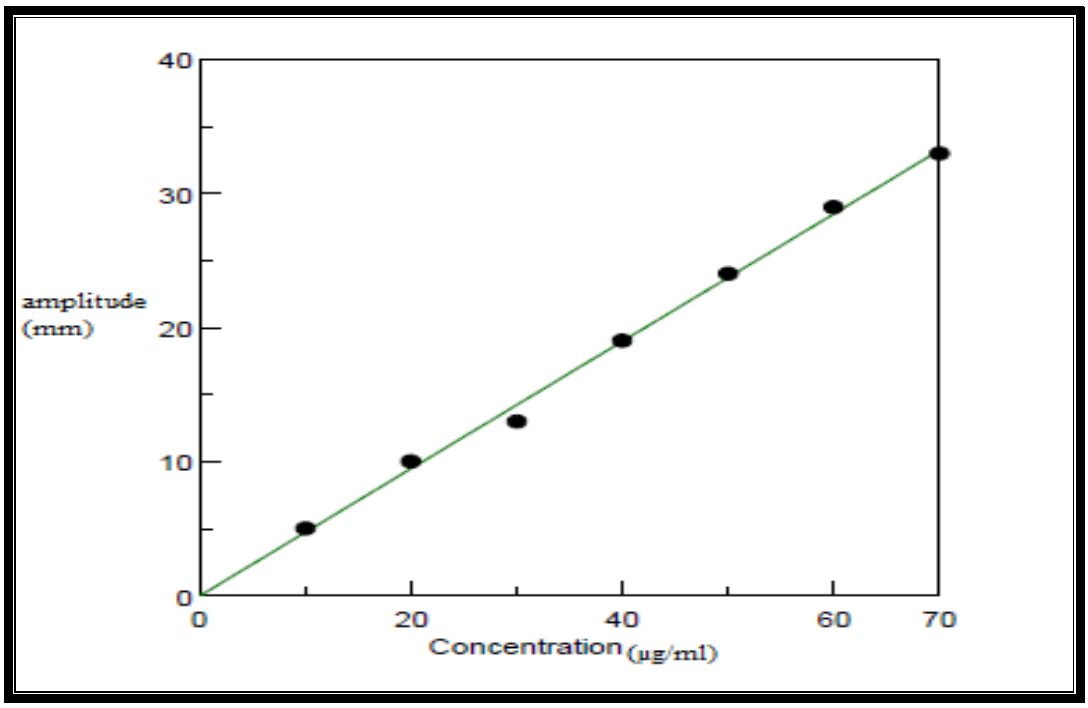


Table 2b: Calibration data for Second order derivative of Sofosbuvir (10-70 µg/ml)

S.No	Concentration(µg/ml)	Amplitude(mm)
1.	10	5
2.	20	10
3.	30	13
4.	40	19
5.	50	24
6.	60	29
7.	70	33

Figure 7b: Linear graph for second order derivative of Sofosbuvir



Precision:

For both zero and second order derivative methods %RSD values were less than 2 for repeatability, intraday precision, and interday precision, which indicated the methods were more precise and the values are listed in the table 3a & 3b to 5a & 5b.

Table 3a: Precision studies of Ledipasvir

(i)Repeatability (Normal method)

Concentration taken (µg/ml)	Absorbance	%RSD*
30	0.7490	0.649
	0.7432	
	0.7461	
	0.7472	
	0.7371	
	0.7385	

*** Average six determination**

(ii)Repeatability (second order)

Concentration taken(µg/ml)	Amplitude(mm)	%RSD*
30	24	0.022
	24	
	24	
	25	
	25	
	25	

*** Average six determination**

Table 3b: Precision studies of Sofosbuvir

(i)Repeatability (Normal method)

Concentration taken (µg/ml)	Absorbance	%RSD*
30	0.4404	0.880
	0.4415	
	0.4412	
	0.4426	
	0.4439	
	0.4329	

*** Average six determination**

(ii)Repeatability (second order)

Concentration taken(µg/ml)	Amplitude(mm)	%RSD*
30	13	0.038
	14	
	13	
	13	
	13	
	14	

*** Average six determination**

Table 4a: Intraday precision of Ledipasvir

(i)Normal method

Concentration taken (µg/ml)	Absorbance	%RSD*
20	0.4892	0.971
	0.4801	
	0.4826	
30	0.7495	0.634
	0.7401	
	0.7456	

***Average of three determination**

(ii) Intraday precision (Second order derivative)

Concentration taken(µg/ml)	Amplitude(mm)	%RSD*
20	16	0.035
	16	
	17	
30	24	0.023
	25	
	25	

***Average of three determination**

Table 4b: Intraday precision of Sofosbuvir

i. Normal method

Concentration taken (µg/ml)	Absorbance	%RSD*
20	0.3984	0.824
	0.3919	
	0.3956	
30	0.4402	0.643
	0.4459	
	0.4431	

***Average of three determination**

ii. Intraday precision (Second order derivative)

Concentration taken (µg/ml)	Amplitude(mm)	%RSD*
20	10	0.043
	11	
	10	
30	13	0.043
	13	
	14	

***Average of three determination**

Table 5a: Interday Precision of ledipasvir

i. Normal method

Concentration taken(µg/ml)	Absorbance	%RSD*
20	0.4806	1.841
	0.4896	
	0.4719	
30	0.7495	0.693
	0.7418	
	0.7397	

***Average of three determination**

ii. Interday precision (second order derivative)

Concentration taken(µg/ml)	Amplitude(mm)	%RSD
20	16	0.034
	17	
	17	
30	24	0.024
	23	
	23	

***Average of three determination**

Table 5b: Inter day Precision of Sofosbuvir

i. Normal method

Concentration taken(µg/ml)	Absorbance	%RSD*
20	0.3984	1.355
	0.3907	
	0.3882	
30	0.4402	0.500
	0.4432	
	0.4389	

***Average of three determination**

ii. Interday precision (second order derivative)

Concentration taken(µg/ml)	Amplitude(mm)	%RSD*
20	10	0.056
	11	
	10	
30	14	0.042
	14	
	13	

***Average of three determination**

Recovery:

In order to demonstrate the suitability, reliability, accuracy and applicability of the proposed methods, recovery studies were conducted for pre – analysed sample formulation by standard addition method. The percentage recovery was close to 100 proved that the methods are highly accurate. The results are given in the table 6a &6b.

Table 6a: Recovery study for ledipasvir

i. Normal method

Level	% Recovery	%RSD*
50%	99.41%	0.01
100%	98.37%	0.12
150%	98.15%	0.14

*** Average of six determination**

ii. Second order

Level	% Recovery	%RSD*
50%	101.84%	0.13
100%	103.71%	0.38
150%	101.65%	0.15

*** Average of six determination**

Table 6b: Recovery study for Sofosbuvir

i. Normal method

Level	% Recovery	%RSD*
50%	97.38%	1.21
100%	98.15%	0.35
150%	97.21%	0.04

*** Average of six determination**

ii. Second order

Level	% Recovery	%RSD*
50%	101.86%	0.84
100%	99.20%	0.15
150%	102.79%	0.95

***Average of six determination**

LOD & LOQ:

The sensitivity of the method is proved by limit of detection and limit of quantification that were calculated from the equation . Ledopasvir LOD and LOQ were found to be 0.24246 µg/ml and 0.7347µg/ml for normal and 0.004454µg/ml and 0.0134µg/ml for second order derivative. sofosbuvir LOD and LOQ were found to be 0.24246 µg/ml and 0.7347µg/ml for normal and 0.004454µg/ml and 0.0134µg/ml for second order derivative.

Stability studies:

Stability of ledipasvir and sofosbuvir in solution was about 6hours in normal room temperature and about 3 days at refrigeration that was shown in table 7a & 7b and 8a & 8b.

Table 7a: Stability of the solution at normal room temperature Ledipasvir

Concentration (µg/ml)	Time (hours)	Absorbance
15	1	0.3565
	2	0.3548
	3	0.3536
	4	0.3528
	5	0.3564
	6	**0.3514**
	7	0.3426
	8	0.3415

Table 7b: Stability of the solution at refrigeration Ledipasvir

Concentration (µg/ml)	Time (hours)	Absorbance
15	1	0.3566
	2	0.3549
	3	0.3568
	4	0.3578
	5	0.3548
	6	0.3565
	7	0.3545
	8	0.3515
	24	0.3526
	48	0.3545
	72	**0.3512**
	96	0.3015

Table 8a: Stability of the solution at normal room temperature Sofosbuvir

Concentration (µg/ml)	Time (hours)	Absorbance
20	1	0.3984
	2	0.3982
	3	0.3978
	4	0.3961
	5	0.3961
	6	**0.3936**
	7	0.3611
	8	0.3548

Table 8b: Stability of the solution at refrigeration sofosbuvir

Concentration (µg/ml)	Time (hours)	Absorbance
20	1	0.3956
	2	0.3945
	3	0.3925
	4	0.3912
	5	0.3915
	6	0.3910
	7	0.3909
	8	0.3905
	24	0.3901
	48	0.3900
	72	**0.3255**
	96	0.3122

The proposed method was successfully applied to formulation. The spectrum of analysis of formulation both normal and second order was shown in figure 8 and 9.The estimated amount and % label claim are shown in table 11.

Figure 8: Normal spectrum of formulation of ledipasvir(9µg/ml) and sofosbuvir(40µg/ml)

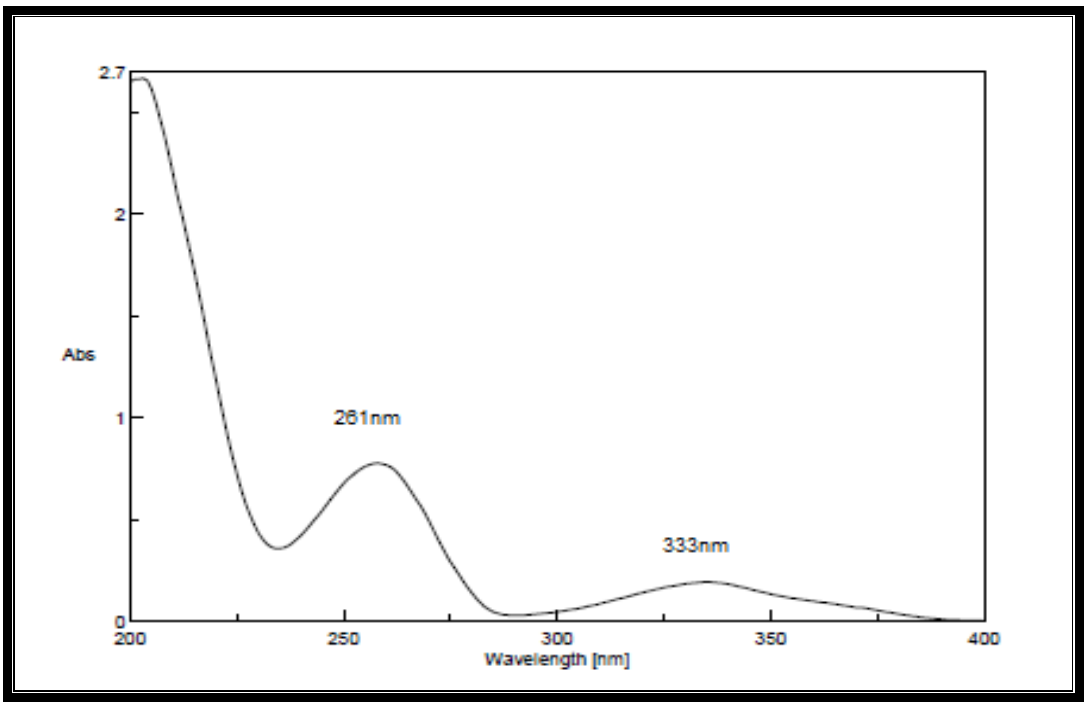


Figure 9: Second order derivative spectrum of formulation of Ledipasvir(9mcg/ml) & Sofosbuvir (40mcg/ml)

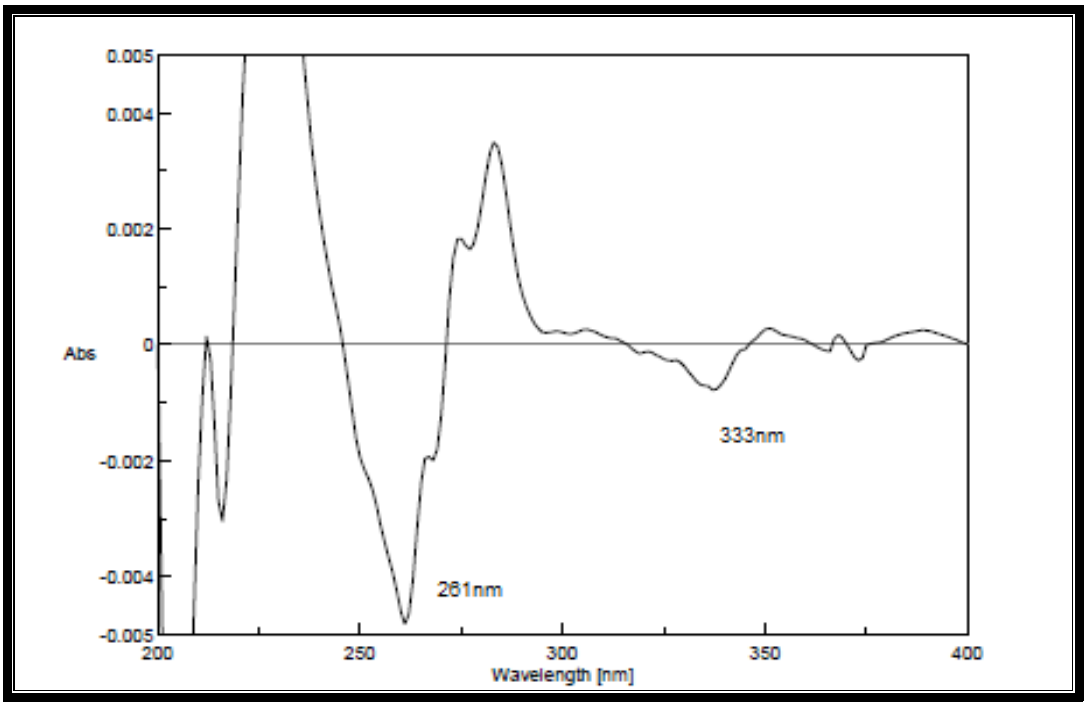


Table 11: Analysis of formulation

i. Simultaneous equation method

Methods	Cimivir-L			
	Amount of drug(mg/tab)		% Label Claim	% RSD*
	Labelled	Estimated		
Ledipasvir	90	89.21	99.12%	0.28
Sofosbuvir	400	398.43	99.60%	0.94

***Average of six observation**

ii. Second order derivative

Methods	Cimivir-L			
	Amount of drug(mg/tab)		% Label Claim	% RSD*
	Labelled	Estimated		
Ledipasvir	90	92.02	102.24%	0.54
Sofosbuvir	400	398.97	99.74%	0.74

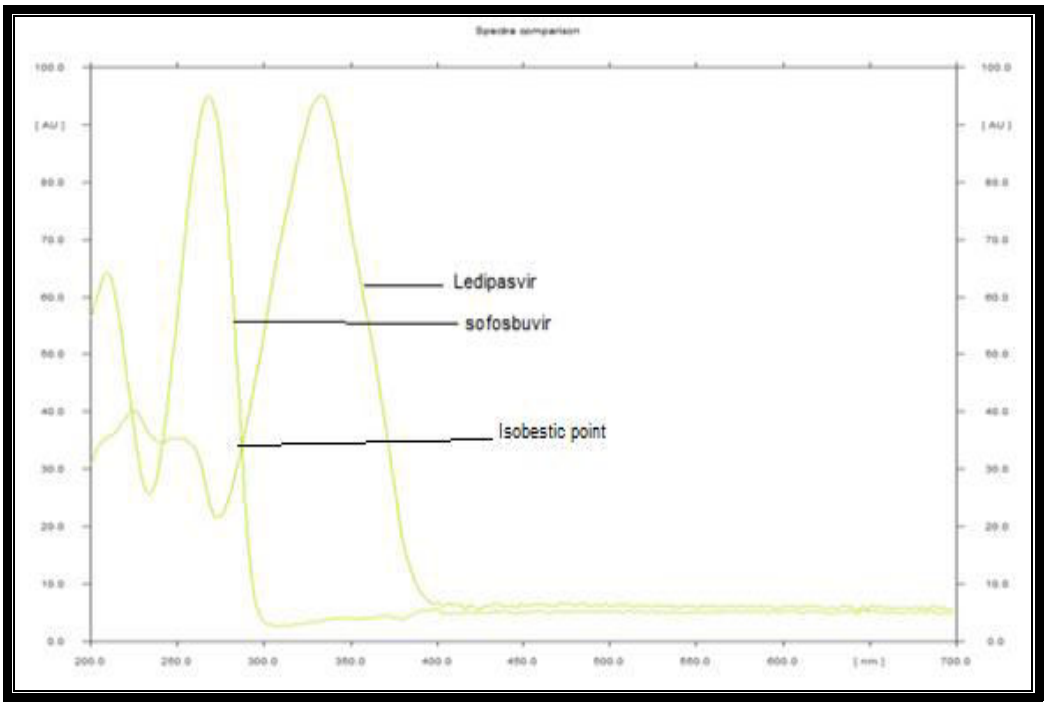
***Average of six observation**

DEVELOPMENT AND VALIDATION OF HPTLC METHOD FOR THE SIMULTANEOUS DETERMINATION OF LEDIPASVIR AND SOFOSBUVIR IN BULK AND FORMULATION

The pre – coated silica gel $G_{60}F_{254}$ in aluminium sheet was selected as stationary phase. In this acetonitrile was the solvent selected for the study because it completely solubilise the drug and show good stability of the drug in its solution form with acceptable volatile nature.

An ideal wavelength is the one that gives good response with maximum absorbance for the both drug to be detected. The spectra was recorded for the both ledipasvir and sofosbuvir on TLC scanner. From the spectra the isobestic point (288nm) was selected for further study. Fig 10

Figure10: Overlay spectrum of ledipasvir and sofosbuvir



Solvent system selection

Mobile phase system	Observation
Toluene : ethanol (7:3, v/v)	Poor separation
Toluene: ACN: Ethyl acetate(6:2:2, v/v/v)	Spot did not separate
Toluene : Ethyl acetate (7:3,v/v)	Spot not developed
Toluene : Butyl acetate : methanol (7:2:1,v/v/v)	Poor separation
Hexane: Ethyl acetate : methanol (5:3:2,v/v/v)	**Good separation**

Among these systems, system consisting of hexane : ethyl acetate : methanol was selected.

Fixed Experimental Conditions

Stationary phase : Pre-coated silica gel $G_{60}F_{254}$ on aluminum sheets

Mobile phase : Hexane : Ethyl acetate : methanol (4:5:1, v/v/v), few drops ammonia

Development chamber : Camag twin trough glass chamber

Separation technique : Ascending development

Chamber saturation time : 15 min

Plate migration distance : 80mm

Band width : 6mm

Slit dimension : 5 × 0.45mm

Plate thickness : 250µm

Source of radiation : Deuterium lamp

Detection wavelength : 288nm

R_f value : Sofosbuvir 0.21 ± 0.02

Ledipasvir 0.43 ± 0.02

The best solvent system hexane : ethyl aetate : methanol [4:5:2v/v/v] few drops of ammonia which resulted in good symmetric peak with R_f value sofosbuvir 0.21 ± 0.02 and Ledipasvir 0.43 ± 0.02 was selected as optimized mobile phase to achieve adequate separation of ledipasvir and sofosbuvir peaks. At higher proportion of hexane composition, the drug was retained at the spot of application and the R_f value less than 0.1 which was not ideal for quantitative determination. The blank and the standard chromatogram are shown in figure 11-12.

Figure 11: Chromatogram of Blank

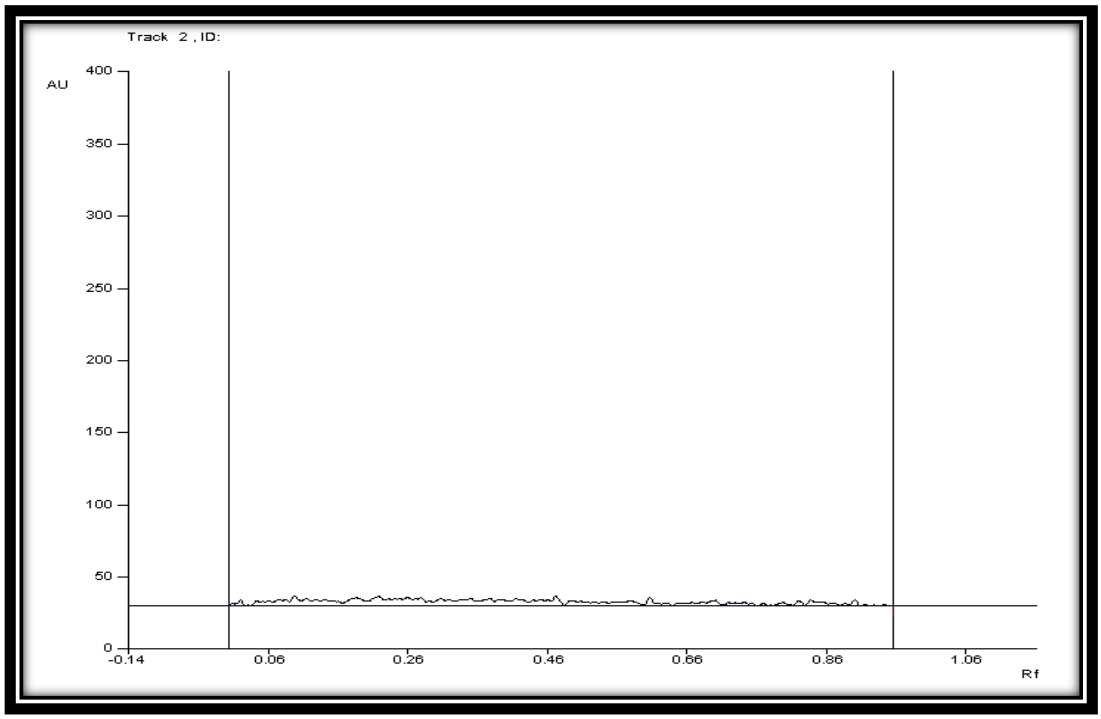
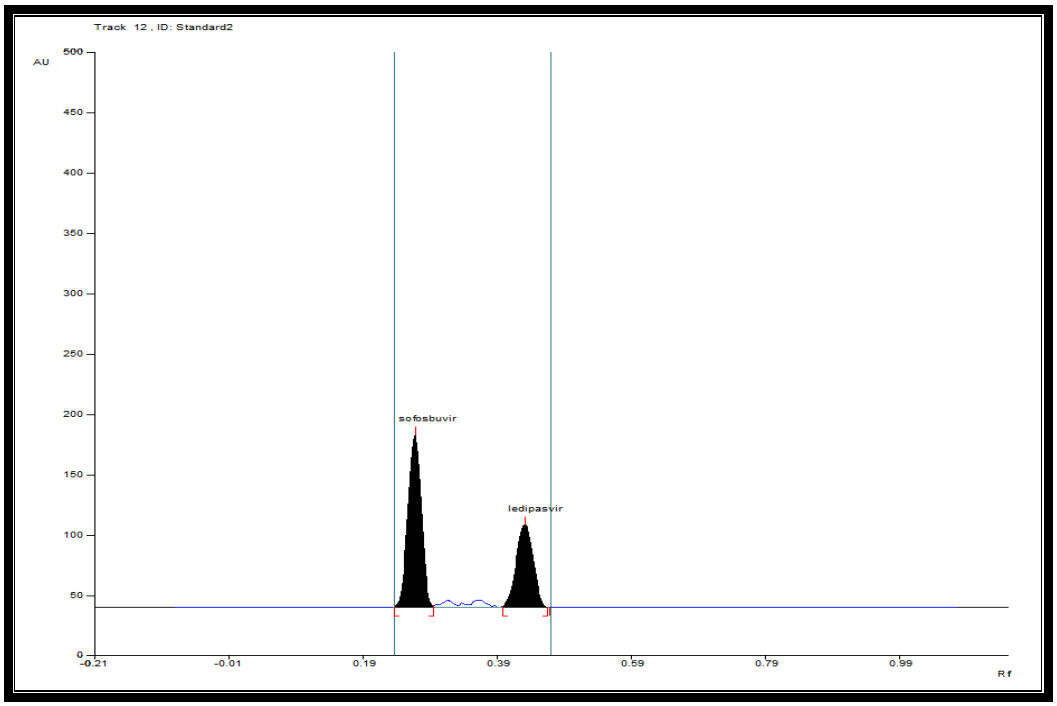


Figure 12: Standard chromatogram of Ledipasvir and sofosbuvir (200ng/spot&880ng/spot)



VALIDATION:

Linearity:

The high correlation coefficient obtained from linear regression analysis showed good linear relationship over the concentration range of ledipasvir 100-700ng/spot and sofosbuvir 440-3080ng/spot that was shown in figure 13 - 19and set of data were tabulated in table 13 &14. The correlation coefficient, slope, and intercept were found (table 12), which was obtained from calibration graph and is shown in figure 20.

Table 12: Regression data

Linear regression parameters	Ledipasvir	Sofosbuvir
Slope	4.847	1.461
Intercept	349.9	870.4
Correlation coefficient	0.994	0.992

Figure 13: Chromatogram of Ledipasvir (100ng/ spot) and Sofosbuvir(440ng/spot)

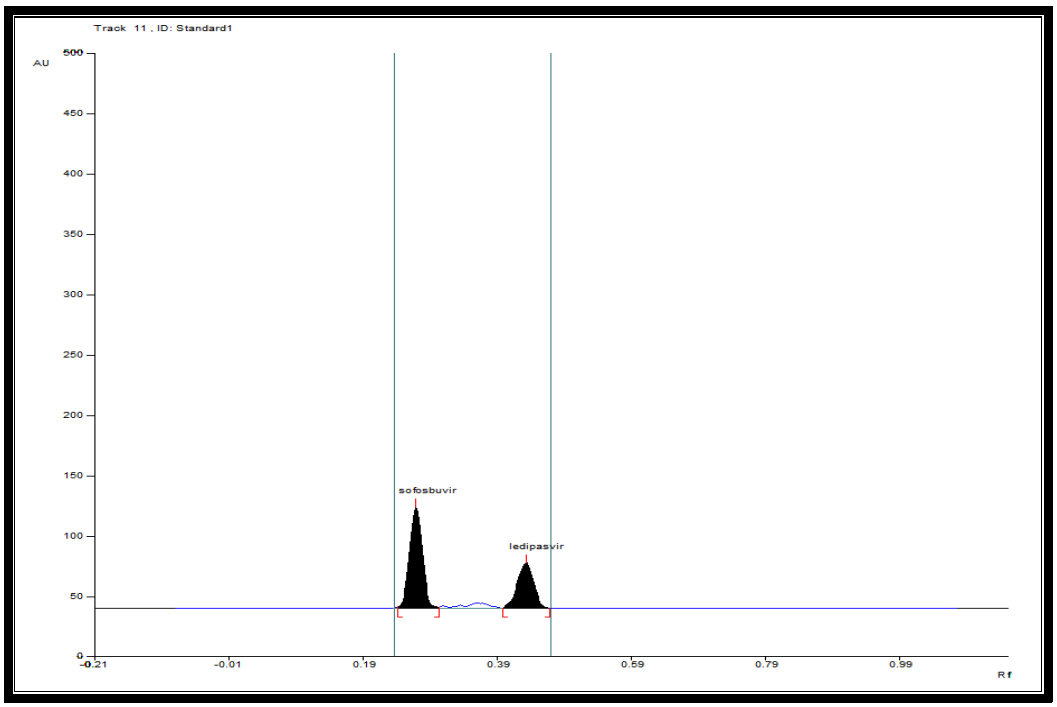


Figure 14: Chromatogram of Ledipasvir (200ng/ spot) and Sofosbuvir(880ng/spot)

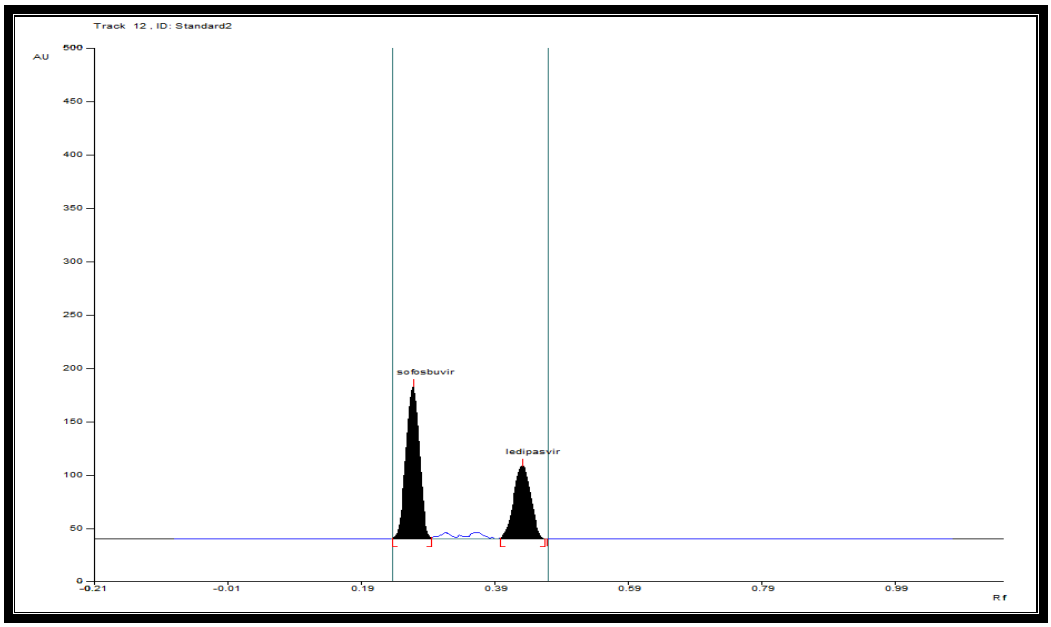


Figure 15: Chromatogram of Ledipasvir (300ng/ spot) and Sofosbuvir(1320ng/spot)

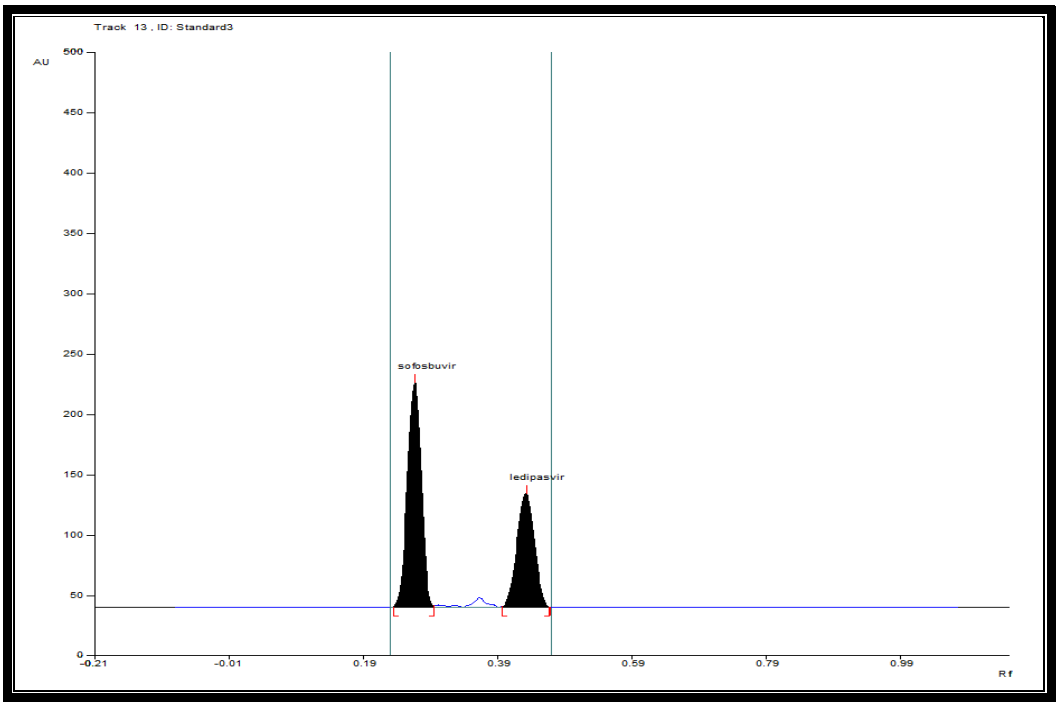


Figure 16: Chromatogram of Ledipasvir (400ng/ spot) and Sofosbuvir(1760ng/spot)

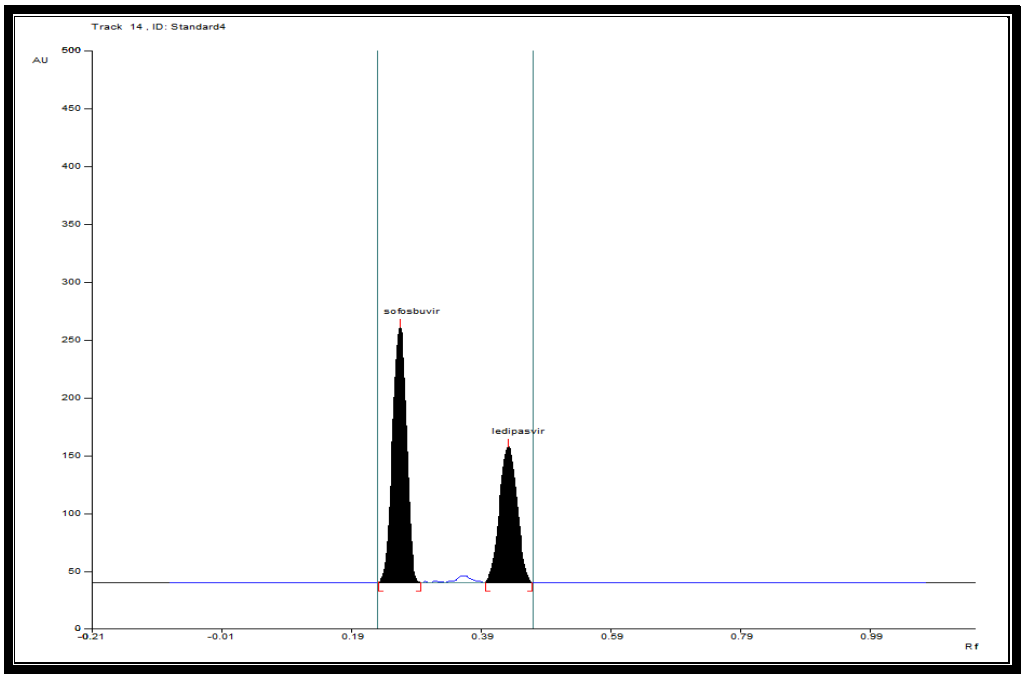


Figure 17: Chromatogram of Ledipasvir (500ng/ spot) and Sofosbuvir(2200ng/spot)

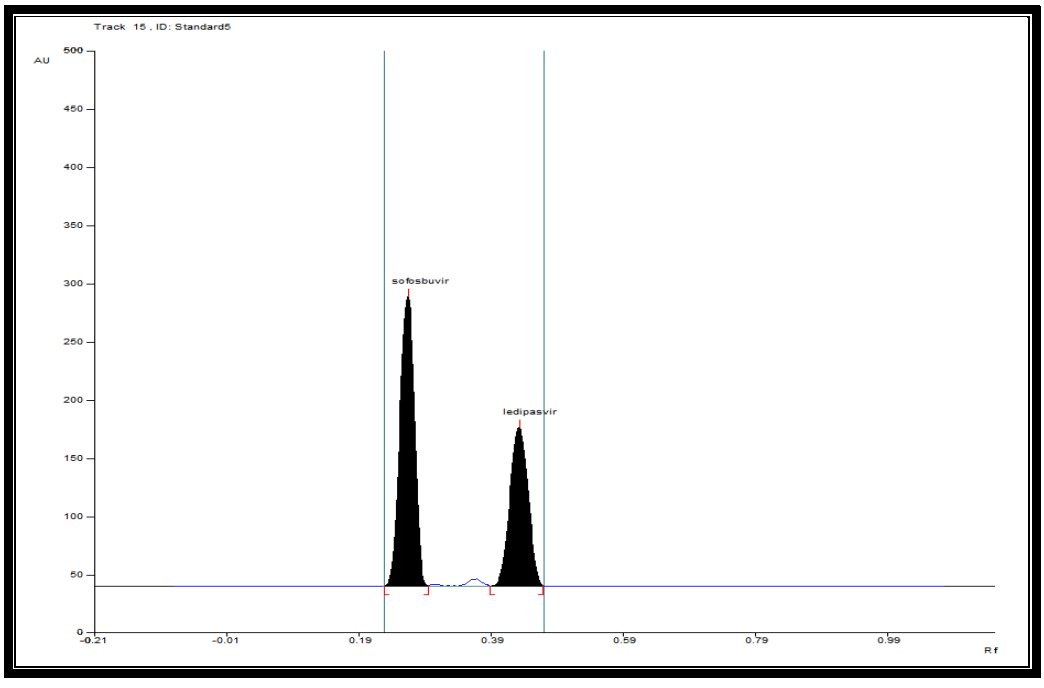


Figure 18: Chromatogram of Ledipasvir (600ng/ spot) and Sofosbuvir (2640ng/spot)

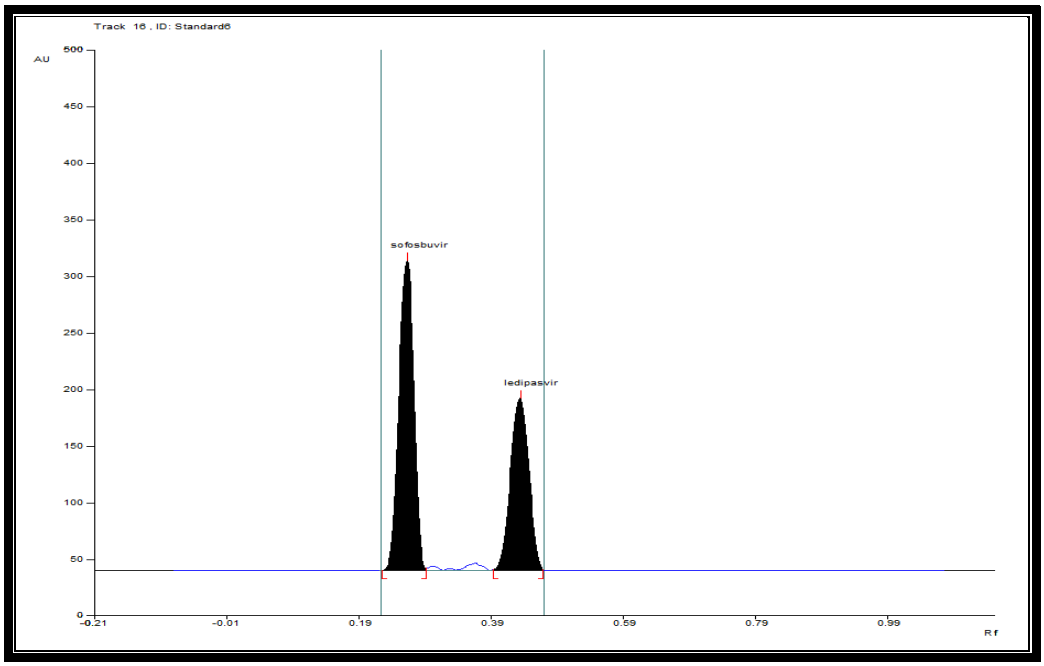


Figure 19: Chromatogram of Ledipasvir (700ng/ spot) and Sofosbuvir(3080ng/spot)

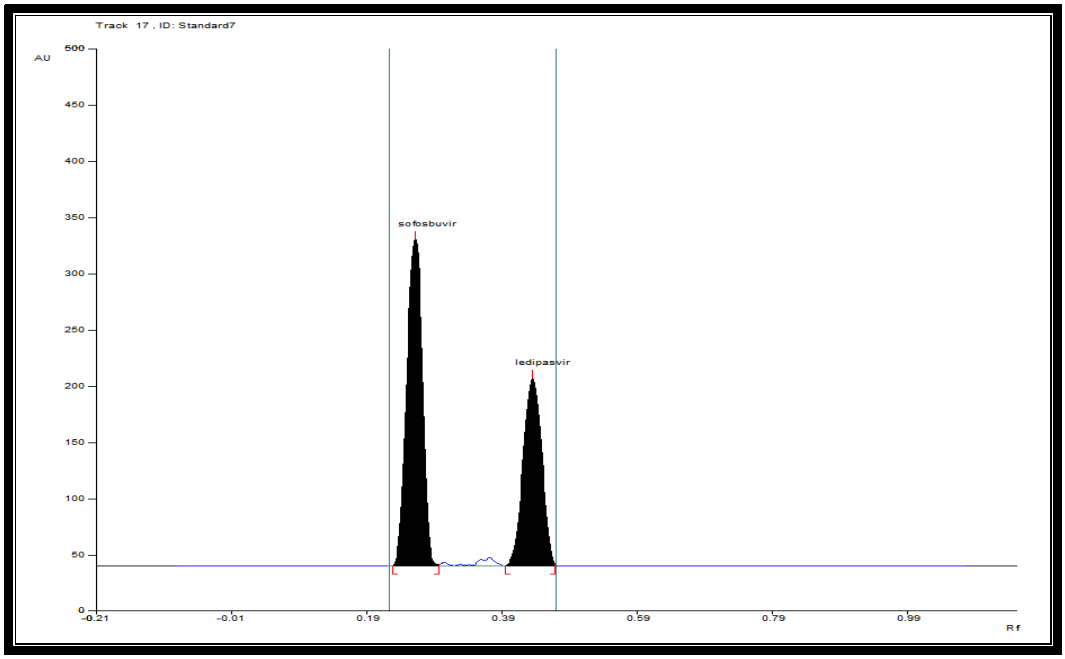


Table 14: Calibration data of Ledipasvir

Concentration (ng/spot)	Peak Area
100	731
200	1375
300	1943
400	2503
500	3044
600	3844
700	4422

Table 15: Calibration data of sofosbuvir

Concentration (ng/spot)	Peak Area
440	1373
880	2244
1320	3018
1760	3721
2200	4342
2640	4841
3080	5385

Figure 21: Calibration graph of Ledipasvir

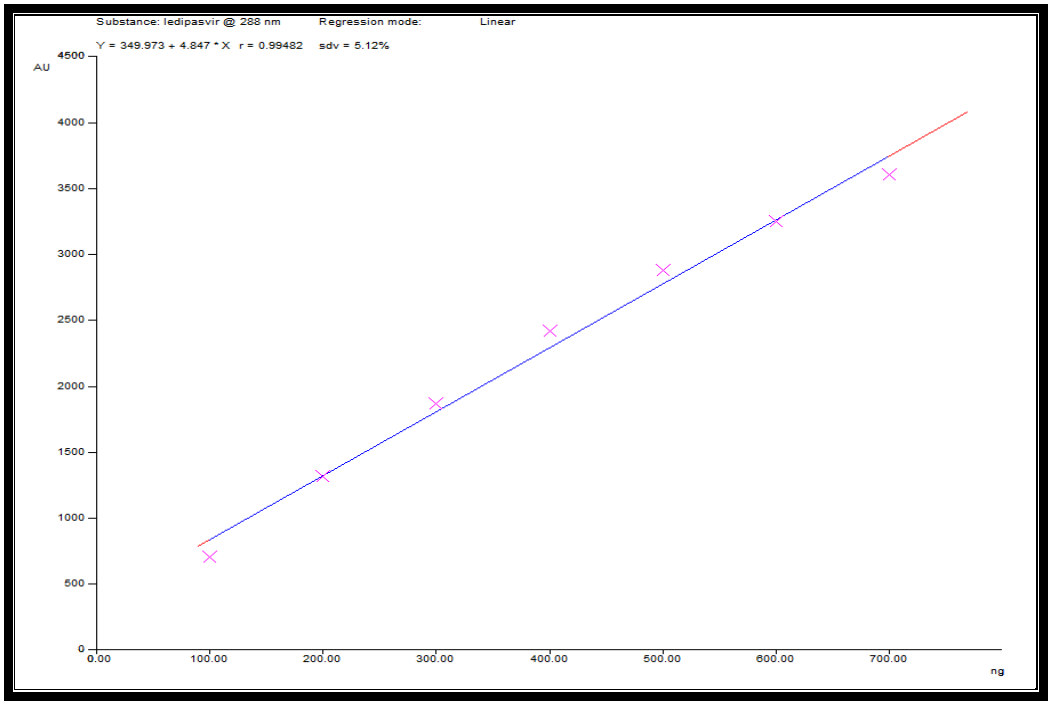
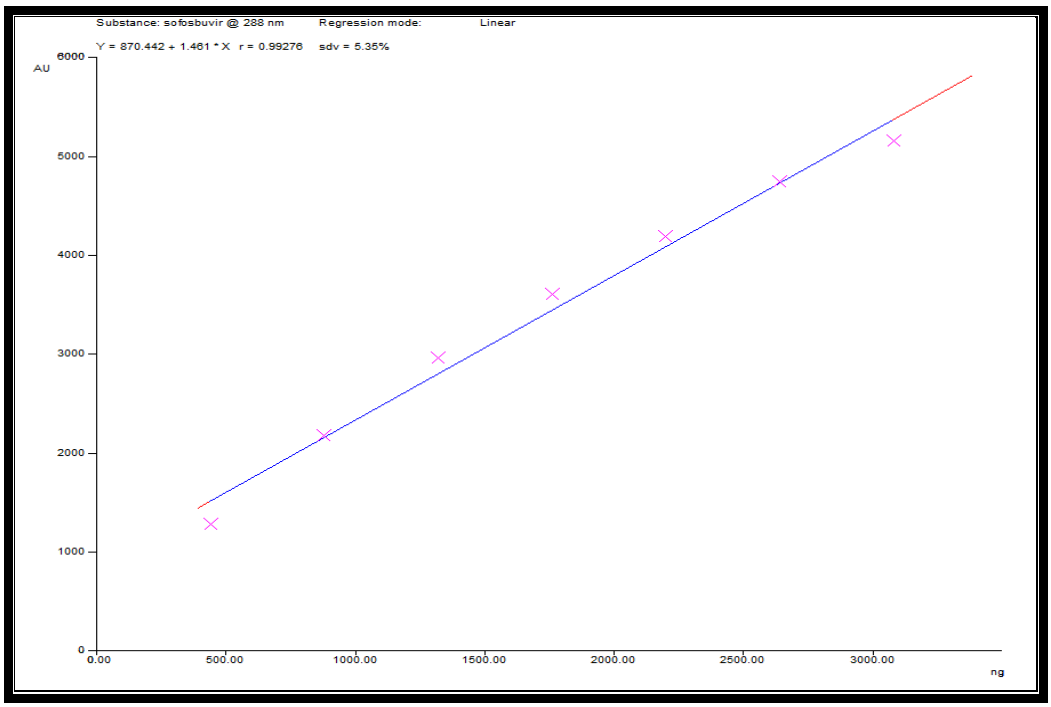


Figure 21: Calibration graph of Sofosbuvir



ACCURACY:

Recovery studies were done for determining accuracy parameter. It was done by mixing known quantity of standard drug with the analysed sample formulation and the contents were reanalysed by the proposed method. Recovery studies were carried out at 50,100, and 150% levels. The percentage recovery and its %RSD were calculated, shown in table 16a and 16b.

Table 16a :Recovery study for ledipasvir

Level	% Recovery	%RSD*
50%	99.41%	0.25
100%	98.25%	0.52
150%	101.84%	1.24

*RSD of six determination

Table 16b :**Recovery study for sofosbuvir**

Level	% Recovery	%RSD*
50%	100.25%	0.45
100%	101.85%	0.89
150%	100.89%	0.21

*RSD of six determination

PRECISION

The method precision was obtained by determining the assay by preparing six samples of selected concentration (200 & 400ng/spot) of Ledipasvir and (880 & 1760ng/spot) of sofosbuvir. The % RSD calculated for method precision of inter-day, intra-day (table 17,18 and repeatability (repeatability of sample measurement and sample application respectively) are shown in table 19,20.

Table 17: Intra-day precision

Concentration (ng/spot)	Peak area		%RSD*	
	Ledipasvir	Sofosbuvir	Ledipasvir	Sofosbuvir
Ledipasvir (200ng/band) & Sofosbuvir (880ng/band)	1375	2243	0.344	1.049
	1368	2251		
	1377	2207		
Ledipasvir (400ng/band) & Sofosbuvir (1760ng/band)	2503	3722	0.426	0.215
	2517	3775		
	2496	3706		

*RSD of three determination

Table 18:Inter-day precision

Concentration (ng/spot)	Peak area		%RSD*	
	Ledipasvir	Sofosbuvir	Ledipasvir	Sofosbuvir
Ledipasvir (200ng/band) & Sofosbuvir (880ng/band)	1365	2201	0.476	0.391
	1358	2212		
	1371	2195		
Ledipasvir (400ng/band) & Sofosbuvir (1760ng/band)	2576	3710	0.427	1.601
	2586	3728		
	2564	3618		

*RSD of three determination

Table 19: Repeatability of sample measurement

Concentration (ng/spot)	Peak area		% RSD*	
	Ledipasvir	Sofosbuvir	Ledipasvir	Sofosbuvir
Ledipasvir (200ng/band) & Sofosbuvir (880ng/band)	1389	2296	0.615	1.517
	1370	2269		
	1390	2258		
	1385	2205		
	1381	2218		
	1394	2233		

*RSD six determination

Table 20: Repeatability sample application

Concentration (ng/spot)	Peak area		% RSD*	
	Ledipasvir	Sofosbuvir	Ledipasvir	Sofosbuvir
Ledipasvir (200ng/band) & Sofosbuvir (880ng/band)	1368	2216	0.393	0.334
	1371	2208		
	1364	2227		
	1378	2212		
	1366	2223		
	1375	2211		

*RSD six determination

LOD and LOQ:

Limit of detection and limit of quantification that were calculated from the equation. The result was shown in table 21a and 21b , that proved the sensitivity of the method.

Table 21a: Results of LOD & LOQ of ledipasvir

Correlation coefficient±SD*	0.9948 ±0.0004590
Slope ± SD*	4.847 ±0.001049
Intercept ± SD*	349.9 ±0.1941
LOD(ng / spot)	0.13 ng / spot
LOQ(ng / spot)	0.40 ng / spot

*RSD of six determinations.

Table 21b: Results of LOD & LOQ of Sofosbuvir

Correlation coefficient ± SD*	0.9927 ±0.0003435
Slope ± SD*	1.461 ±0.001581
Intercept ± SD*	870.4 ±0.1924
LOD (ng / spot)	0.4345 ng / spot
LOQ (ng / spot)	1.316 ng / spot

*RSD of six determinations.

Robustness:

The study was confirmed to be robust as the R_f value and peak area was not much affected by introducing slight changes in the experimental conditions, that was shown in table 22 .

Table 22: Robustness evaluation

(200 Ledipasvir and 440 Sofosbuvir ng/spot)

MOBILE PHASE COMPOSITION:					
FACTOR	**LEVEL**	**PEAK AREA**		**% RSD ***	
		Ledipasvir	**Sofosbuvir**	**Ledipasvir**	**Sofosbuvir**
4:5:1(10ml)	-0.1	1370	2241	1.02	0.84
	0	1375	2244	0.96	0.48
	+0.1	1360	2254	0.85	0.45
MOBILE PHASE VOLUME:					
FACTOR	**LEVEL**	**PEAK AREA**		**% RSD ***	
		Ledipasvir	**Sofosbuvir**	**Ledipasvir**	**Sofosbuvir**
10ml	-0.1	1350	2247	0.78	1.44
	0	1380	2265	0.65	1.04
	+0.1	1375	2278	0.89	0.89
CHAMBER SATURATION TIME:					
FACTOR	**LEVEL**	**PEAK AREA**		**% RSD***	
		Ledipasvir	**Sofosbuvir**	**Ledipasvir**	**Sofosbuvir**
20 minutes	-5	1358	2304	0.36	0.69
	0	1369	2245	0.89	0.80
	+5	1398	2278	0.78	0.92
SOLVENT MIGRATION DISTANCE :					
FACTOR	**LEVEL**	**PEAK AREA**		**% RSD***	
		Ledipasvir	**Sofosbuvir**	**Ledipasvir**	**Sofosbuvir**
80mm	+5	1365	2245	0.25	0.12
	0	1368	2244	0.75	0.02
	-5	1369	2249	0.48	0.09

* Mean of six observations

Specificity:

The peak purity index at the peak start, peak apex and peak end was determined and was found to be about 0.999661 and 0.999978(ledipasvir)and 0.999458 and 0.999847 of sofosbuvir that was shown in table 23a and 23b. This proved high specificity of the method thereby developed.

Table 23a: Specificity of ledipasvir

Peak purity	Peak purity index
r(s* , m*)	0.999661
r(m* , e*)	0.999978

s*peak start**,** m*apex and e*peak end

Table 23b: Specificity of Sofosbuvir

Peak purity	Peak purity index
r(s* , m*)	0.999458
r(m* , e*)	0.999847

s*peak start**,** m*apex and e*peak end

STABILITY STUDIES

Stability of chromatographic plate

When the developed chromatographic plate was exposed to atmosphere, the analytes are likely to decomposed. Hence it was necessary to conduct stability studies of the plate.

Stability of the analyte on the plate was studied at different time intervals and peak areas were compared with peak area of freshly scanned plate. The developed plate was found to be stable for upto 24hrs, table 24a and 24b.

Table 24a: Ledipasvir

Time (hr)	Concentration(ng/spot)	Peak area
0	200 400	2244 3721
6	200 400	2201 3608
24	200 400	2017 3415

Table 24b: sofosbuvir

Time (hr)	Concentration(ng/spot)	Peak area
0	880 1760	1375 2503
6	880 1760	1310 2445
24	880 1760	1125 2278

ANALYSIS OF TABLET FORMULATION

The % label claim was calculated and the estimated amount was close to the labelled value that was shown in figure 22 and table 25.

Figure 22: Chromatogram of Ledipasvir and Sofosbuvir

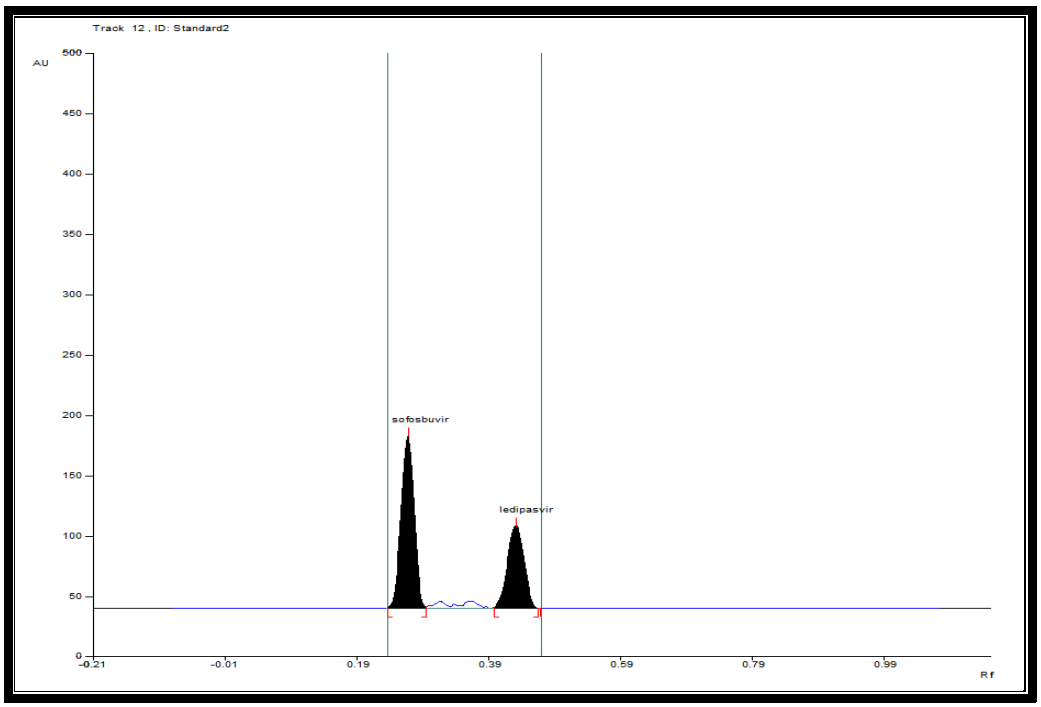


Table 25: Analysis of formulation

Drug Name (cimivir-L)	Amount (mg/tab)		% Label claim	%RSD*
	Labelled	Estimated		
Ledipasvir	90	91.45	101.61	0.25
Sofosbuvir	400	399.05	99.76	0.38

***RSD of six observations**

FORCED DEGRADATION STUDIES: LEDIPASVIR

Acid hydrolysis:

Ledipasvir was subjected to acid hydrolysis, an additional peak was observed along with standard, R_f value of 0.34, which is shown in figure 23a &23b.

Figure 23a: Chromatogram of acid hydrolysis of Ledipasvir

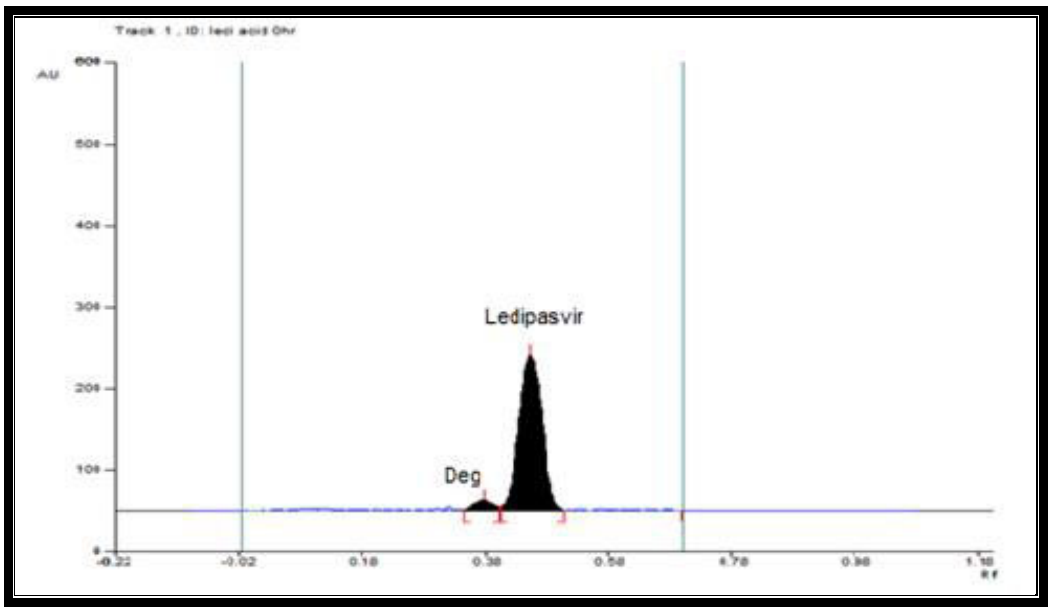
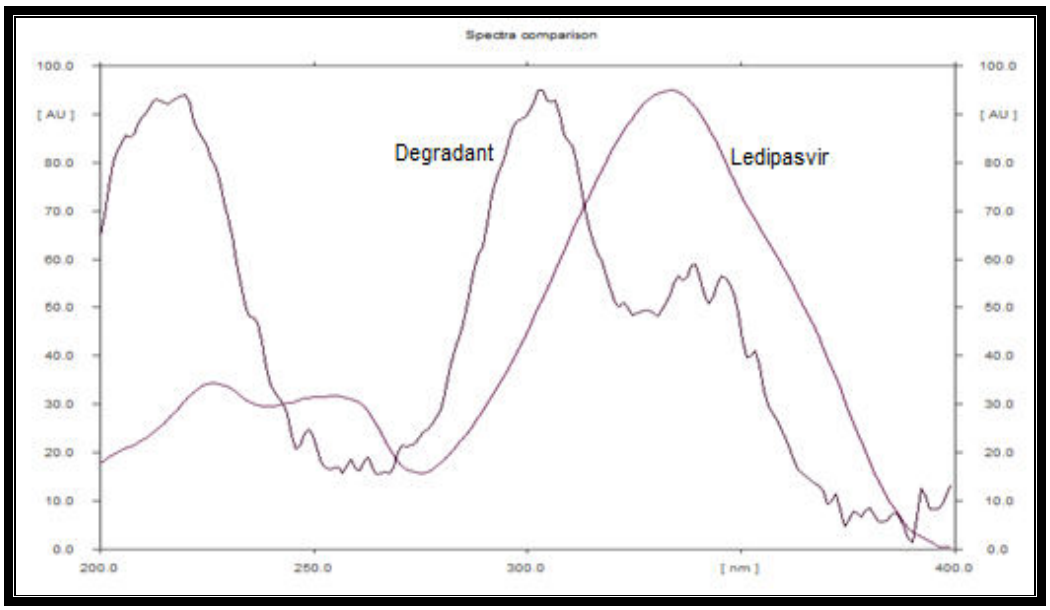


Figure 23b: Spectrum of Ledipasvir and Degradant



Alkaline hydrolysis:

The drug was subjected to alkaline hydrolysis additional peaks were observed at R_f value 0.34, which was shown in figure 24a &24b.

Figure 24a: Chromatogram of base hydrolysis of Ledipasvir

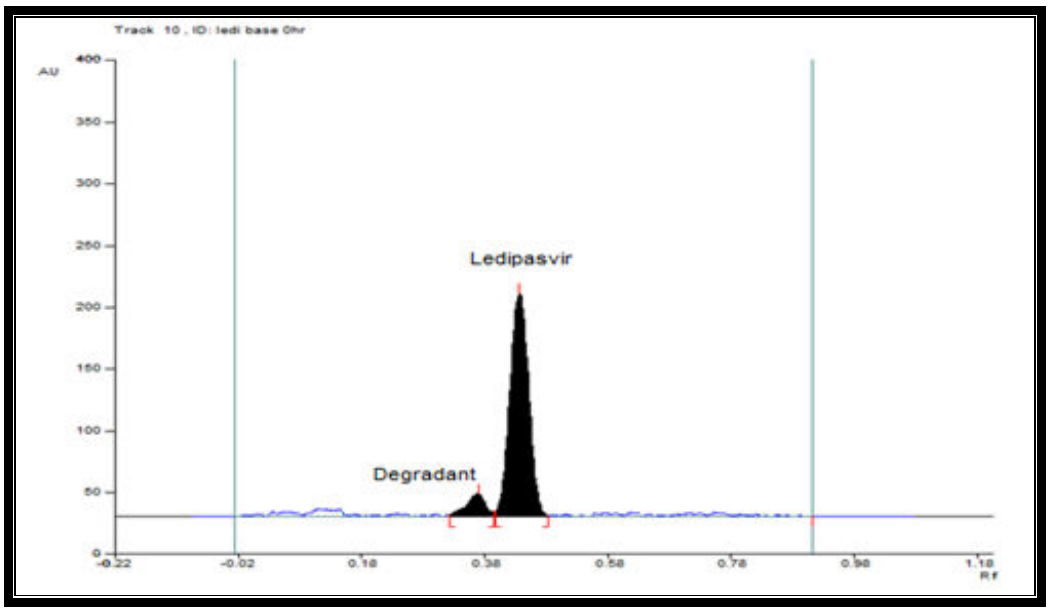
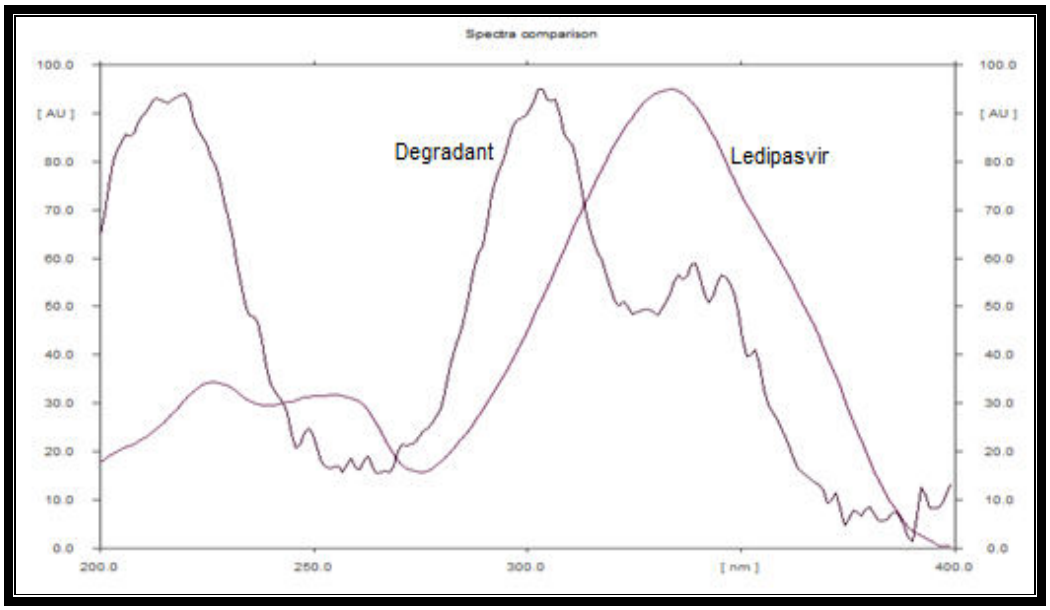


Figure 24b: Spectrum of ledipasvir and degradant



Neutral hydrolysis:

The drug was subjected to neutral hydrolysis no additional peaks were observed, which was shown in figure 25a & 25b.

Figure 25a: Chromatogram of neutral hydrolysis of Ledipasvir

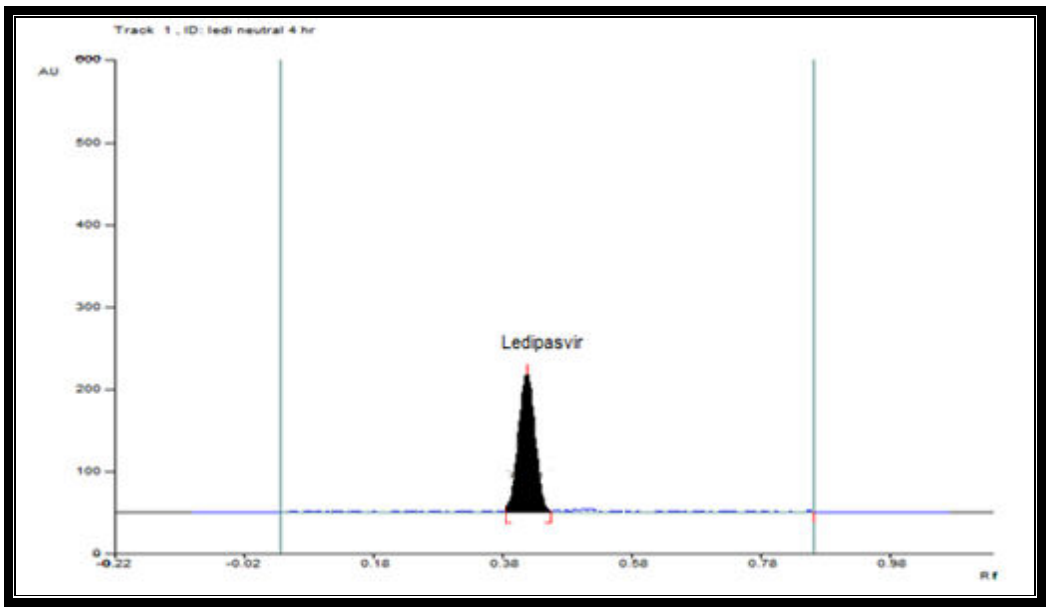
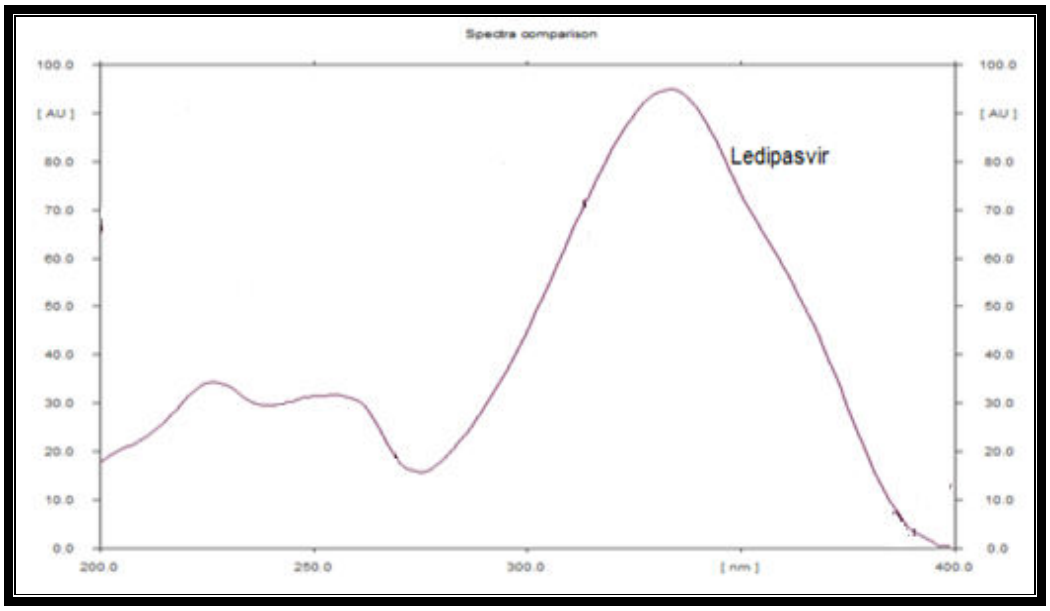


Figure 25b: Spectrum of ledipasvir



Oxidative degradation:

The drug was subjected to oxidation additional peaks were observed at R_f value 0.34, which was shown in figure 26a & 26b.

Figure 26a: Chromatogram of oxidative degradation of Ledipasvir

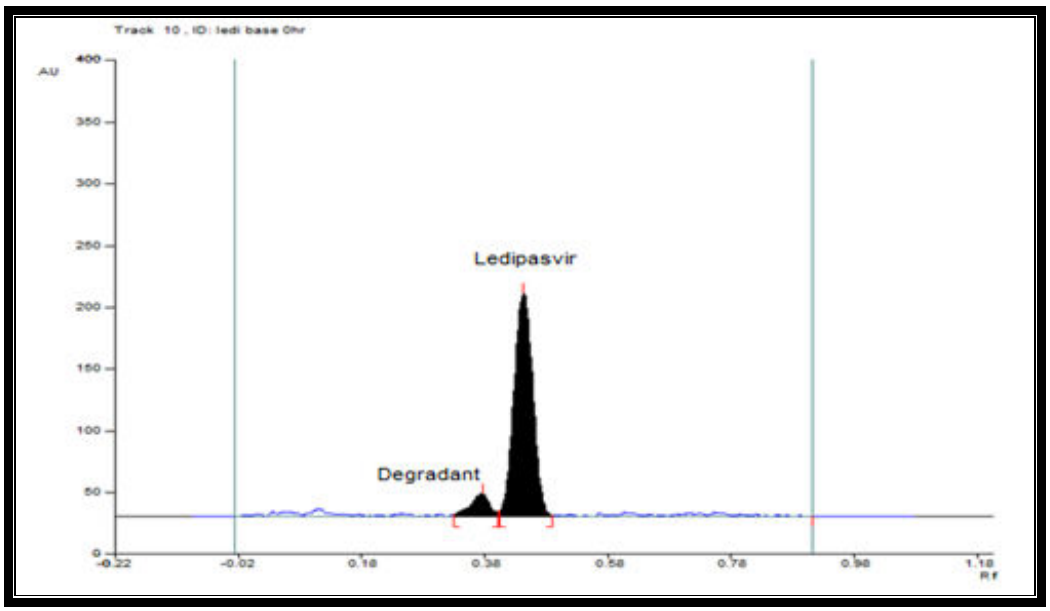
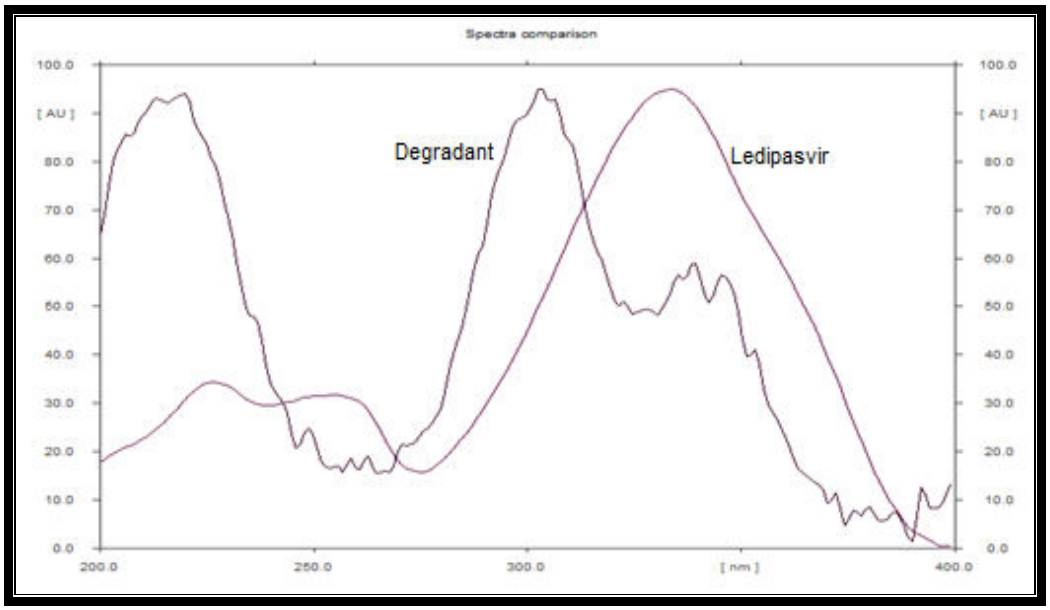


Figure 26b: Spectrum of ledipasvir and degradant



Photolytic degradation:

The drug was subjected to photolytic degradation additional peaks were observed at R_f value 0.34, which was shown in figure 27a & 27b.

Figure 27a: Chromatogram of photolytic degradation of ledipasvir

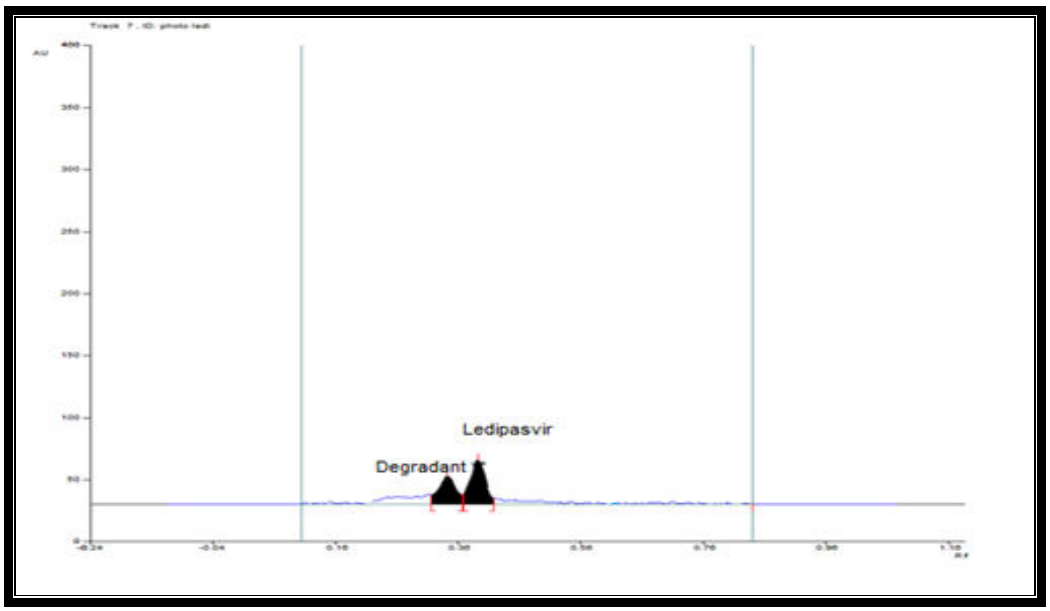
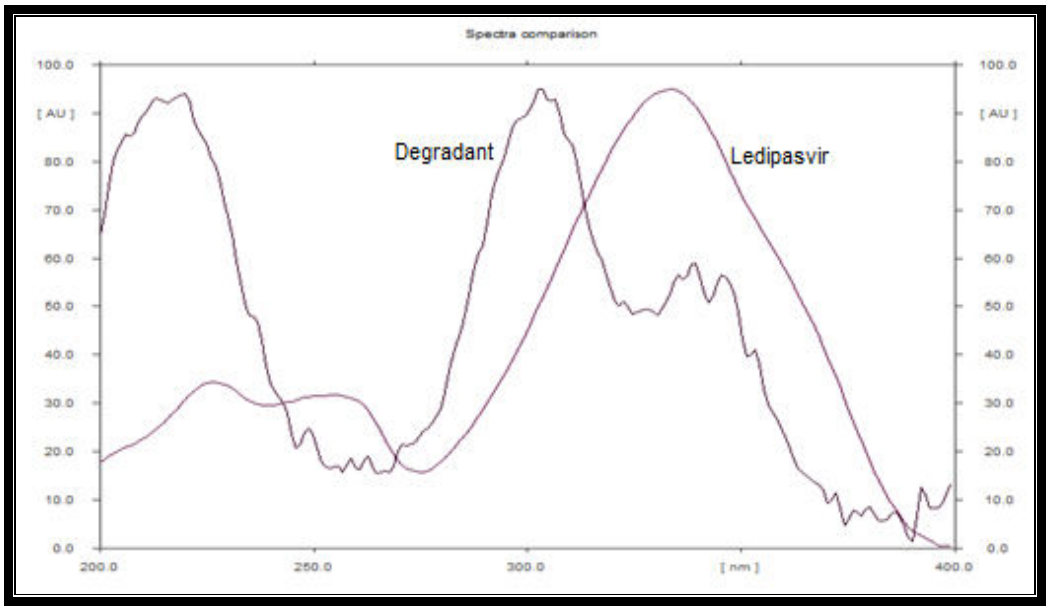


Figure 27b: Spectrum of ledipasvir and degradant



Thermal degradation

The drug was subjected to thermal degradation additional peaks were observed at R_f value 0.34, which was shown in figure 28a & 28b.

Figure 28a: Chromatogram of thermal degradation of Ledipasvir

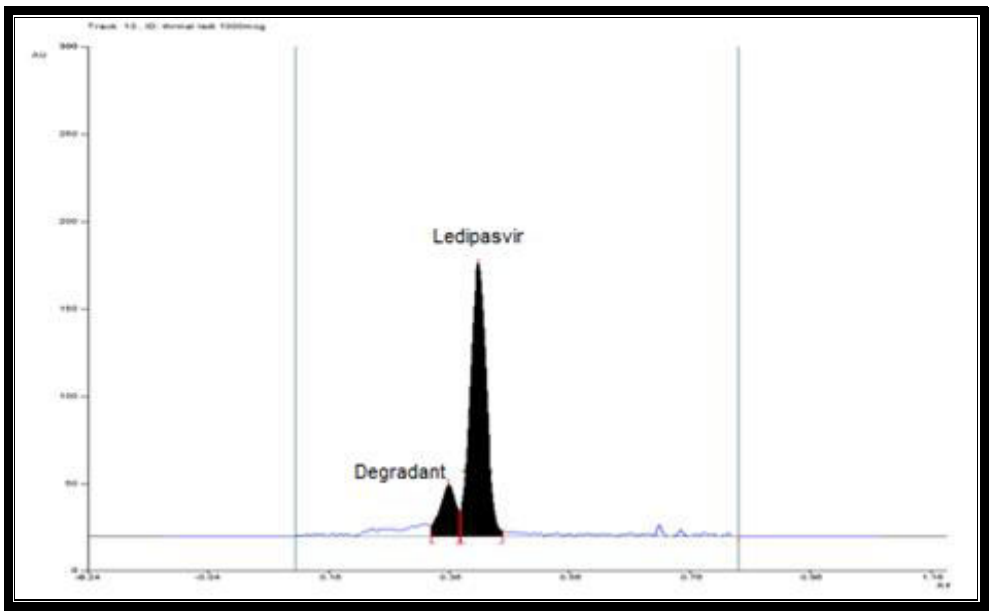
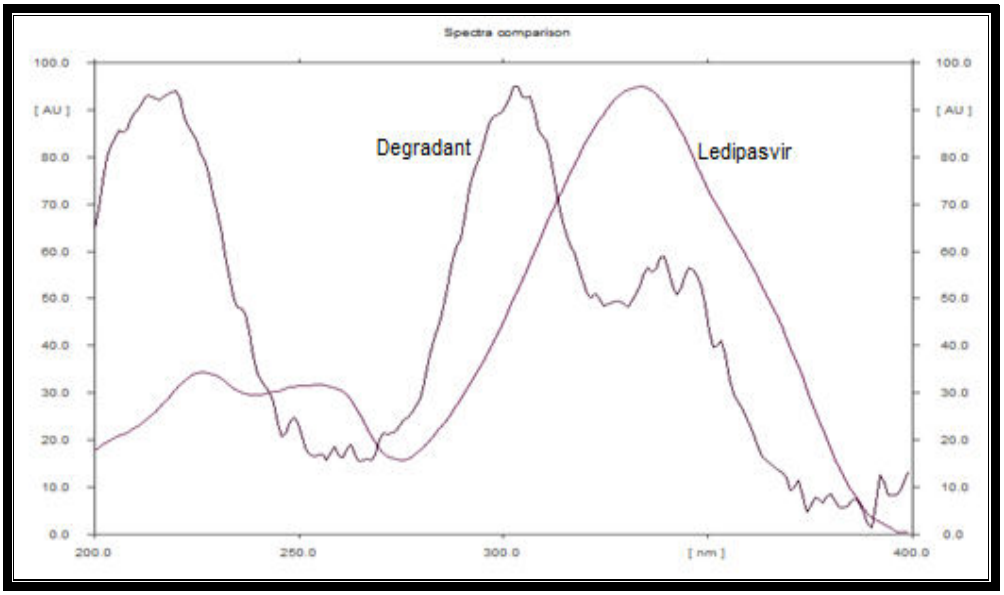


Figure 28b: Spectrum of ledipasvir and degradant



Summary of forced degradation was shown in the table 26.

Table 26 Summary of forced degradation studies for Ledipasvir:

Type of stress	Stress condition	R_f value	
		Drug	Degraded Product
Acid hydrolysis	Drug refluxed with 0.1M HCl for about 5 hours	0.43±0.02	0.34 ± 0.02
Basic hydrolysis	Drug refluxed with 0.1M NaoH for about 5 hours	0.43±0.02	0.34 ± 0.02
Neutral hydrolysis	Drug treated with water and refluxed for about 5 hours	0.43±0.02	No additional peaks
Oxidative degradation	Drug treated with 6 % hydrogen peroxide at normal room temperature	0.43±0.02	0.34± 0.02
Photo degradation	Drug exposed to Sunlight for 5 hours	0.43±0.02	0.34± 0.02
Thermal degradation	Drug introduced in Hot air oven for 5 hours	0.43±0.2	0.34± 0.02

FORCED DEGRADATION STUDIES: SOFOSBUVIR

Acid hydrolysis:

Sofosbuvir was subjected to acid hydrolysis, no additional peak was observed along with standard, that is shown in figure 29a & 29b.

Figure 29a: Chromatogram of acid hydrolysis of Sofosbuvir

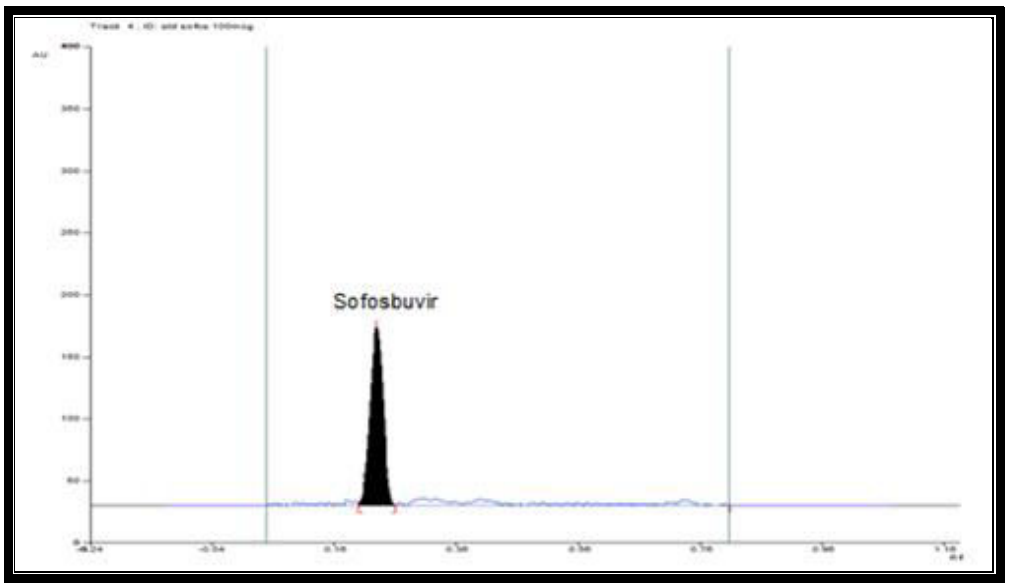
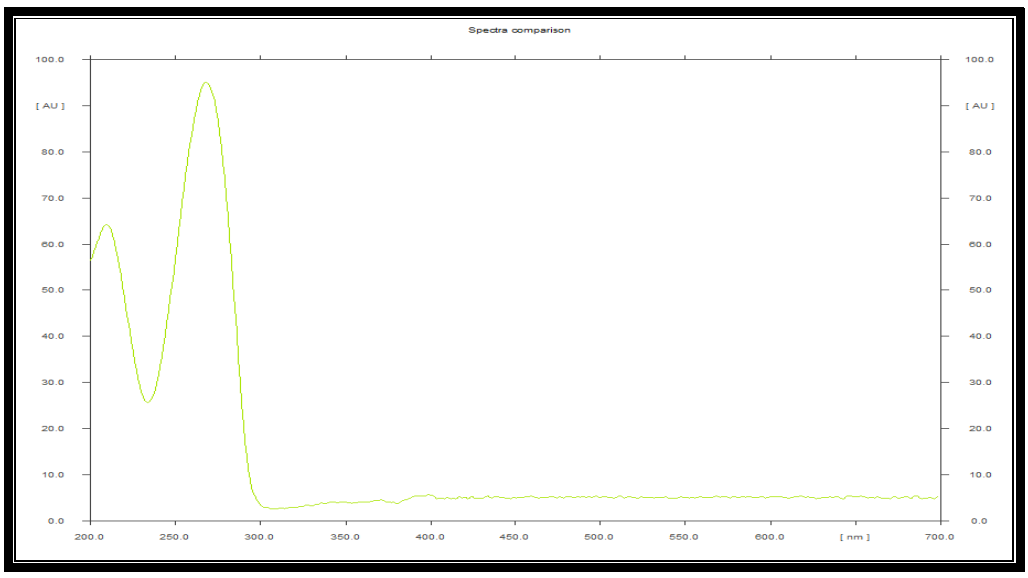


Figure 29b: Spectrum of Sofosbuvir



Alkaline hydrolysis:

The drug was subjected to alkaline hydrolysis three additional peaks were observed at R_f 0.12, 0.28 and 0.33 value which was shown in figure 30a & 30b.

Figure 30a: Chromatogram of base hydrolysis of Sofosbuvir

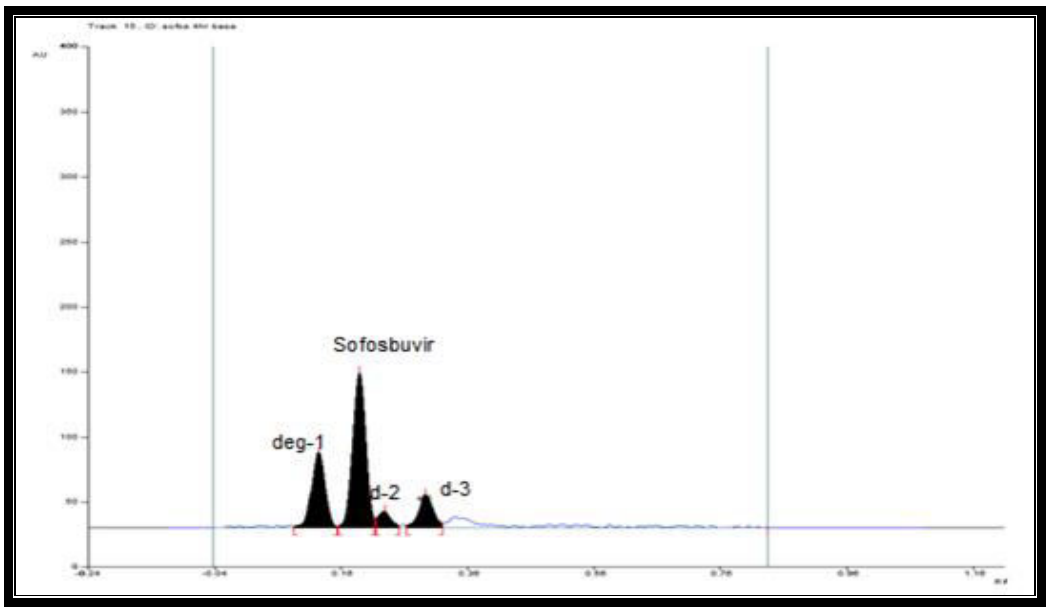
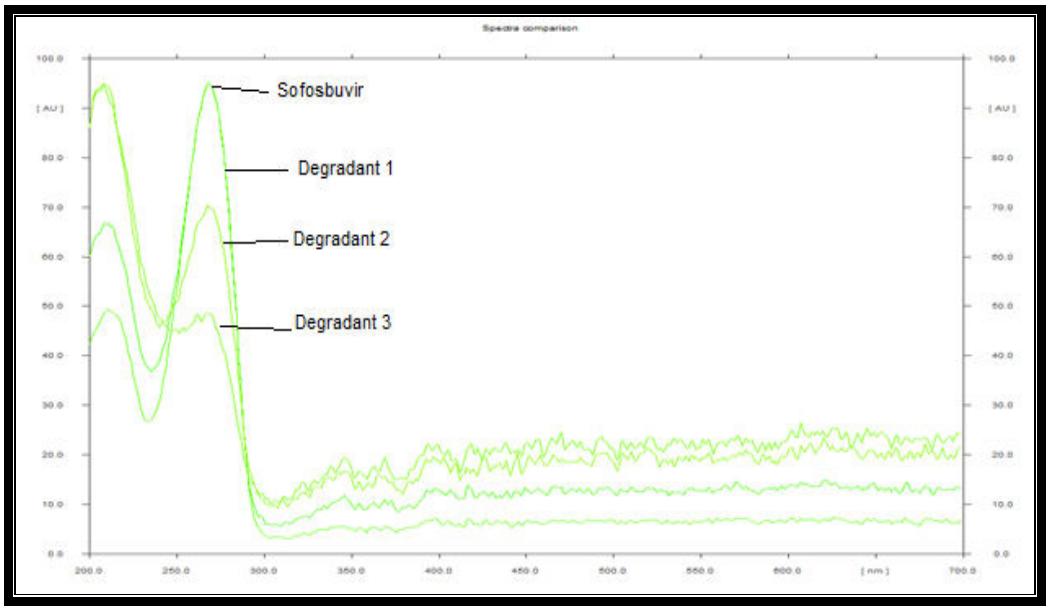


Figure 30b: Spectrum of Sofosbuvir and degradant



Neutral hydrolysis:

The drug was subjected to neutral hydrolysis no additional peaks were observed, which was shown in figure 31a & 31b

Figure 31a: Chromatogram of neutral hydrolysis of Sofosbuvir

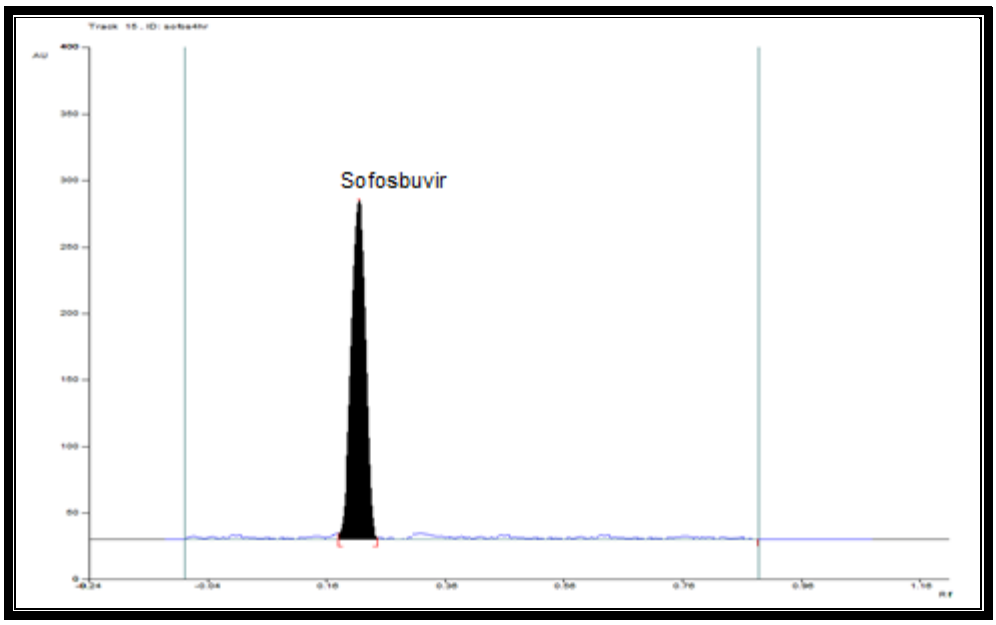
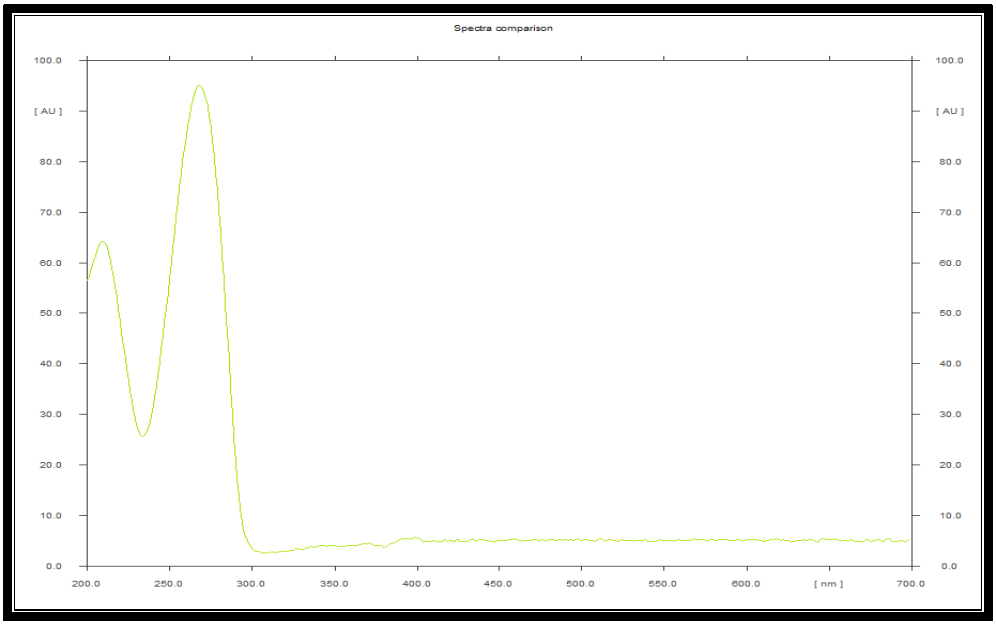


Figure 31b: Spectrum of Sofosbuvir



Oxidative degradation:

The drug was subjected to oxidation no additional peaks were observed at R_f value 0.04, which was shown in figure 32a & 32b.

Figure 32a: Chromatogram of oxidative degradation of Sofosbuvir

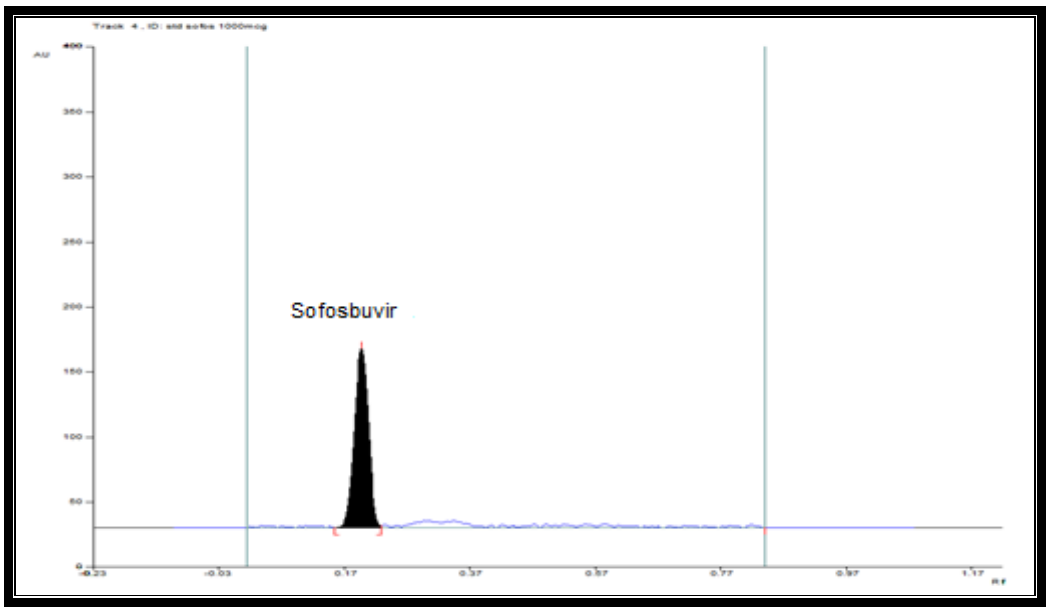
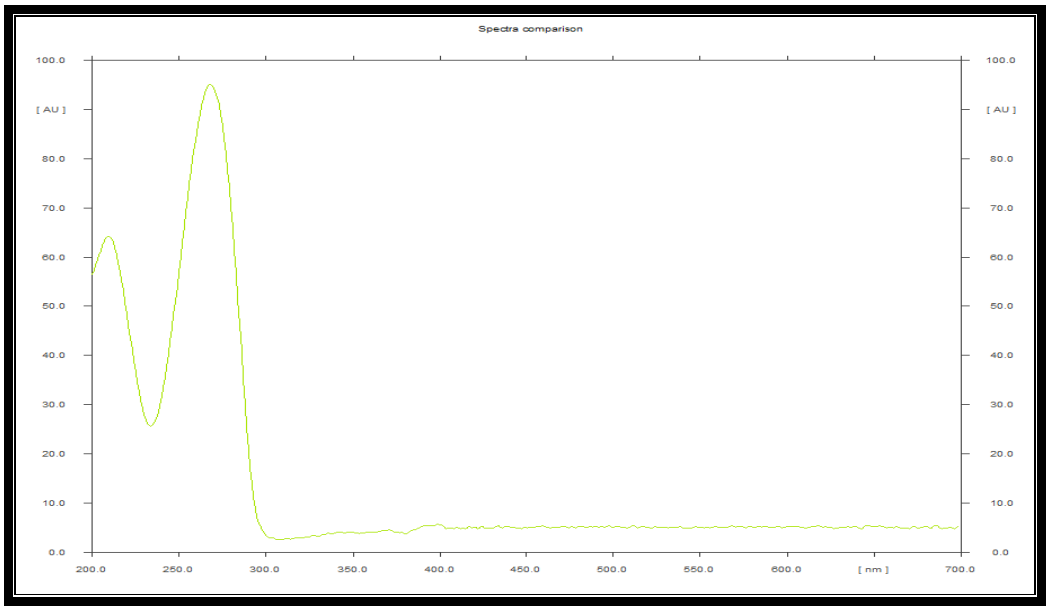


Figure 32b: Spectrum of Sofosbuvir and



Photolytic degradation:

The drug was subjected to photolytic degradation no additional peaks were observed, which was shown in figure 33a & 33b.

Figure 33a: Chromatogram of photolytic degradation of Sofosbuvir

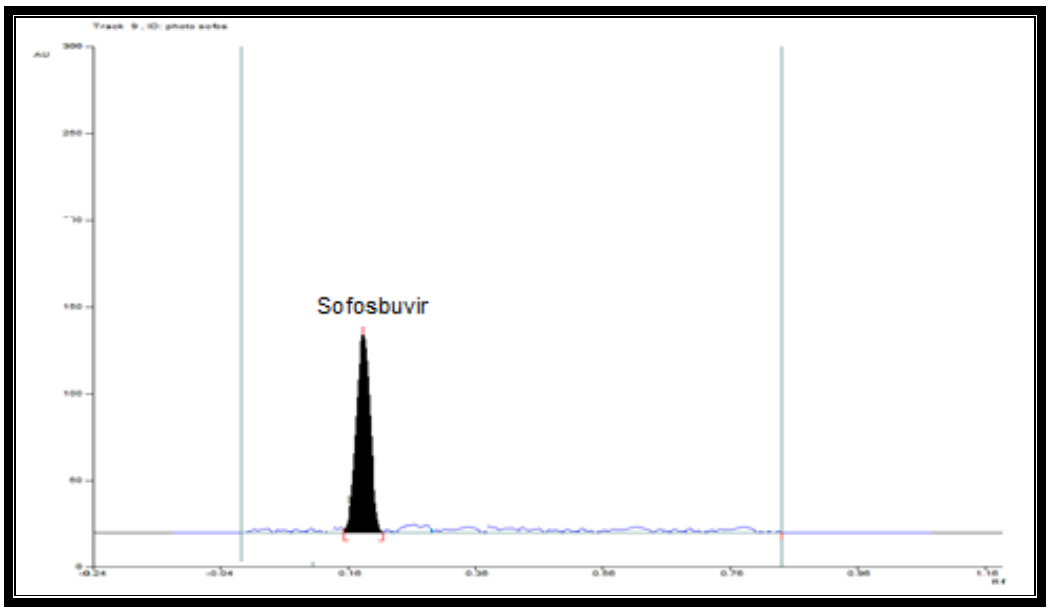
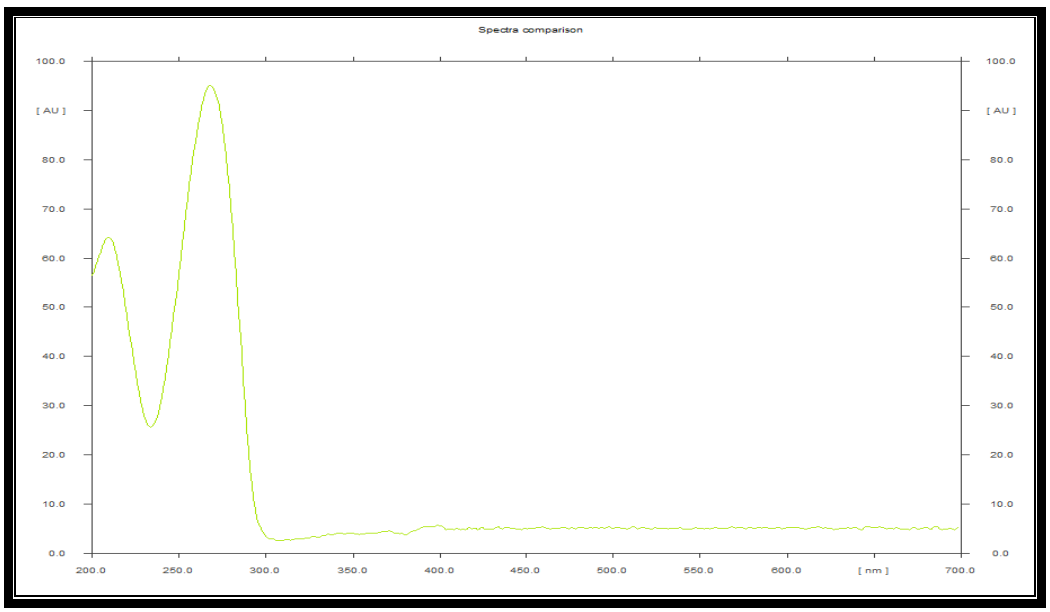


Figure 33b: Spectrum of Sofosbuvir



Thermal degradation

The drug was subjected to thermal degradation no additional peaks were observed, which was shown in figure 34a & 34b.

Figure 37: Chromatogram of thermal degradation of Sofosbuvir

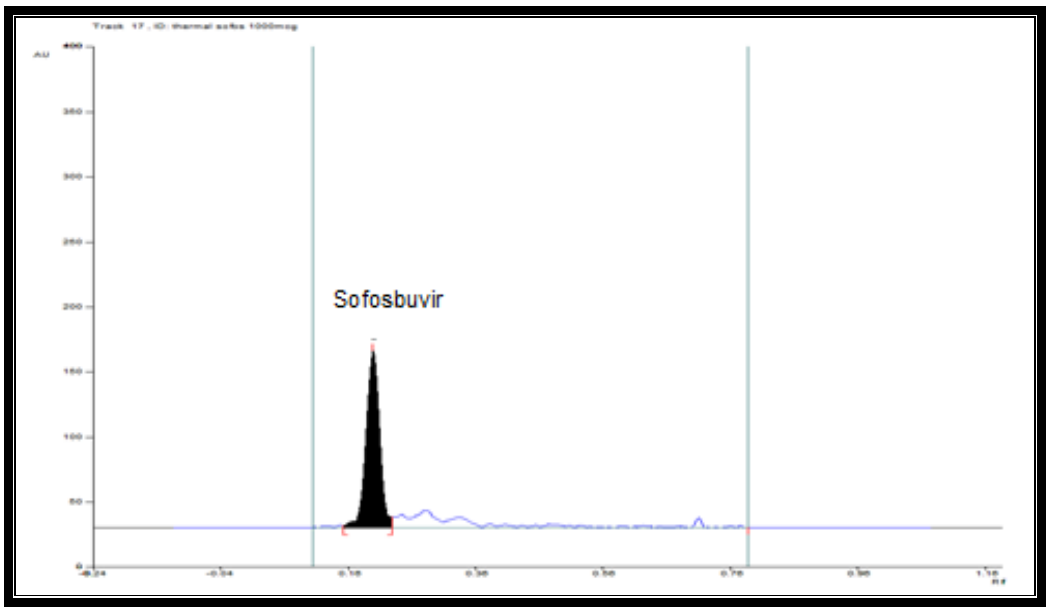
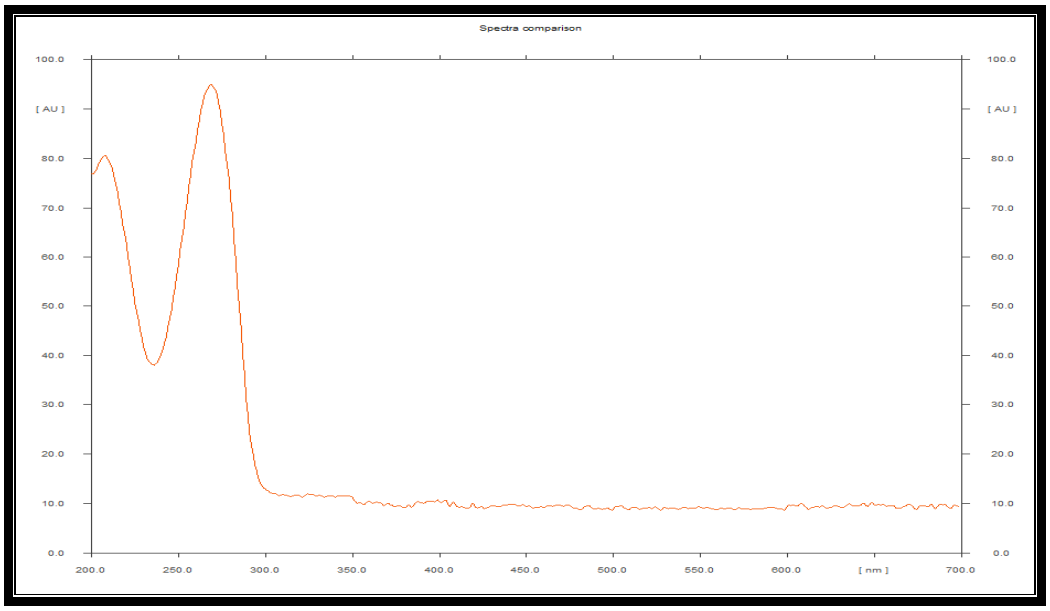


Figure 34b: Spectrum of Sofosbuvir and degradant



Summary of forced degradation was shown in the table 27.

Table 27 Summary of forced degradation studies for Sofosbuvir:

Type of stress	Stress condition	R_f value	
		Drug	Degraded Product
Acid hydrolysis	Drug refluxed with 0.1M HCl for about 5 hours	0.21±0.02	No additional peaks
Basic hydrolysis	Drug refluxed with 0.1m NaoH for about 5 hours	0.21±0.02	0.12 ± 0.02 0.28 ± 0.02 0.33± 0.02
Neutral hydrolysis	Drug treated with water and refluxed for about 5 hours	0.21±0.02	No additional peaks
Oxidative degradation	Drug treated with 6% hydrogen peroxide at normal room temperature	0.21±0.02	No additional peaks
Photo degradation	Drug exposed to Sunlight for 5 hours	0.21±0.02	No additional peaks
Thermal degradation	Drug introduced in Hot air oven for 5 hours	0.21±0.2	No additional peaks

FORCED DEGRADATION STUDIES: LEDIPASVIR AND SOFOSBUVIR

Acid hydrolysis:

Ledipasvir and sofosbuvir were subjected to acid hydrolysis, an additional peak was observed along with standards, R_f value of 0.34 that is shown in figure 35a & 35b.

Figure 35a: Chromatogram of acid hydrolysis of ledipasvir and sofosbuvir

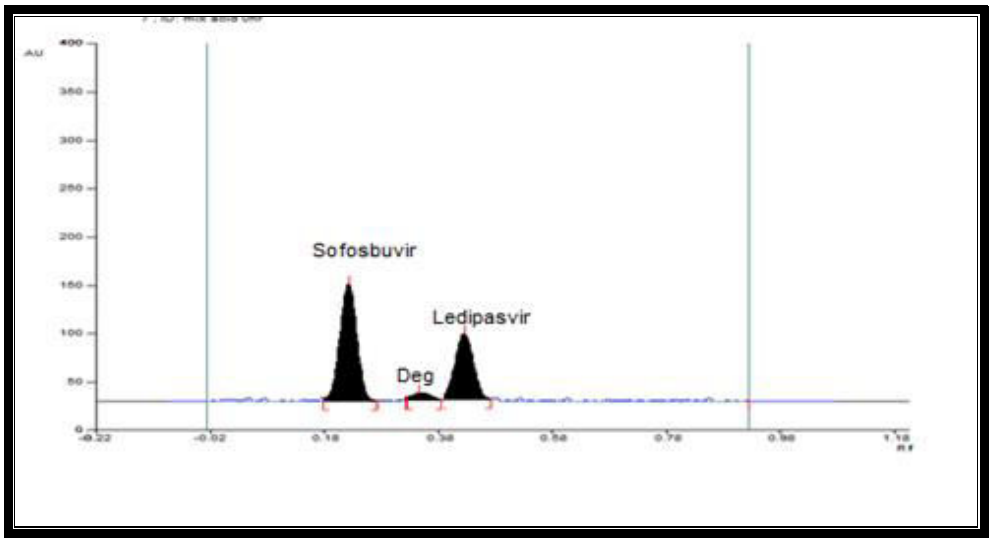
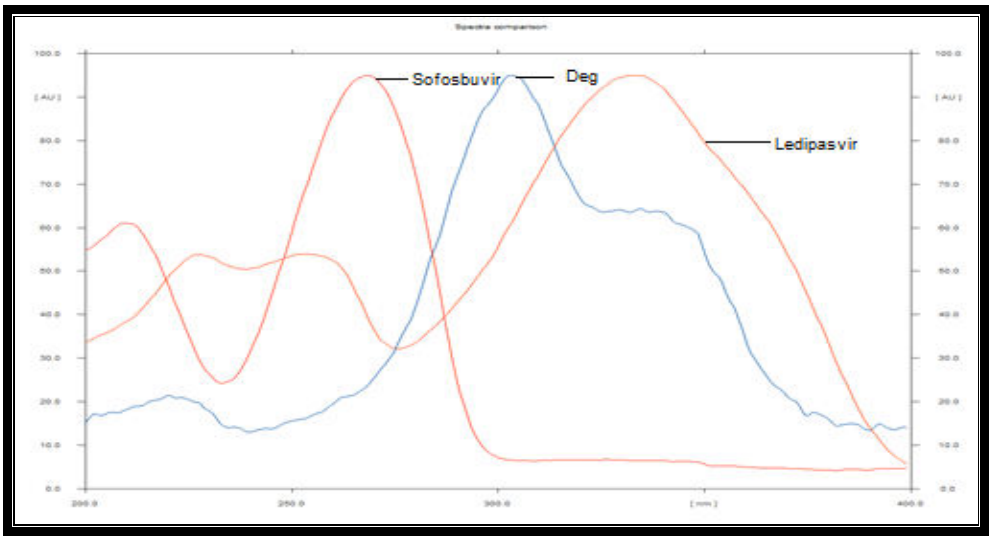


Figure 35b: Spectrum of Ledipasvir, Sofosbuvir and degradants



Alkaline hydrolysis:

The drug was subjected to alkaline hydrolysis two additional peaks were observed at R_f 0.12, 0.28 and 0.34value which was shown in figure 36a & 36b.

Figure 36a: Chromatogram of alkaline hydrolysis of ledipasvir and sofosbuvir

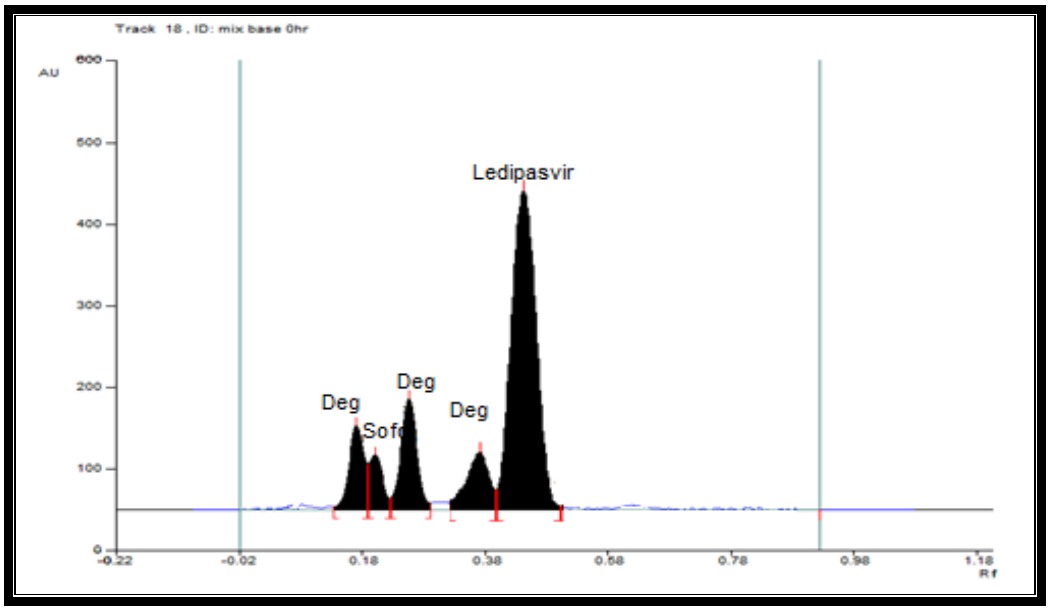
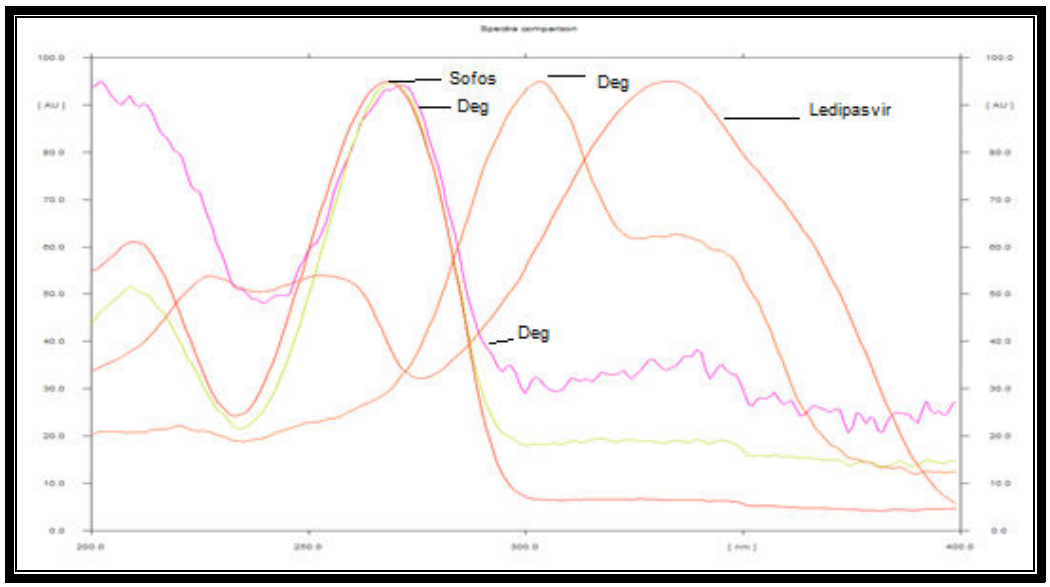


Figure 36b: Spectrum of Ledipasvir, Sofosbuvir and degradants



Neutral hydrolysis:

Ledipasvir and sofosbuvir were subjected to neutral hydrolysis no additional peaks were observed, which was shown in figure 37a & 37b.

Figure 37a: Chromatogram of neutral hydrolysis of ledipasvir and sofosbuvir

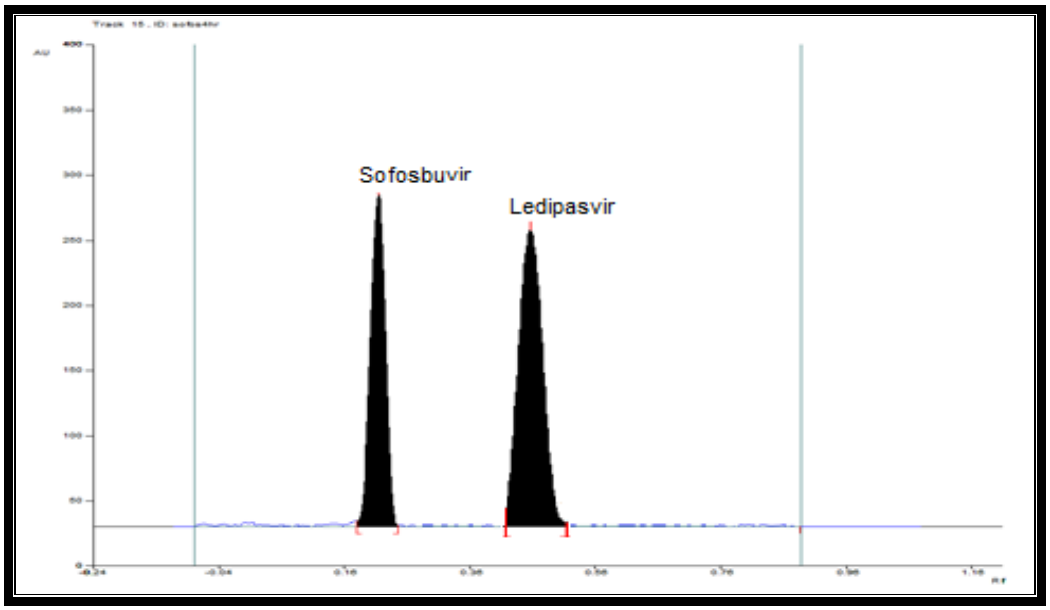
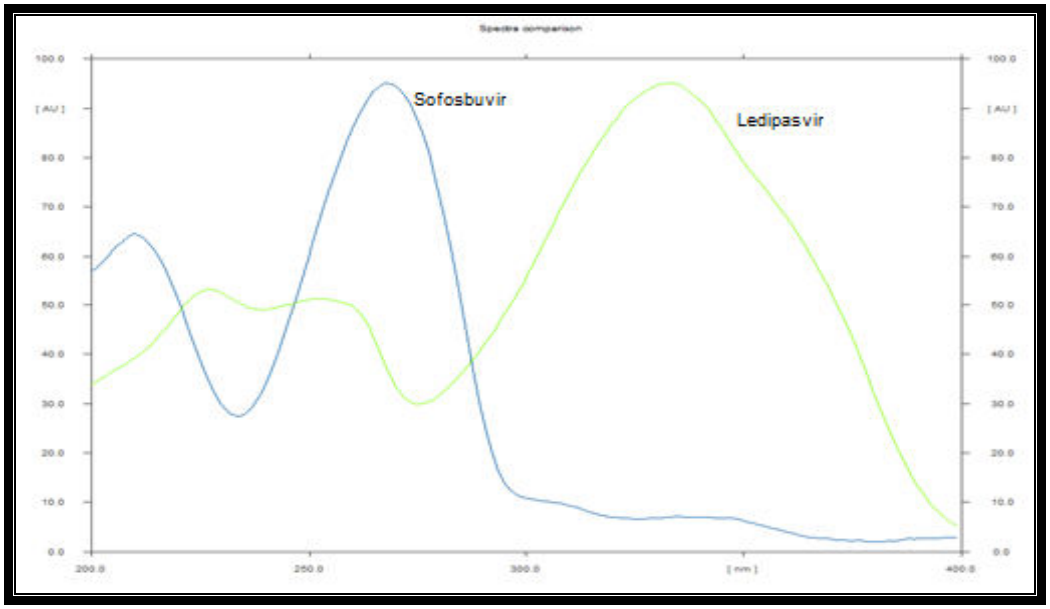


Figure 37b: Spectrum of Ledipasvir and Sofosbuvir



Oxidative degradation:

Ledipasvir and sofosbuvir were was subjected to oxidation additional peaks were observed at R_f value 0.34 , which was shown in figure 38a & 38b.

Figure 38a: Chromatogram of Oxidative degradation of ledipasvir and sofosbuvir

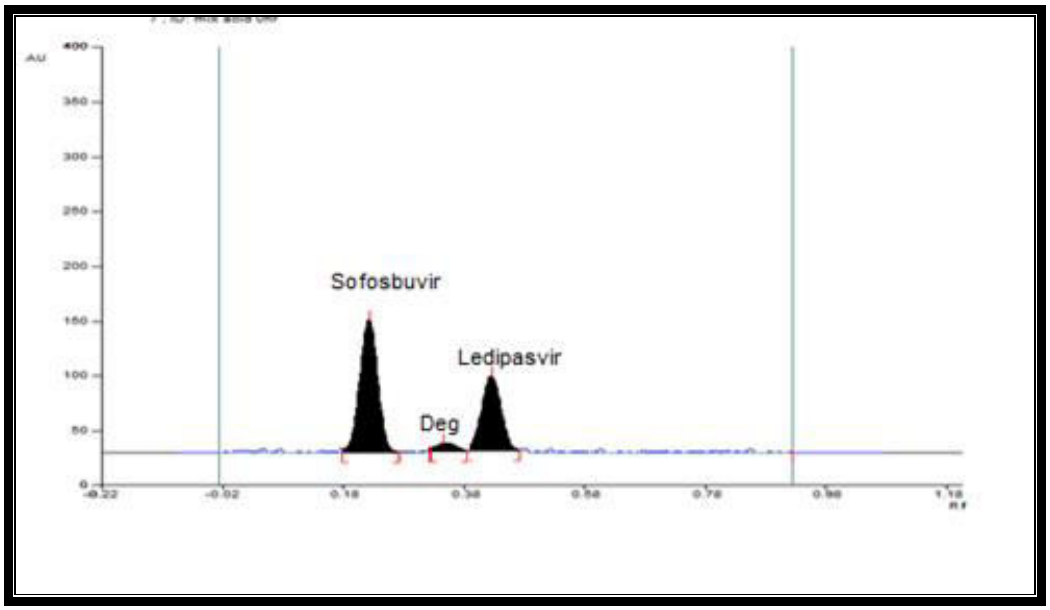
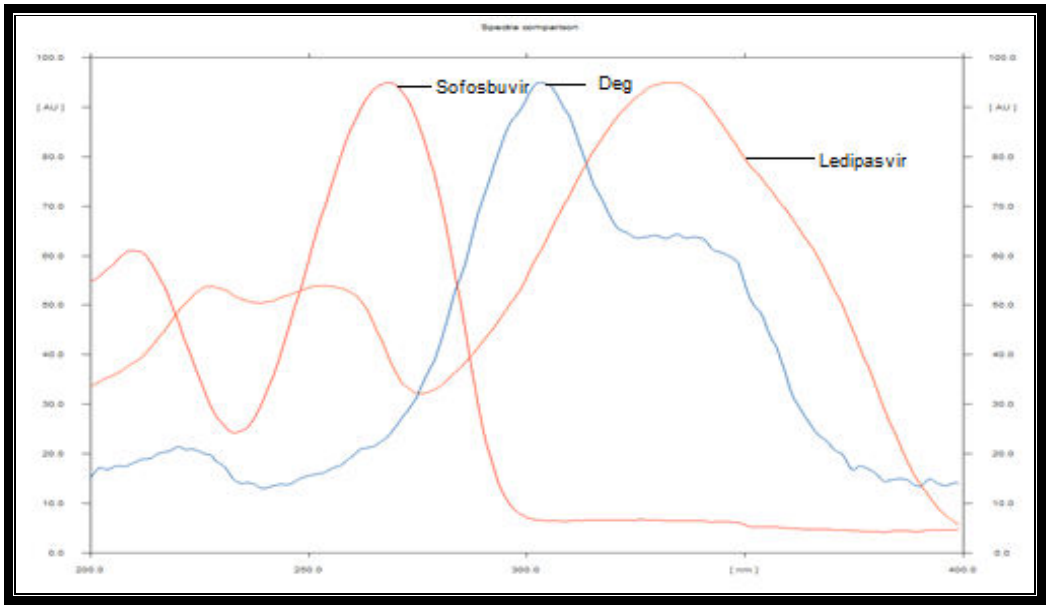


Figure 38b: Spectrum of Ledipasvir, Sofosbuvir and degradants



Photolytic degradation:

Ledipasvir and sofosbuvir were was subjected to photolytic degradation additional peaks were observed at R_f value 0.34, which was shown in figure 39a &39b.

Figure 39a: Chromatogram of photolytic degradation of ledipasvir and sofosbuvir

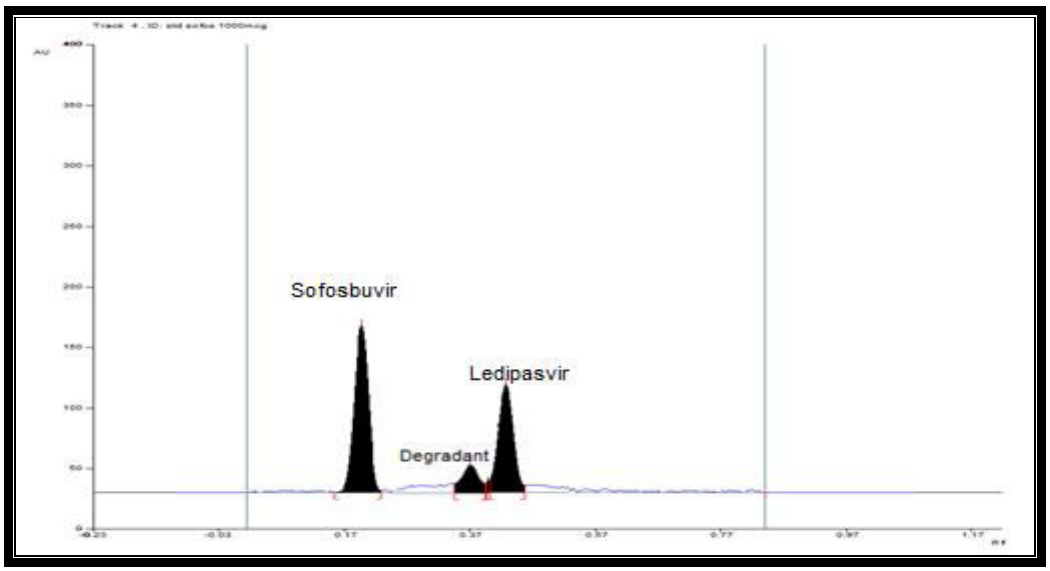
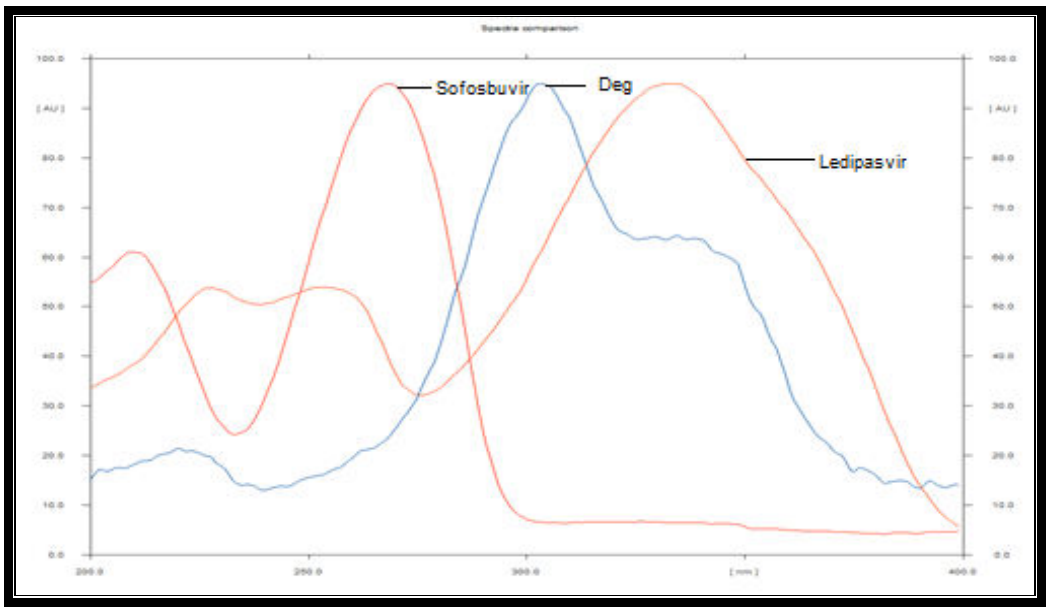


Figure 39b: Spectrum of Ledipasvir, Sofosbuvir and degradants



Thermal degradation

The drug was subjected to thermal degradation additional peaks were observed at R_f value 0.34, which was shown in figure 40a & 40b.

Figure 40a: Chromatogram of thermal degradation of ledipasvir and sofosbuvir

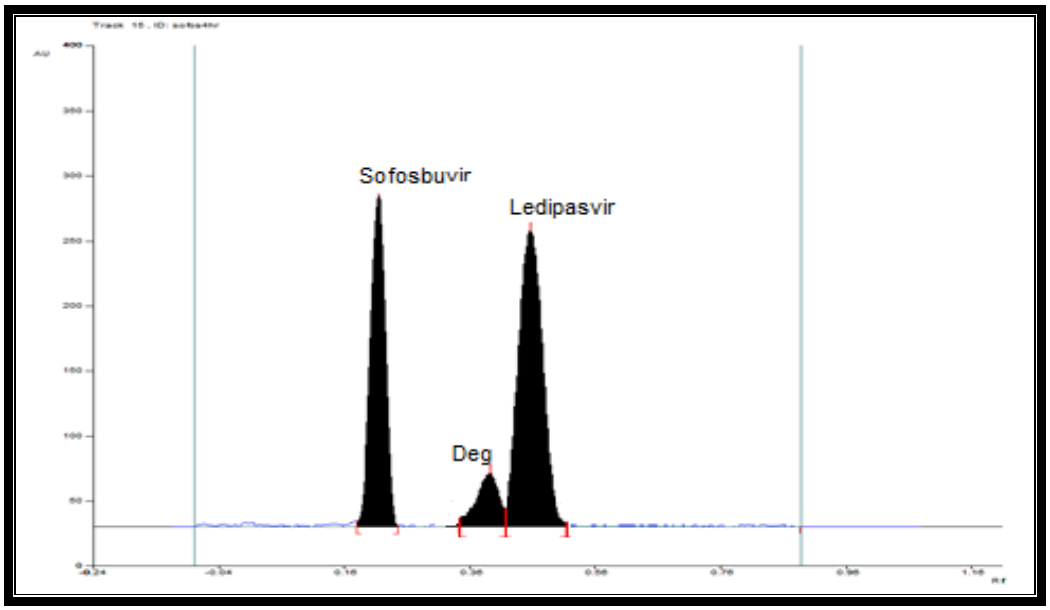


Figure 340b: Spectrum of Ledipasvir, Sofosbuvir and degradant

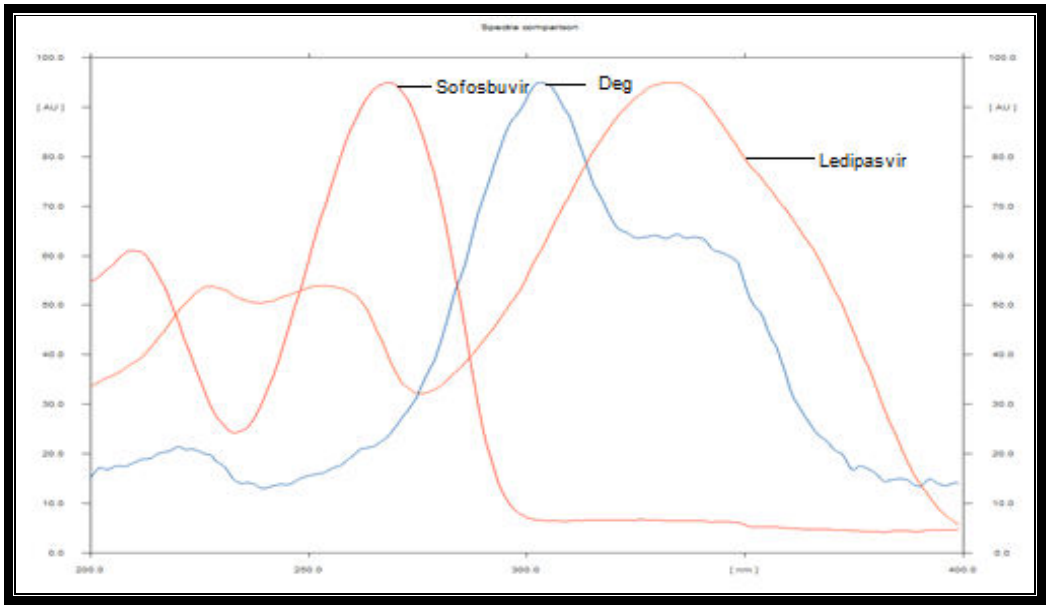


Table 28: Summary of forced degradation studies for Ledipasvir and Sofosbuvir:

Type of stress	Stress condition	R_f value			
		Ledipasvir	Sofosbuvir	Ledipasvir Degradant	Sofosbuvir Degradant
Acid hydrolysis	Drugs refluxed with 0.1M HCl for about 5 hours	0.43±0.02	0.21 ± 0.02	0.34 ± 0.02	No additional peaks
Basic hydrolysis	Drugs refluxed with 0.1m NaoH for about 5 hours	0.43±0.02	0.21 ± 0.02	0.34 ± 0.02	0.12 ± 0.02 0.28 ± 0.02
Neutral hydrolysis	Drugs treated with water and refluxed for about 5 hours	0.43±0.02	0.21± 0.02	No additional peaks	No additional peaks
Oxidative degradation	Drugs treated with 6% hydrogen peroxide at normal room temperature	0.43±0.02	0.21± 0.02	0.34± 0.02	No additional peaks
Photo degradation	Drugs exposed to Sunlight for 5 hours	0.43±0.02	0.21± 0.02	0.34± 0.02	No additional peaks
Thermal degradation	Drugs introduced in Hot air oven for 5 hours	0.43±0.2	0.21± 0.02	0.34± 0.02	No additional peaks

DEVELOPMENT AND VALIDATION OF RP-HPLC METHOD FOR THE SIMULTANEOUS DETERMINATION OF LEDIPASVIR AND SOFOSBUVIR IN BULK AND FORMULATION

The Ledipasvir and Sofosbuvir is a polar in nature hence RP-HPLC method and C_{18} column was used for the HPLC method development and stability indicating for the ledipasvirand sofosbuvir.

Selection of wavelength:

Good analytical separation can be obtained only by careful selection of wavelength for the detection (Fig. 41-43). The smooth obtained from which 254nm was select for the both drug was recorded on HPLC system.

Fig. 41 Spectrum of Ledipasvir

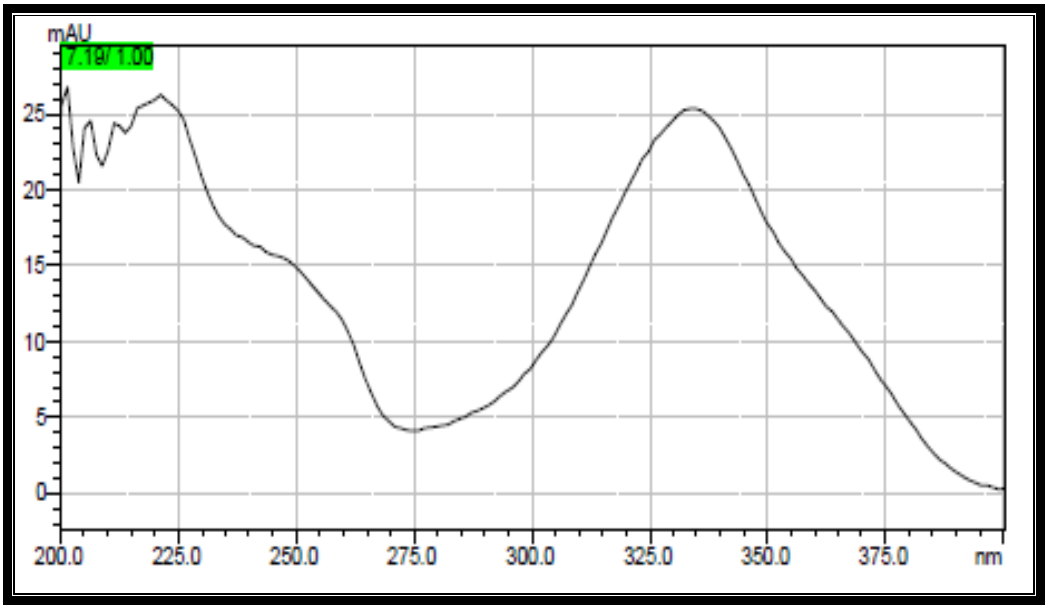


Fig. 42 Spectrum of Sofosbuvir

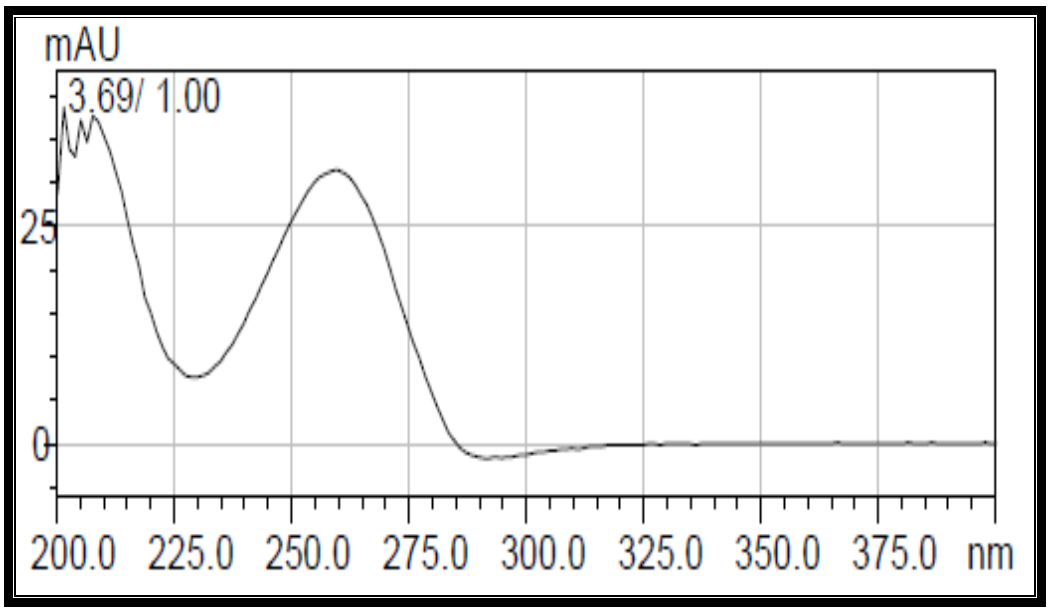
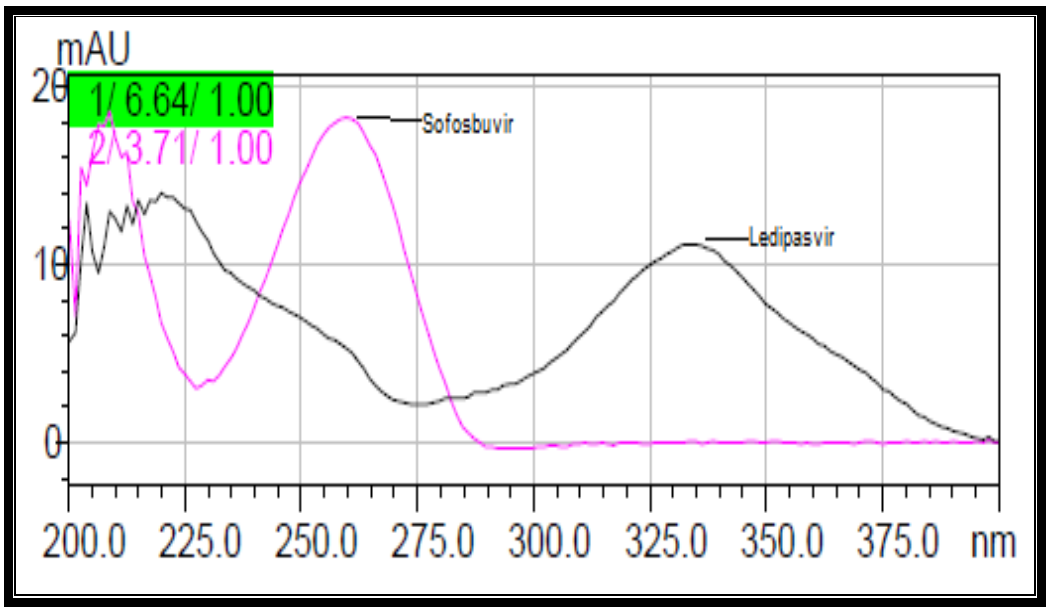


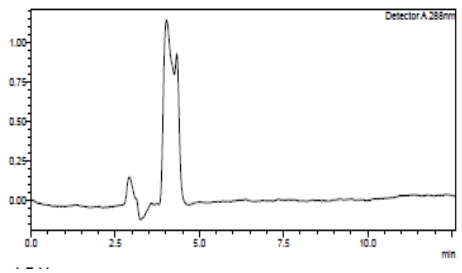
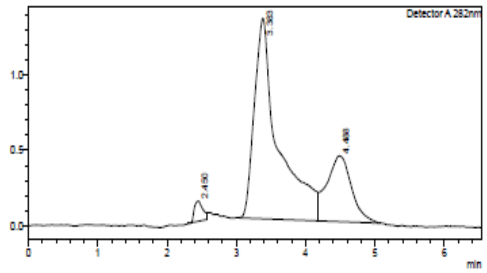
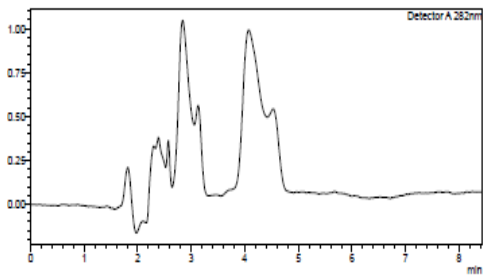
Fig. 43 Overlay Spectrum of Ledipasvar and Sofosbuvir



SELECTION OF THE MOBILE PHASE

Based on the solubility and polarity, different mobile phases were tried and the chromatograms were recorded and results are displayed in table:29

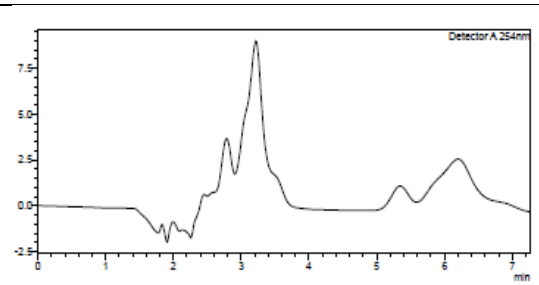
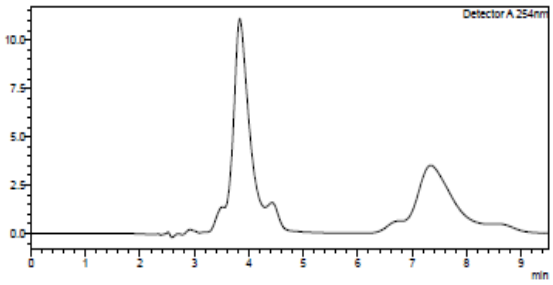
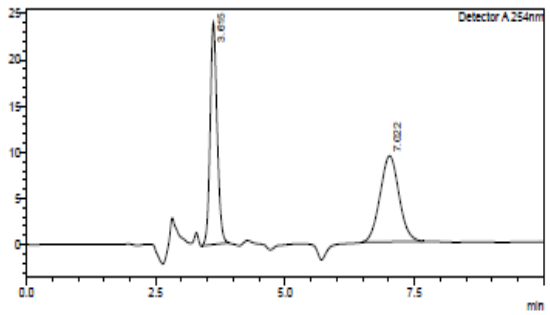
Table 29: Selection of Mobile Phase

Mobile phase	Ratio (%)	Chromatograms	Observation
ACN	100		Both drugs not separate
Water : Methanol	80:20		Broad peak and Less resolution
Water : ACN	80:20		Good resolution

SELECTION OF BUFFER:

In the mobile phase system consisting of 1% orthophosphoric acid: acetonitrile, different buffers like phosphate and acetate. The chromatogram were recorded and results are given in table 30.

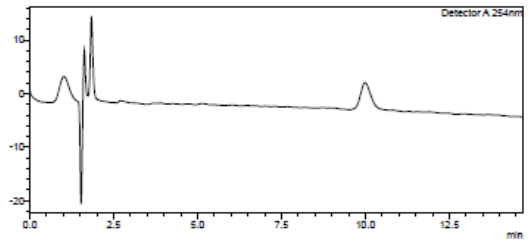
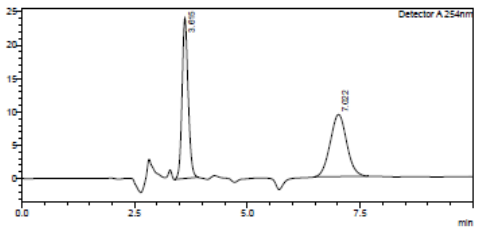
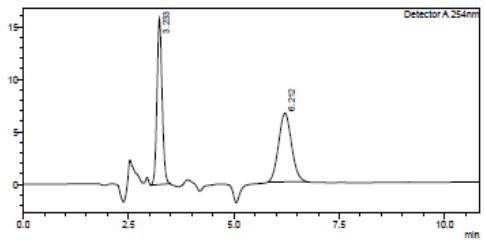
Table30 : Selection of Buffer

S. no	Buffer	Chromatograms	Observation
1.	Photassium di hydrogen Phosphate: ACN		Split peak
2.	Ammonium acetate: ACN		Peak tailing
3.	1% ortho phosphoric acid: ACN		Good peak shape & good resolution

Selection of flow rate:

Keeping the ratio of mobile phase constant (30;70 v/v), the chromatogram were recorded with different pH like 3.4, 6.0, 6.4 and 6.9 were tried. For pH 6.4, good resolution and symmetrical peak was obtained and hence selected for further studies table 31.

Table 31:Selection of Flow Rate

S. no	Flow rate (ml/min)	Chromatogram	Observation
1	0.5ml/min		Ledipasvir Rt> 10min
2	0.8ml/min		Symmetric peak & Good resolution
3	0.9ml/min		Medium resolution

FIXED CHROMATOGRAPHIC CONDITIONS

Chromatographic method : RP-HPLC

Column (stationary phase) : Hibar, C_{18} column (250mmx 4.0mm, 5µm)

Mobile phase : 1% orthophosphoric acid (pH6.4): Acetonitrile

Ratio of mobile phase	:	30:70 v/v
Detection of wavelength	:	254nm
Flow rate	:	0.8ml/min
Retention time	:	Sofosbuvir 3.7 ± 0.02
		Ledipasvir 7.1 ± 0.02
Temperature	:	Room temperature

Linearity and range:

A calibration graph was plotted with measured peak areas against concentration. From the graph it was found that ledipasvir and sofosbuvir shows good linearity in the concentration range 1-10µg/ml and 1-10µg/ml. The peak area of these solutions was measured at 254nm. The slope, intercept, and correlation coefficient values were calculated respectively (table 32). The linear graph and standard chromatogram obtained are shown in fig 44 to 56. The linearity table is shown in table 33 and 34.

Table 32: Regression data

Linear regression	Ledipasvir	Sofosbuvir
Slope	5976.13	0.13807.7
Intercept	14699.1	24637.8
Correlation coefficient	0.9987	0.9995

Figure 44: Chromatogram of Blank

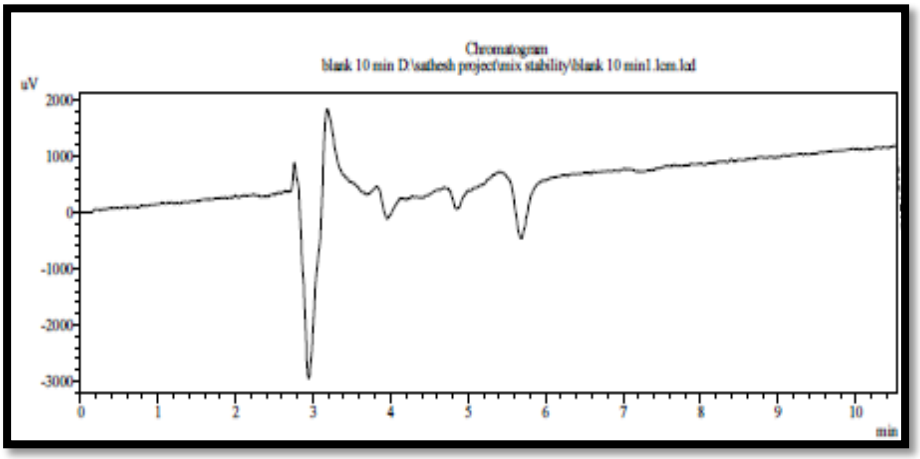


Fig 45: Chromatogram of ledipasvir (1µg/ml)and sofosbuvir (1µg/ml)

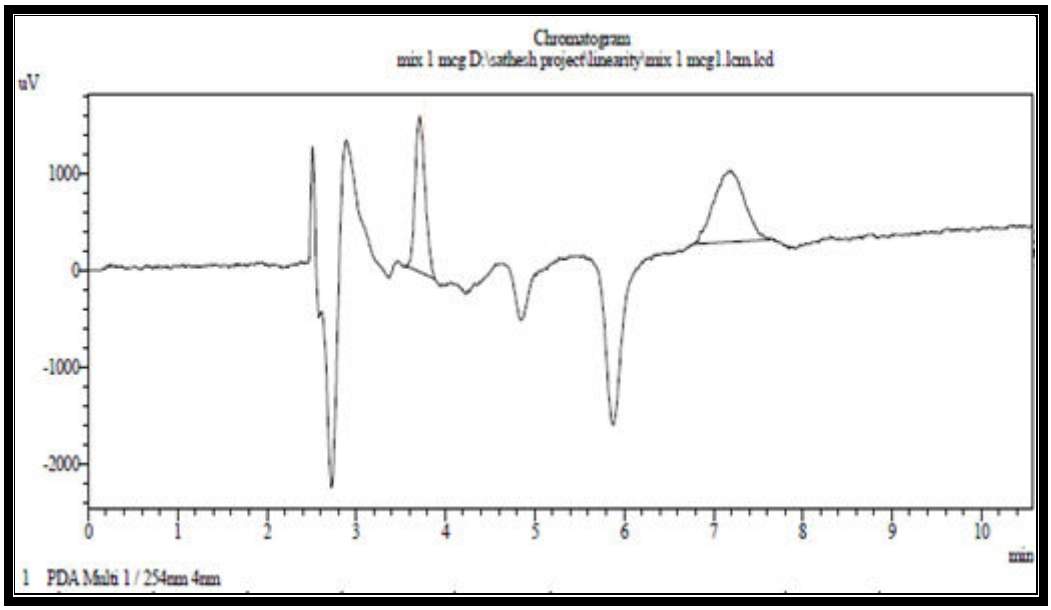


Fig 46: Chromatogram of ledipasvir (2µg/ml)and sofosbuvir (2µg/ml)

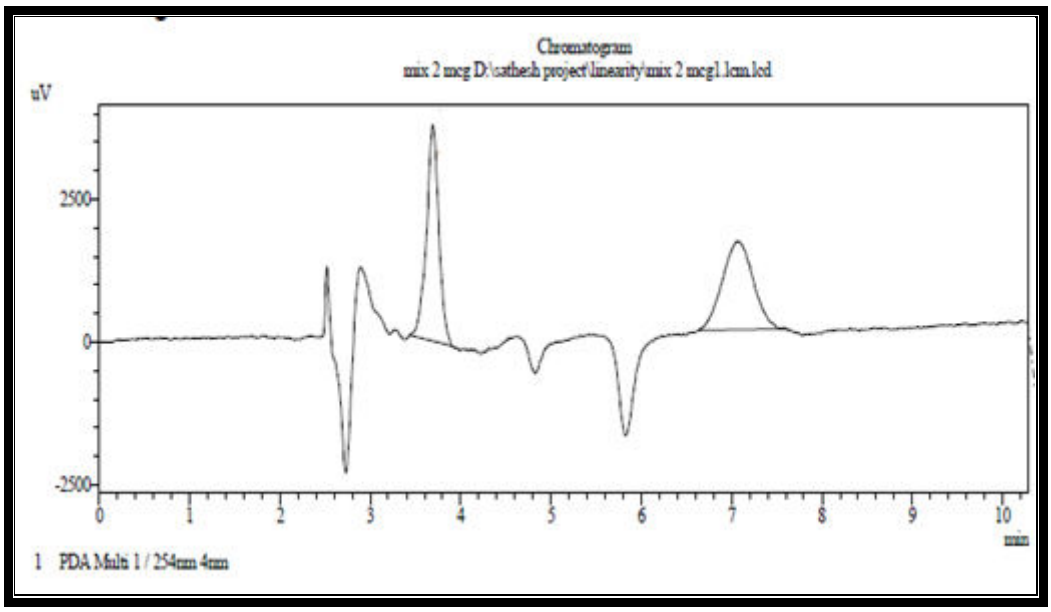


Fig 47: Chromatogram of ledipasvir (3µg/ml)and sofosbuvir (3µg/ml)

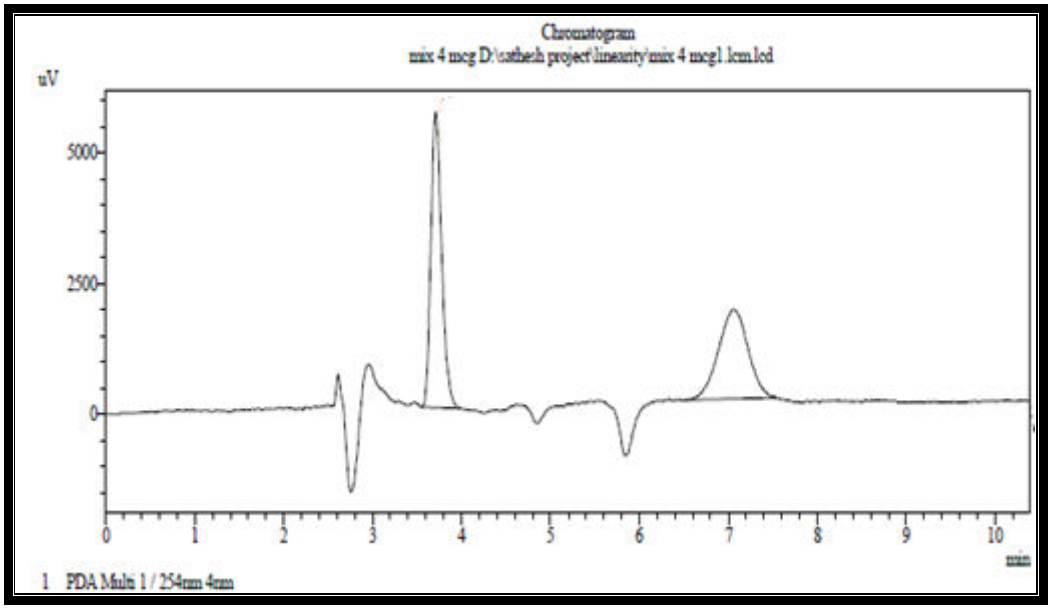


Fig 48: Chromatogram of ledipasvir (4µg/ml)and sofosbuvir (4µg/ml)

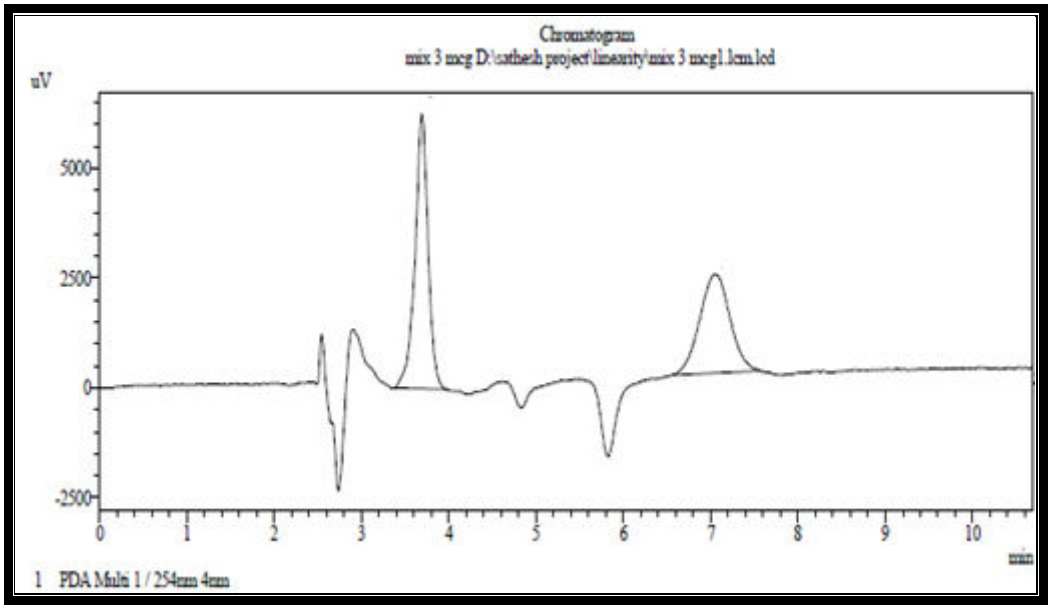


Fig 49: Chromatogram of ledipasvir (5µg/ml)and sofosbuvir (5µg/ml)

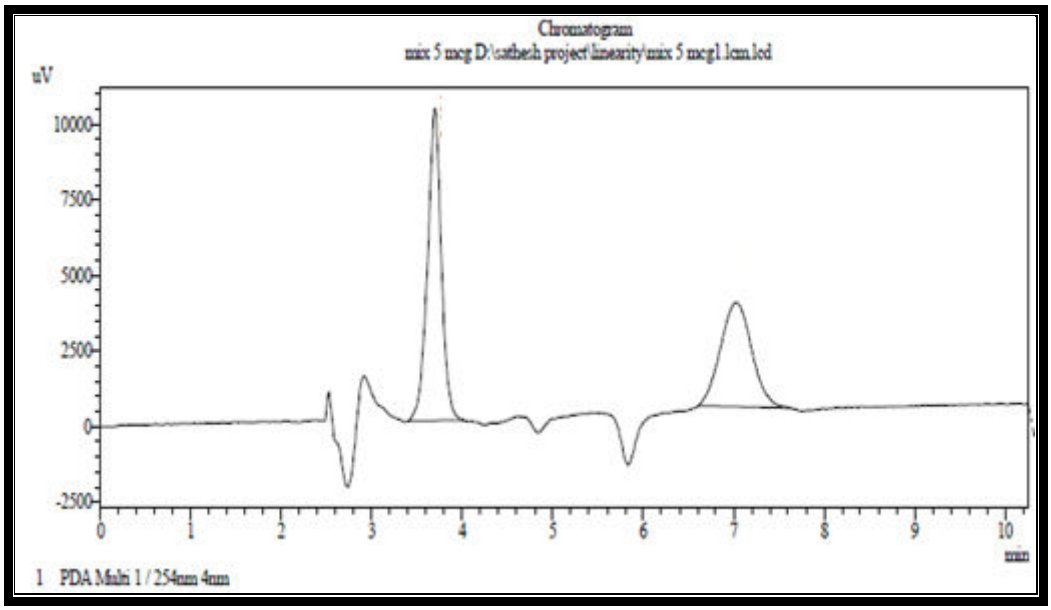


Fig 50: Chromatogram of ledipasvir (6µg/ml)and sofosbuvir (6µg/ml)

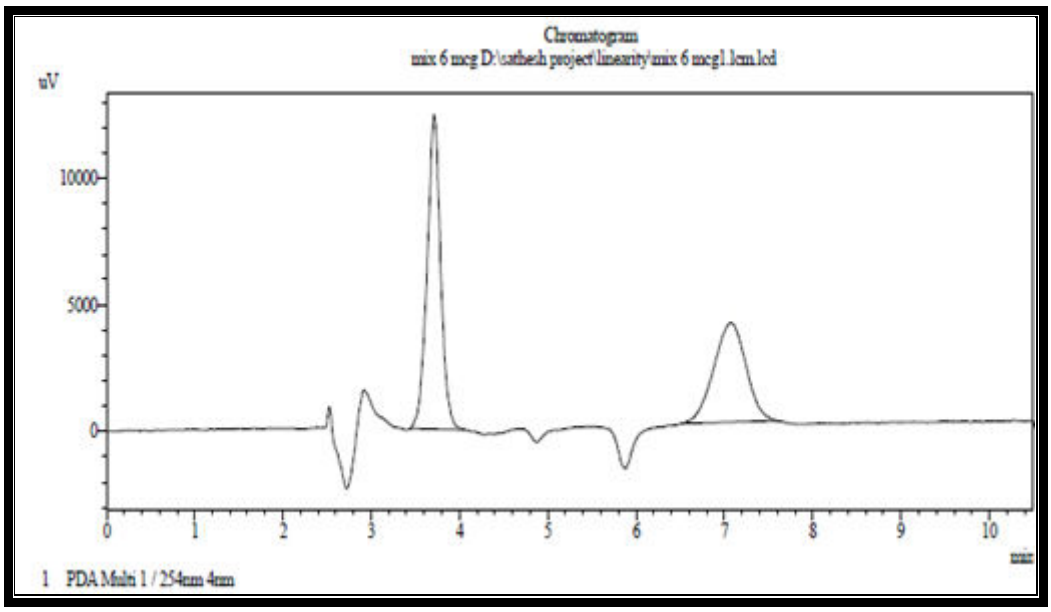


Fig 51: Chromatogram of ledipasvir (7µg/ml)and sofosbuvir (7µg/ml)

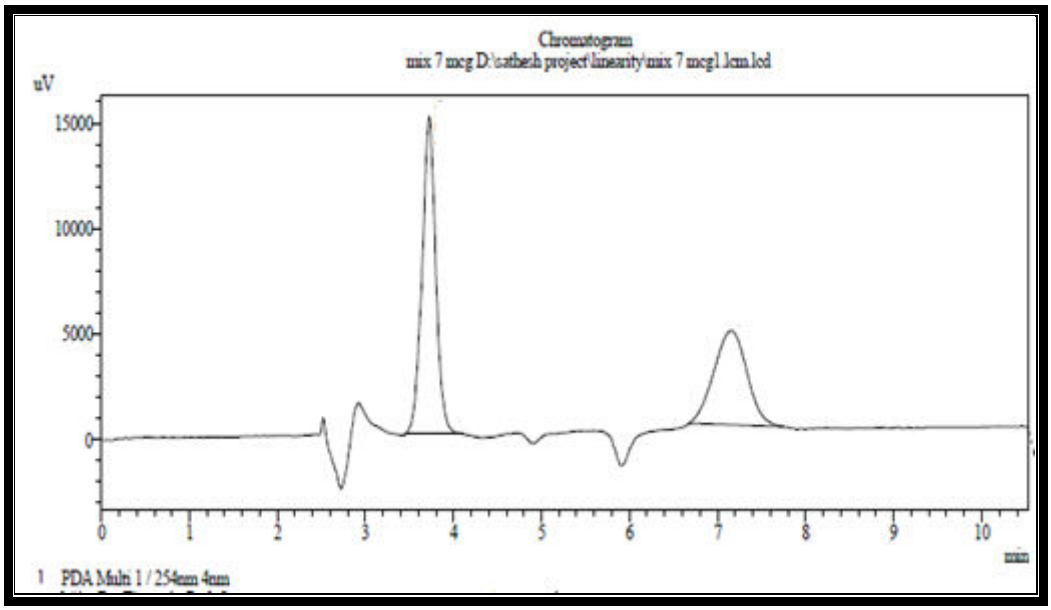


Fig 52: Chromatogram of ledipasvir (8µg/ml)and sofosbuvir (8µg/ml)

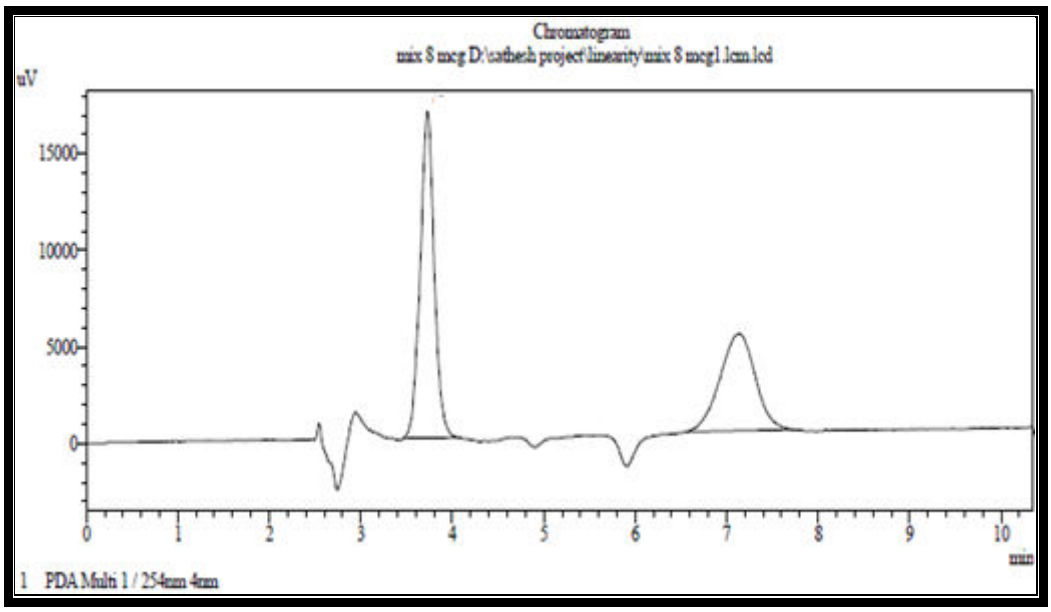


Fig 53: Chromatogram of ledipasvir (9µg/ml)and sofosbuvir (9µg/ml)

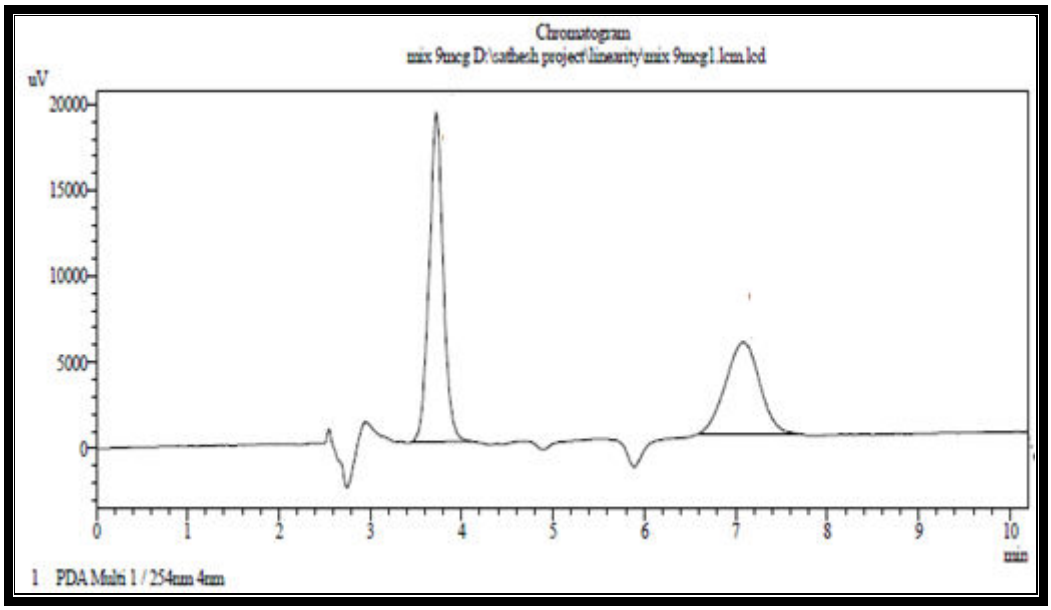


Fig 54: Chromatogram of ledipasvir (10µg/ml)and sofosbuvir (10µg/ml)

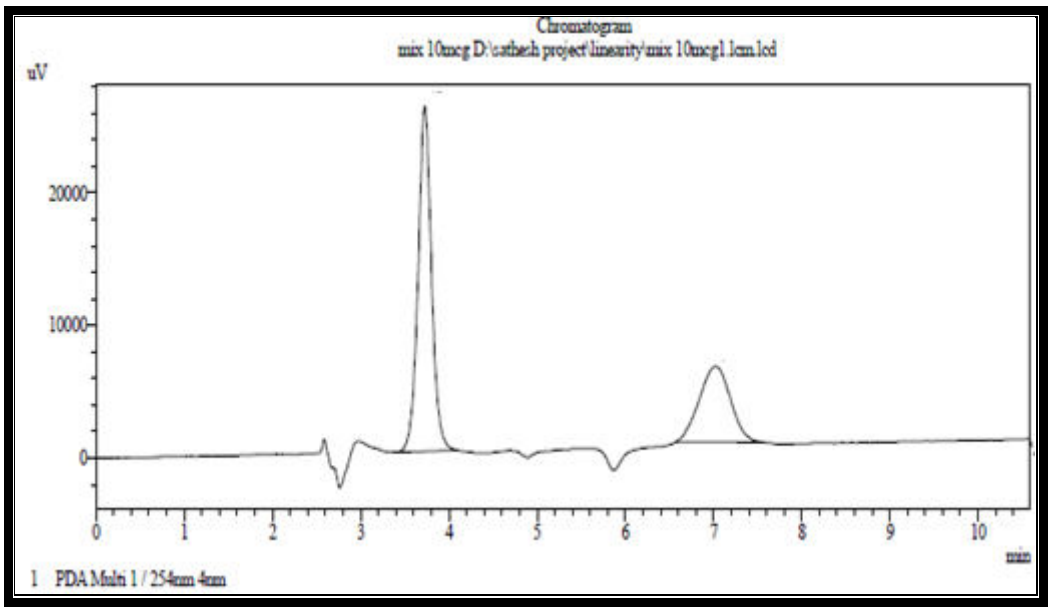


Table 33: Calibration data for Ledipasvir

Concentration(µg/ml)	Peak area
1	17517
2	36796
3	48253
4	65251
5	82453
6	95549
7	110564
8	125468
9	135894
10	150468

Table 33: Calibration data for Sofosbuvir

Concentration(µg/ml)	Peak area
1	12267
2	36018
3	57611
4	84734
5	110043
6	132226
7	162341
8	181393
9	204639
10	235708

Fig 55: Calibration graph of Ledipasvir (1-10µg/ml)

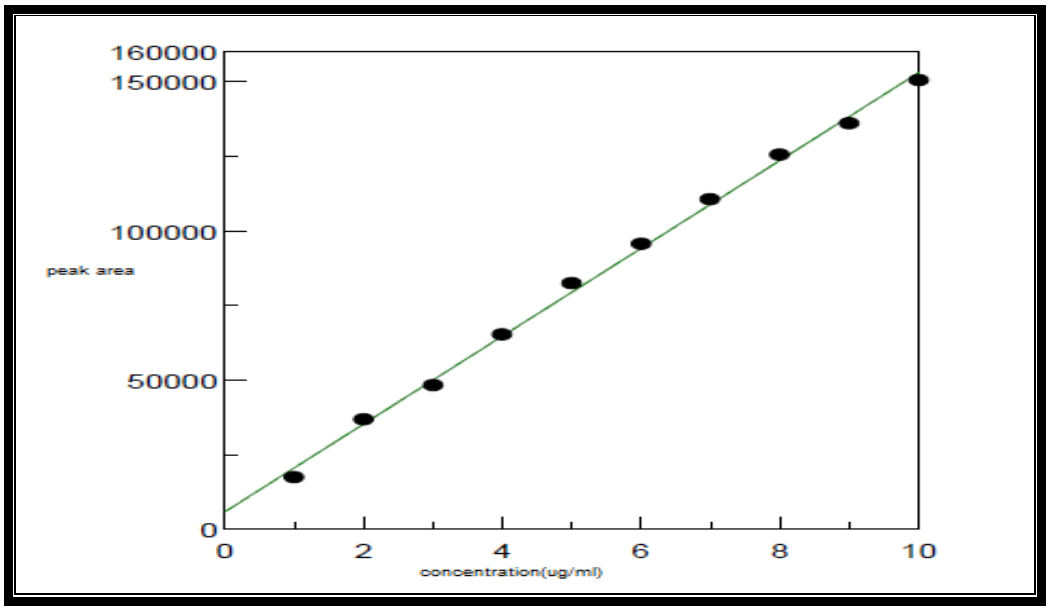
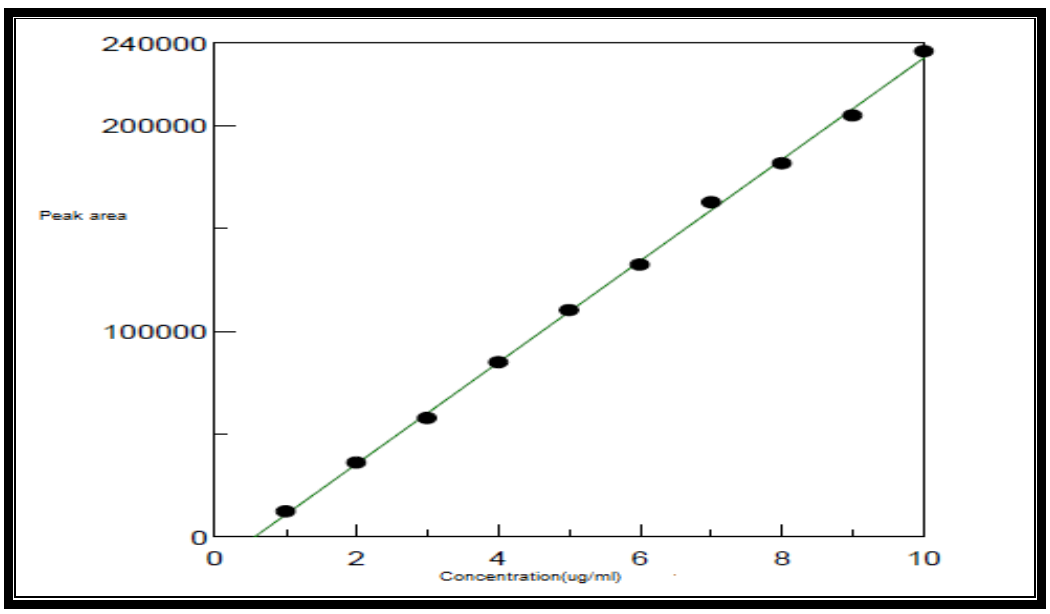


Fig 56: Calibration graph of Sofosbuvir (1-10µg/ml)



Recovery study:

Recovery studies were carried out at 50,100, and 150% levels. The percentage recovery and its %RSD were calculated, shown in table 33a & 33b.

Table 33a :Recovery study for Ledipasvir

Level	% Recovery	%RSD*
50%	98.34%	0.05
100%	98.01%	0.84
150%	99.27%	0.94

*RSD of six determination

Table 33b: Recovery study for Sofosbuvir

Level	% Recovery	%RSD*
50%	99.73%	0.54
100%	100.36%	0.25
150%	101.48%	0.14

*RSD of six determination

Precision:

Intra-day and inter-day precision:

Intra-day and inter-day precision was determined by injecting standard solutions in between linearity range (4 and 8µg/ml for ledipasvir and 4and 8µg/ml for sofosbuvir) were injected for three times and % RSD was calculated (table 34, and 35).

Table 34: Intra-day precision

Concentration (ng/spot)	Peak area		%RSD*	
	Ledipasvir	Sofosbuvir	Ledipasvir	Sofosbuvir
Ledipasvir(4µg/ml) & Sofosbuvir (4 µg/ml)	65221	84645	0.0123	0.0270
	65531	84660		
	65537	84690		
Ledipasvir(8 µg/ml) & Sofosbuvir (8 µg/ml)	125378	181499	0.0300	0.0185
	125319	181491		
	125308	181437		

*RSD of three determinations

Table 35: Inter-day precision

Concentration (ng/spot)	Peak area		%RSD*	
	Ledipasvir	Sofosbuvir	Ledipasvir	Sofosbuvir
Ledipasvir(4µg/ml) & Sofosbuvir (4 µg/ml)	65310	84650	0.0177	0.0343
	65319	84666		
	65333	84609		
Ledipasvir(8 µg/ml) & Sofosbuvir (8 µg/ml)	124219	181409	0.0065	0.0128
	124225	181410		
	124009	181450		

*RSD of three determinations

Repeatability:

Repeatability of injection was determined by injecting standard solutions (4µg/ml) of ledipasvir and (4µg/ml) of sofosbuvir for six times, noted peak areas and % RSD was calculated table 36a & 36b.

Table 36a: Repeatability injection of Ledipasvir

Concentration (µg/ml)	Repeatability	% RSD*
4	65251	0.0215
	65242	
	65255	
	65235	
	65260	
	65275	

*RSD of six determinations

Table 36b: Repeatability injection of Sofosbuvir

Concentration (µg/ml)	Repeatability	% RSD*
4	84734	0.0102
	84712	
	84722	
	84730	
	84725	
	84735	

*RSD of six determinations

Limit of detection and limit of quantification (LOD and LOQ):

Limit of detection and limit of quantification that were calculated from the equation. The result was shown in table 37a & 37b , that proved the sensitivity of the method.

Table 37a: Results of LOD & LOQ of Ledipasvir

Correlation coefficient ±SD*	0.9987 ±0.0001342
Slope ±SD*	3.123 ± 2.608
Intercept ± SD*	1112.65 ±2.436
LOD (ng / spot)	0.008µg / ml
LOQ (ng / spot)	0.27µg / ml

*RSD of six determinations.

Table 37b: Results of LOD & LOQ of Sofosbuvir

Correlation coefficient ±SD*	0.9995 ±0.4080
Slope ±SD*	3.123 ±2.611
Intercept ± SD*	1112.65 ±2.822
LOD (ng / spot)	0.0006µg /ml
LOQ (ng / spot)	0.0020 µg / ml

*RSD of six determinations.

Specificity:

There were no additional peaks observed while injecting solvents or mobile phase alone. The peak purity index of standard ledipasvir 0.9998 and sofosbuvir 0.9995.

Robustness:

In order to demonstrate the robustness of the method, the following optimized conditions were slightly changed.

± 0.1ml flow rate

± 2% organic solvent

± 0.5 P^H

The responses for these changed chromatographic parameters were almost same for the fixed chromatographic parameters and hence the developed method was said to be robustness

Stability of solution:

The solution under room temperature was stable for 24 hours. (Table 38)

Table 38: Stability of solution

Hours	Ledipasvir		Sofosbuvir	
	Concentration (µg/ml)	Peak area	Concentration (µg/ml)	Peak area
0 hrs	5	82453	5	110043
6 hrs		82412		110012
24 hrs		82219		109702

System suitability studies:

The system suitability parameters like peak area, tailing factor, theoretical plate count, resolution, and retention time were calculated from the standard chromatogram (table 39).

Table 39: System suitability studies

Drug	Theoretical plate(N)	Retention time(min)	Tailing factor (10%)	Resolution
Ledipasvir	2703	3.7	1.062	-
Sofosbuvir	1762	7.1	0.994	6.90

ANALYSIS OF TABLET FORMULATION

The % label claim was calculated and the estimated amount was close to the labelled value that was shown in figure 57 and table 40.

Figure 27: Chromatogram of Ledipasvir and Sofosbuvir

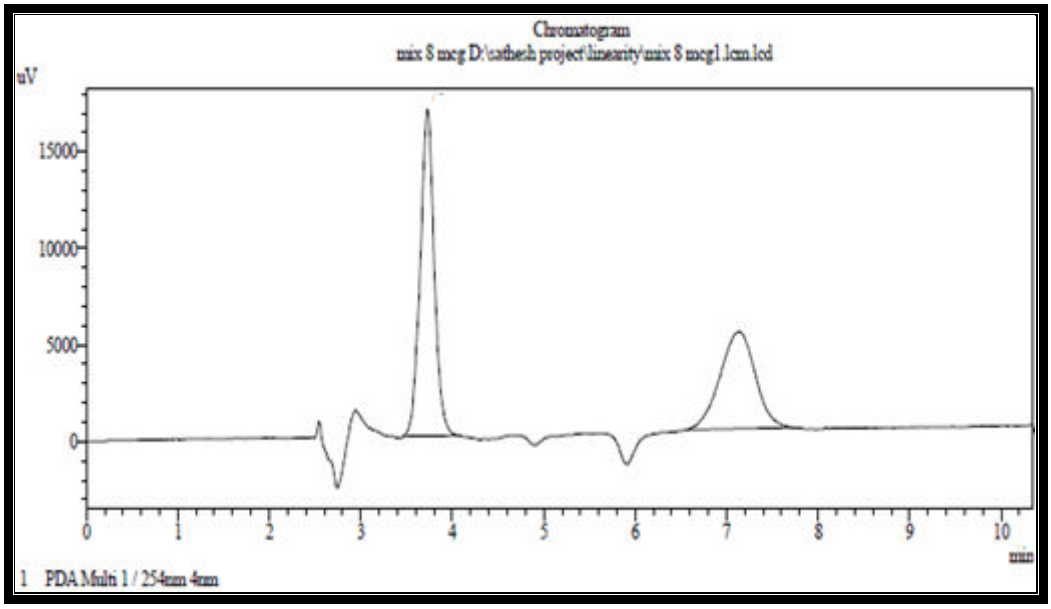


Table 40: Analysis of formulation

Drug Name (cimivir-L)	Amount (mg/tab)		% Label claim	%RSD*
	Labelled	Estimated		
Ledipasvir	90	89.34	99.30	0.41
Sofosbuvir	400	398.17	99.54	0.67

*RSD of six observations

FORCED DEGRADATION STUDIES: LEDIPASVIR

Acid hydrolysis:

Ledipasvir was subjected to acid hydrolysis, no additional peak was observed along with standard, which is shown in figure 58a & 58b.

Figure 58a: Chromatogram of acid hydrolysis of Ledipasvir

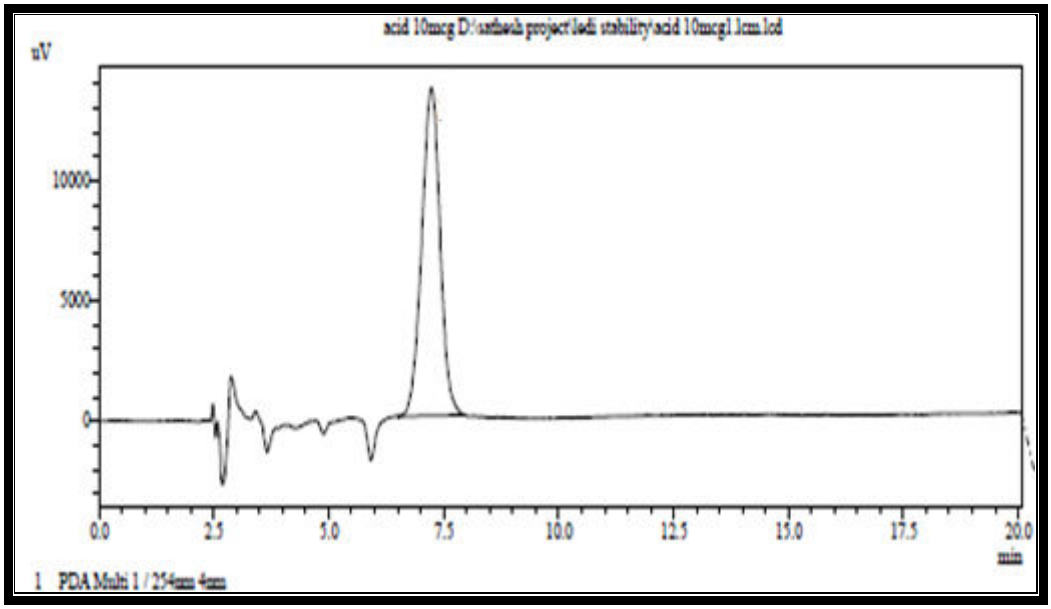
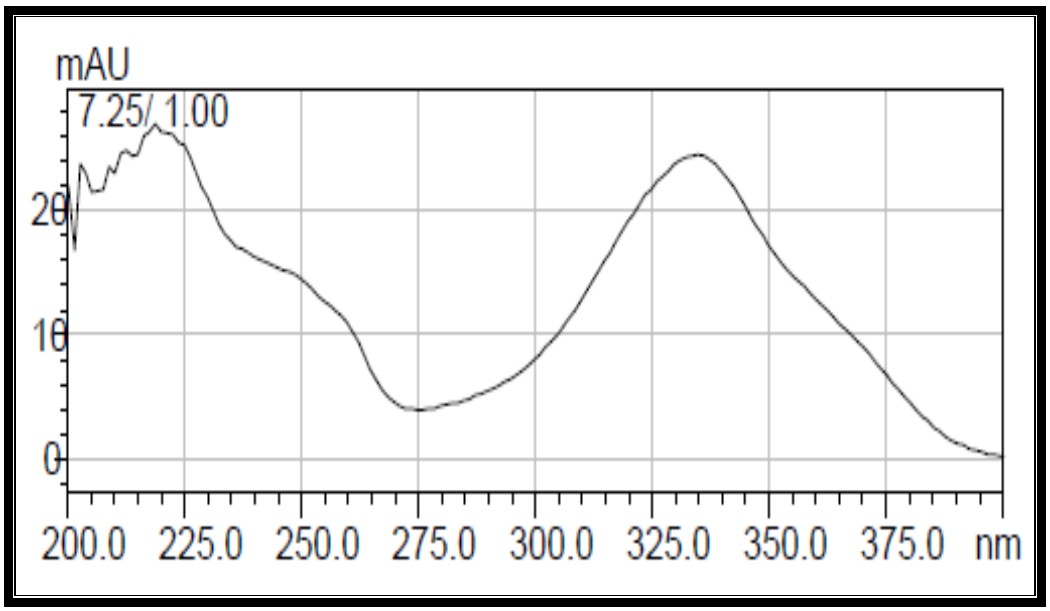


Figure 58b: Spectrum of Ledipasvir



Alkaline hydrolysis:

The drug was subjected to alkaline hydrolysis additional peaks were observed, which was shown in figure 59a & 59b.

Figure 59a: Chromatogram of base hydrolysis of Ledipasvir

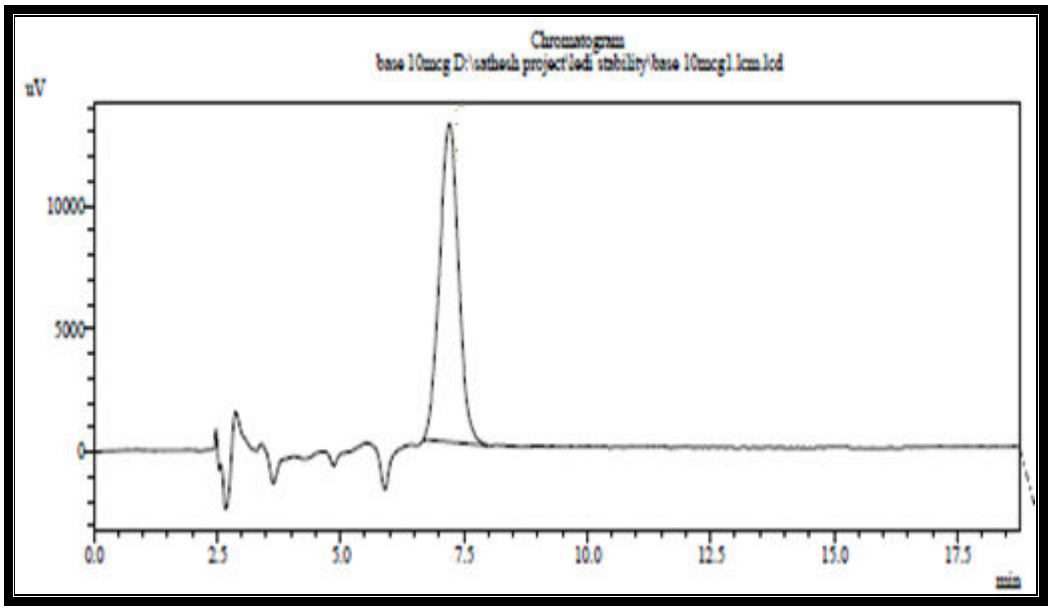
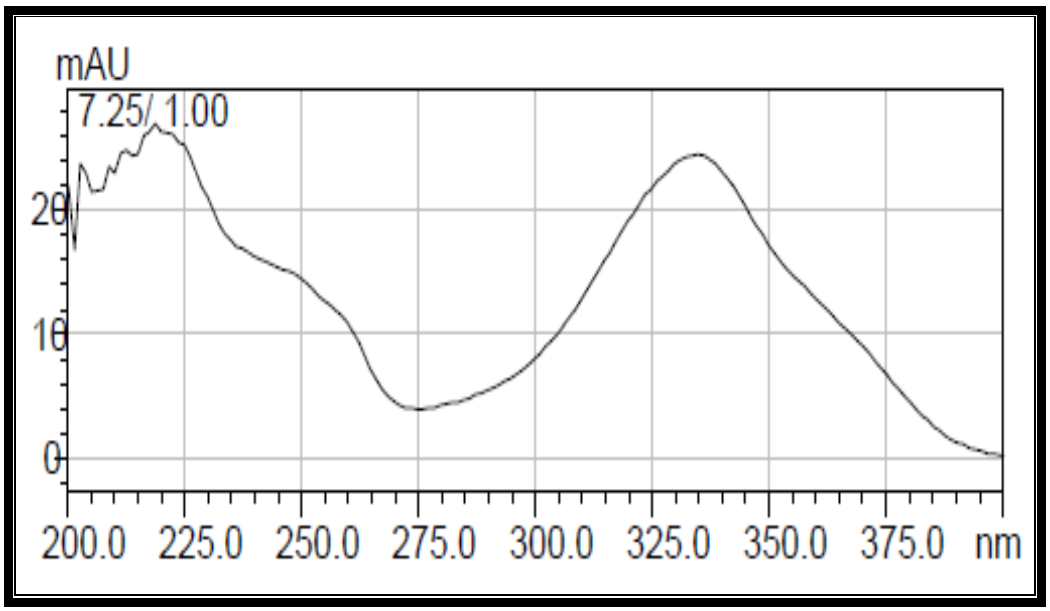


Figure 59b: Spectrum of Ledipasvir



Neutral hydrolysis:

The drug was subjected to neutral hydrolysis no additional peaks were observed, which was shown in figure 60a & 60b.

Figure 60a: Chromatogram of neutral hydrolysis of Ledipasvir

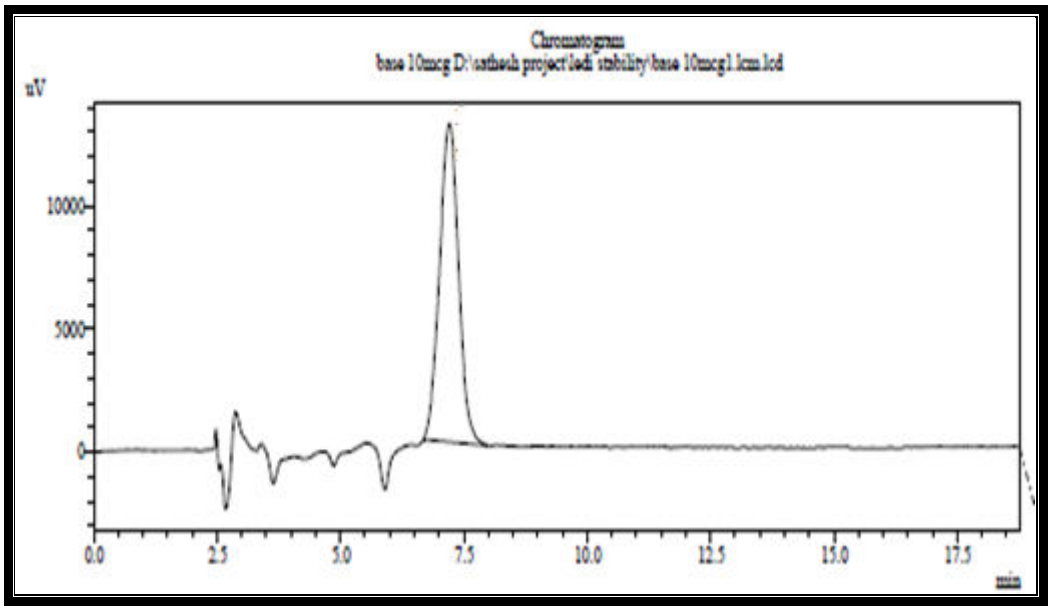
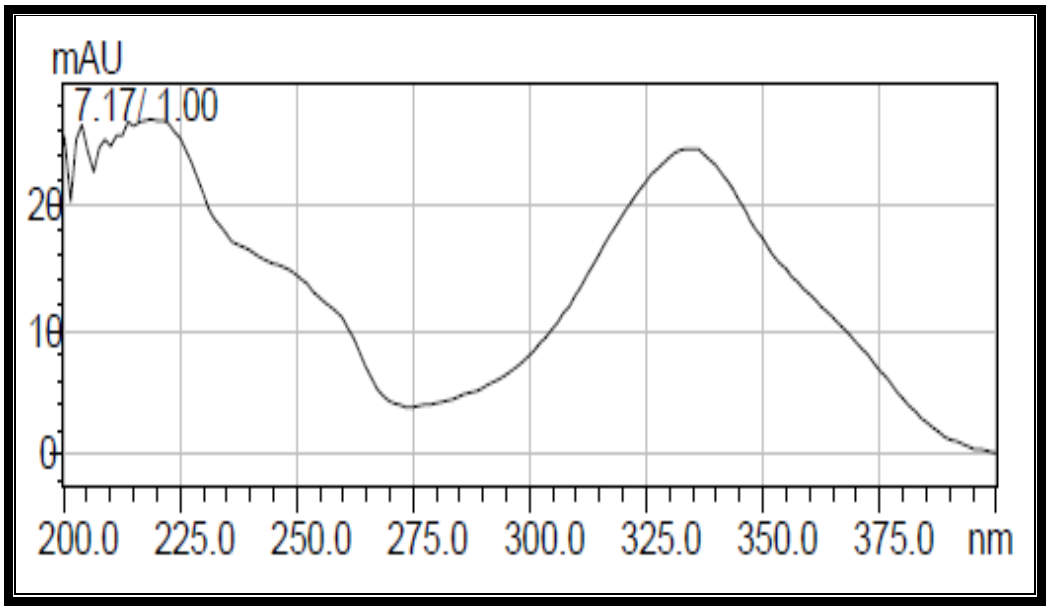


Figure 60b: Spectrum of Ledipasvir



Oxidative degradation:

The drug was subjected to oxidation no additional peaks were observed, which was shown in figure 61a & 61b.

Figure 61a: Chromatogram of oxidative degradation of Ledipasvir

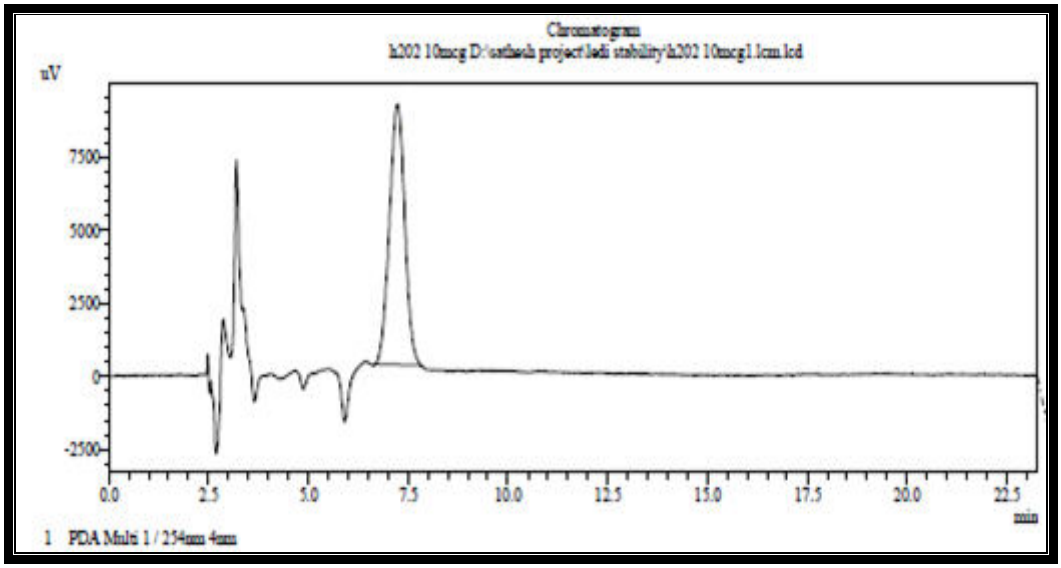
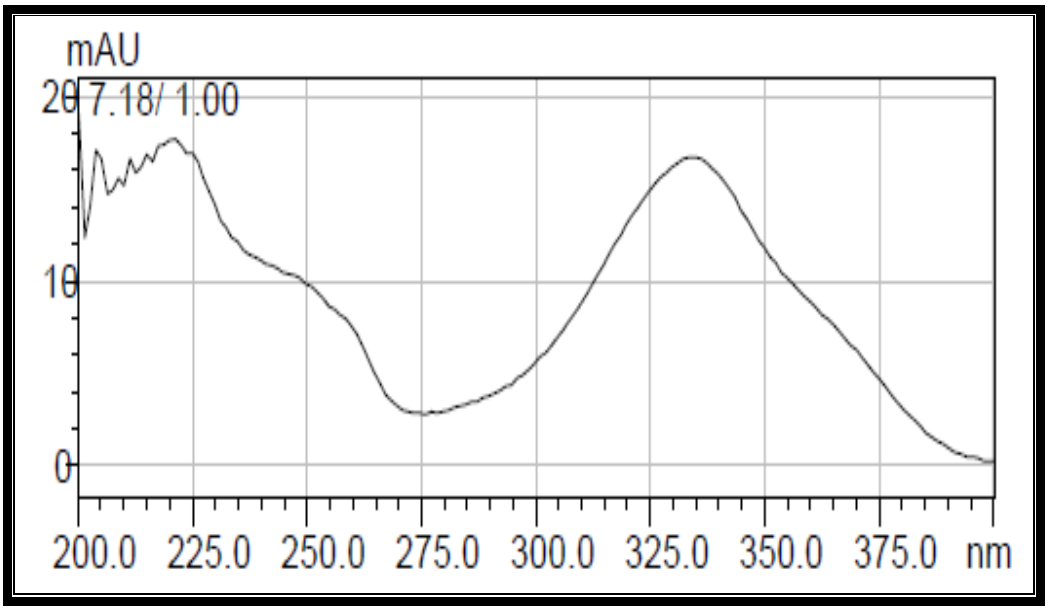


Figure 61b: Spectrum of Ledipasvir



Photolytic degradation:

The drug was subjected to photolytic degradation no additional peaks were observed, which was shown in figure 62a &62b.

Figure 62a: Chromatogram of photolytic degradation of Ledipasvir

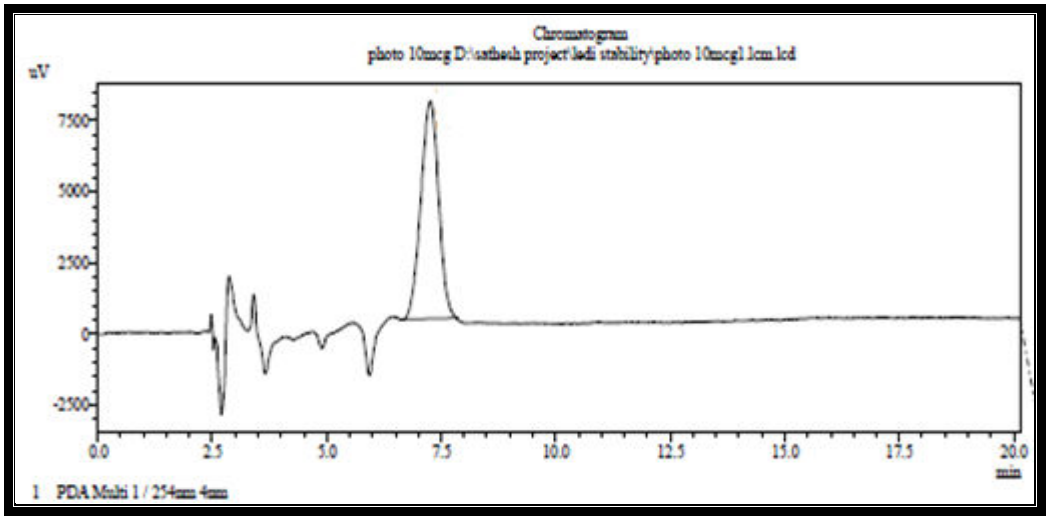
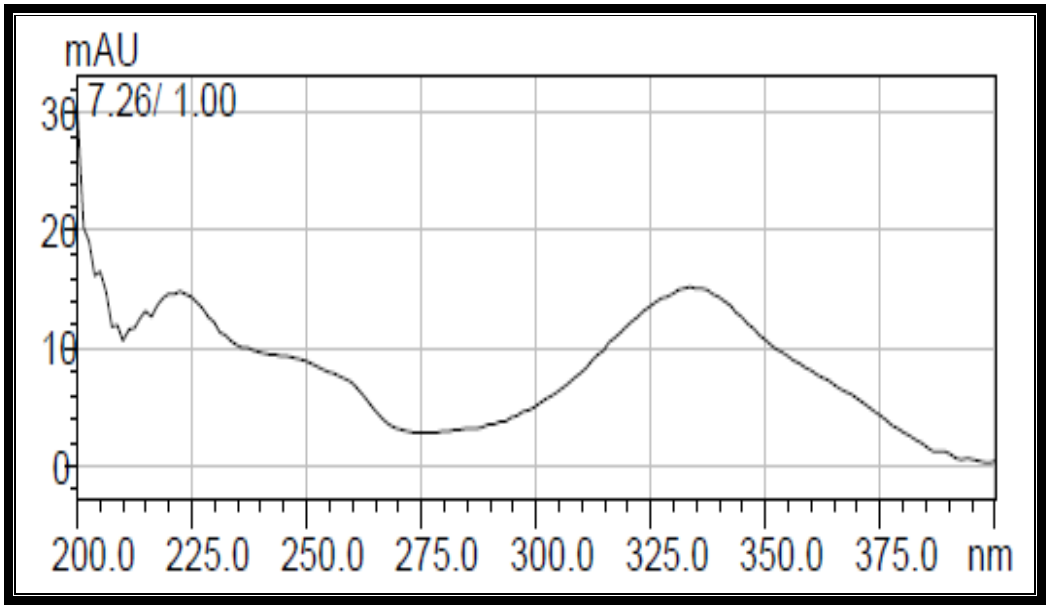


Figure 62b: Spectrum of Ledipasvir



Thermal degradation

The drug was subjected to thermal degradation additional peaks were observed at R_f value 0.34, which was shown in figure 63a & 63b.

Figure 63a: Chromatogram of thermal degradation of Ledipasvir

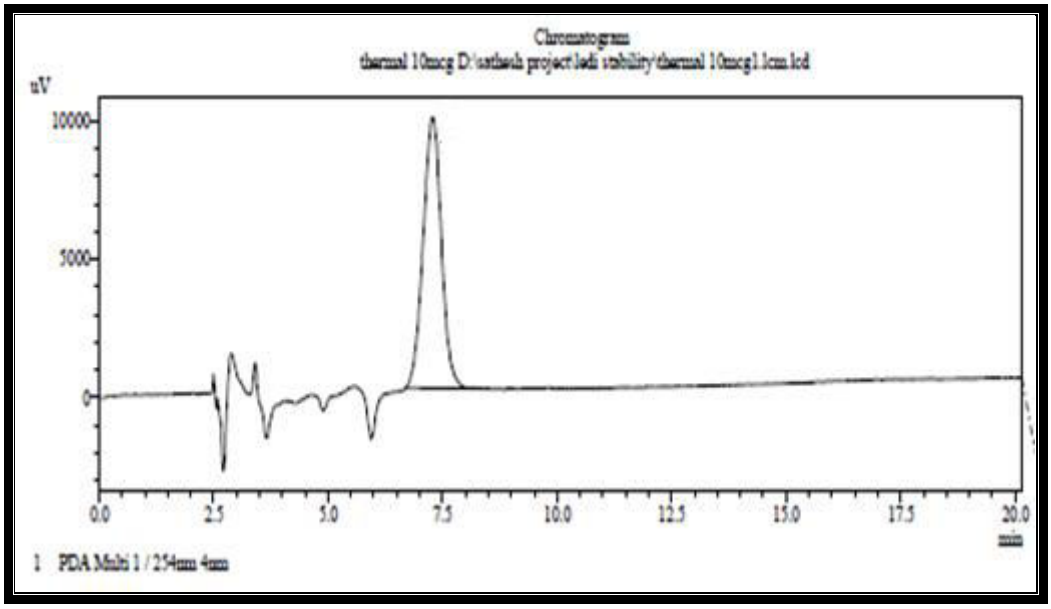
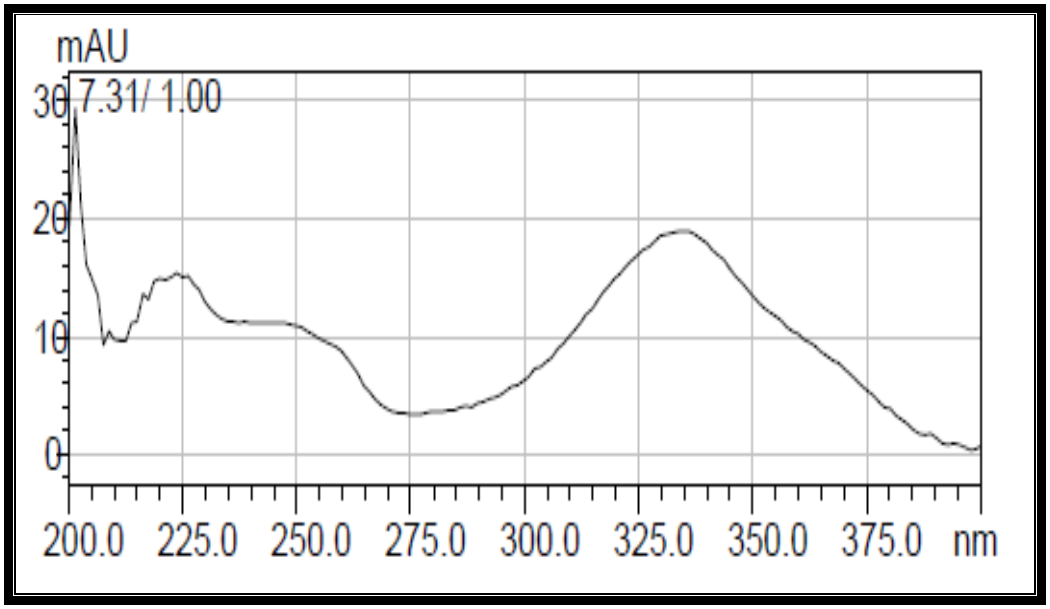


Figure 63b: Spectrum of Ledipasvir



Summary of forced degradation was shown in the table 41.

Table 41 Summary of forced degradation studies for Ledipasvir:

Type of stress	Stress condition	Retention time(min)	
		Drug	Degraded Product
Acid hydrolysis	Drug refluxed with 0.1M HCl for about 5 hours	7.1	No additional peak
Basic hydrolysis	Drug refluxed with 0.1M NaoH for about 5 hours	7.1	No additional peak
Neutral hydrolysis	Drug treated with water and refluxed for about 5 hours	7.1	No additional peak
Oxidative degradation	Drug treated with 6 % hydrogen peroxide at normal room temperature	7.1	No additional peak
Photo degradation	Drug exposed to Sunlight for 5 hours	7.1	No additional peak
Thermal degradation	Drug introduced in Hot air oven for 5 hours	7.1	No additional peak

FORCED DEGRADATION STUDIES: SOFOSBUVIR

Acid hydrolysis:

Sofosbuvir was subjected to acid hydrolysis, no additional peak was observed , that is shown in figure 64a & 64b.

Figure 64a: Chromatogram of acid hydrolysis of Sofosbuvir

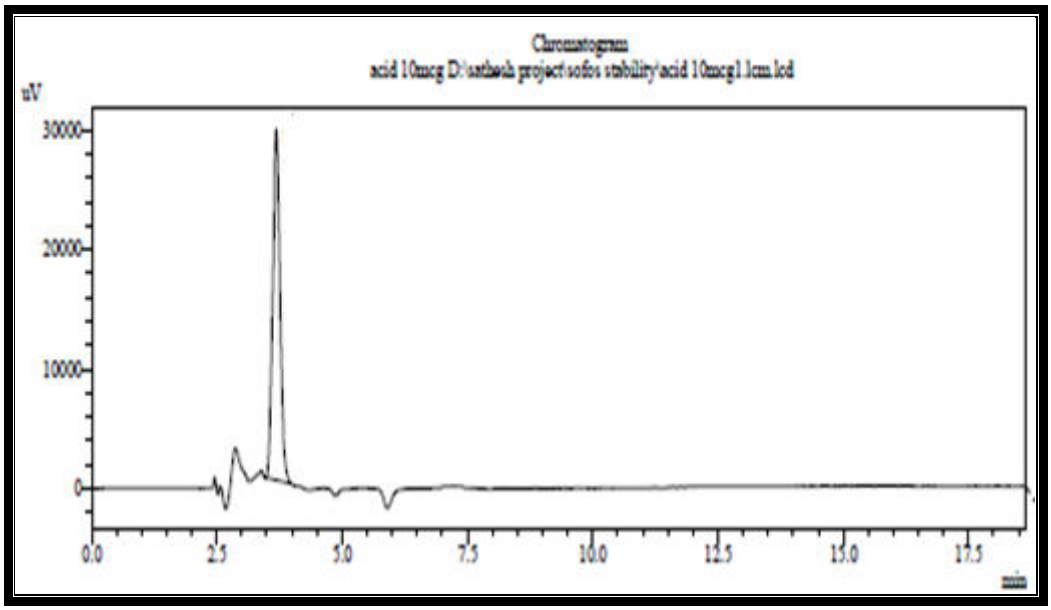
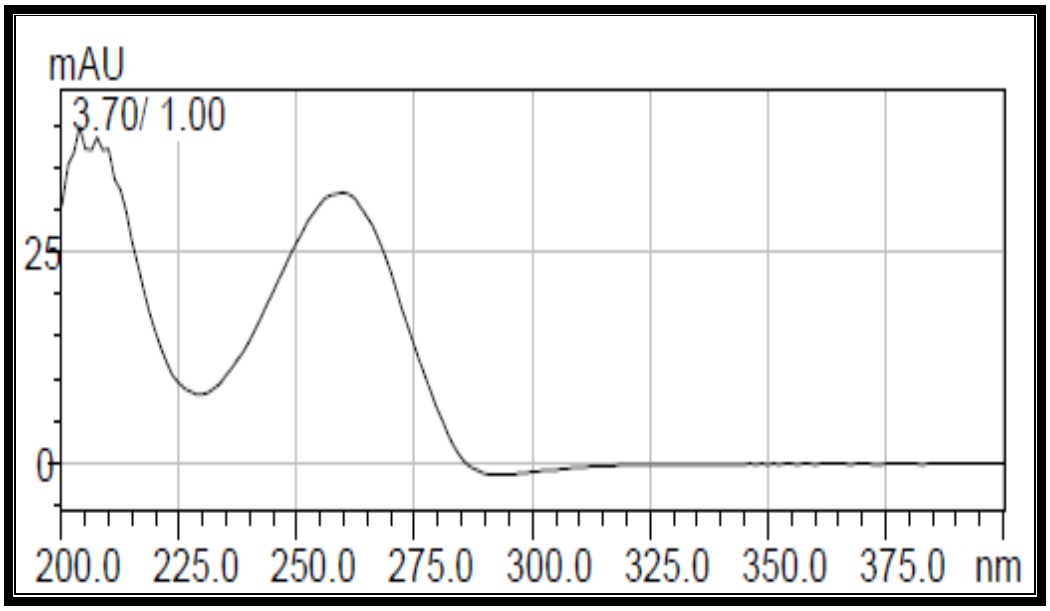


Figure 64b: Spectrum of Sofosbuvir



Alkaline hydrolysis:

The drug was subjected to alkaline hydrolysis three additional peaks were observed at R_t 2.8 and 4.1 value which was shown in figure 65a,65b,66c & 66d.

Figure 65a: Chromatogram of base hydrolysis of Sofosbuvir

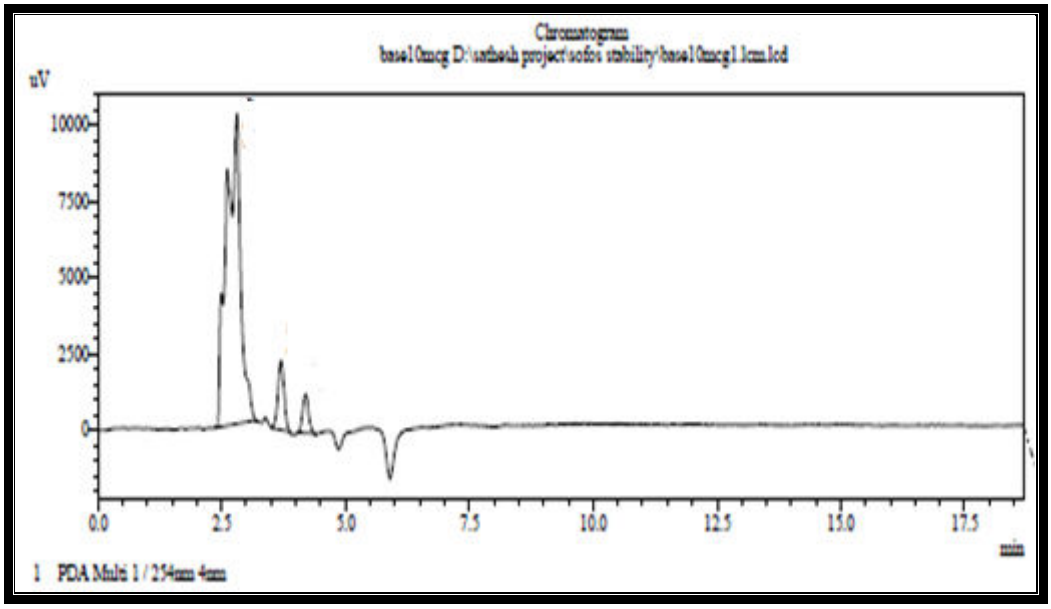


Figure 65b: Spectrum of Sofosbuvir

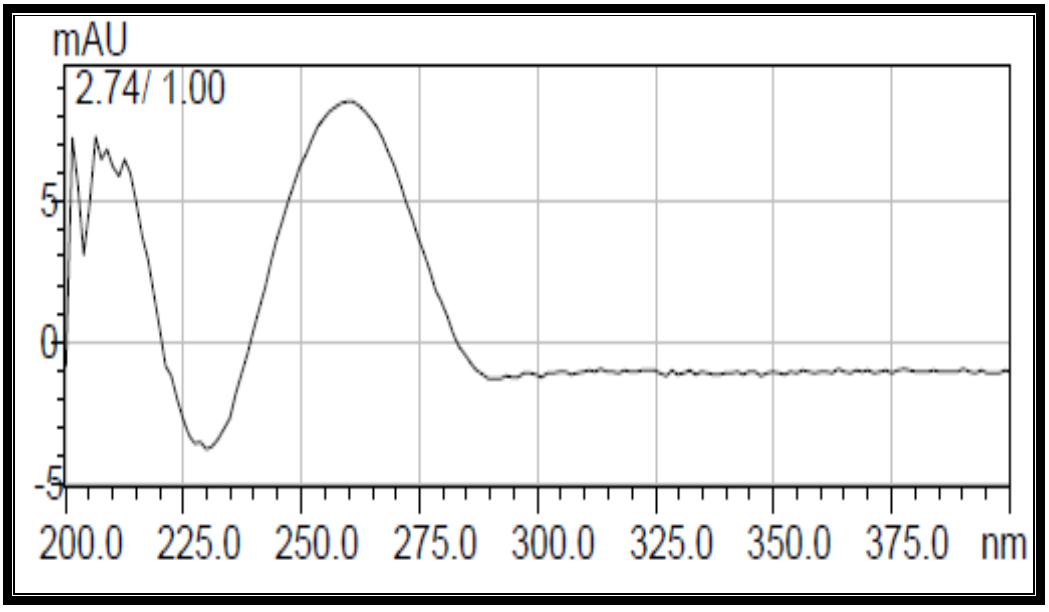


Figure 65c: Spectrum of Sofosbuvir degradant

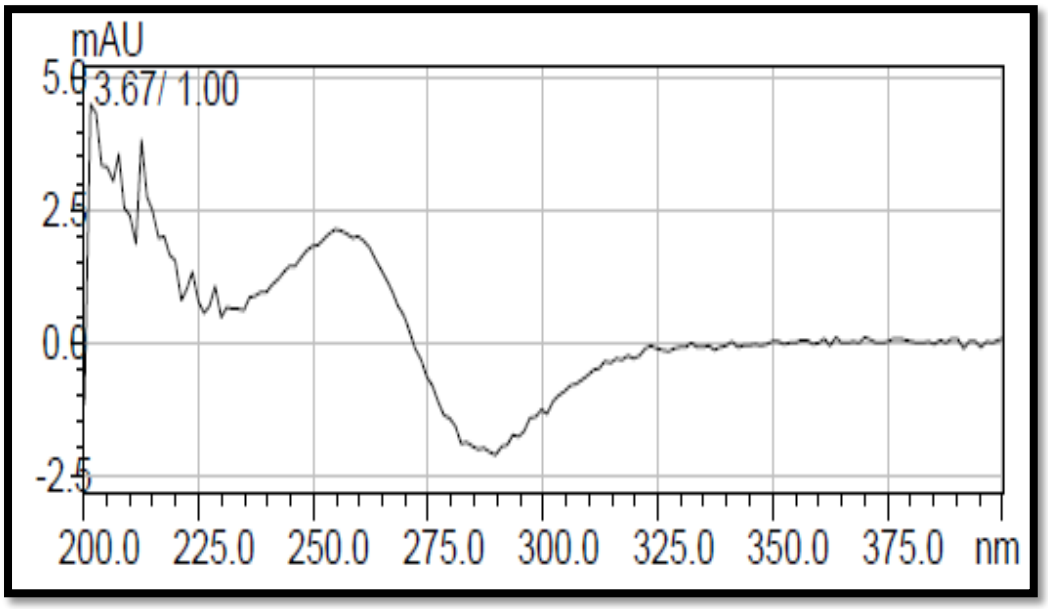
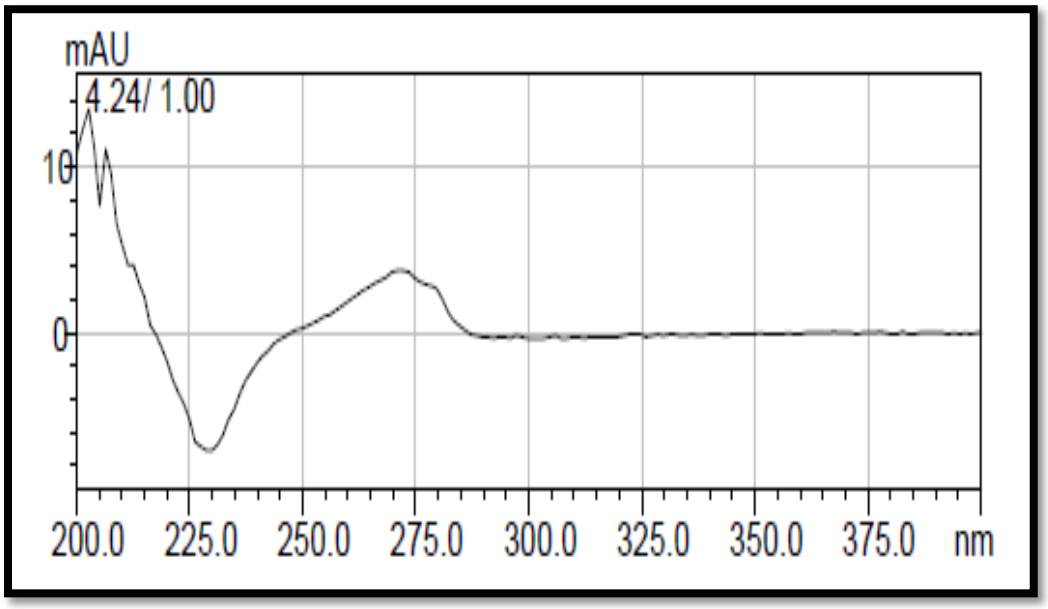


Figure 65d: Spectrum of Sofosbuvir degradant



Neutral hydrolysis:

The drug was subjected to neutral hydrolysis no additional peaks were observed, which was shown in figure 66a &66b.

Figure 66a: Chromatogram of neutral hydrolysis of Sofosbuvir

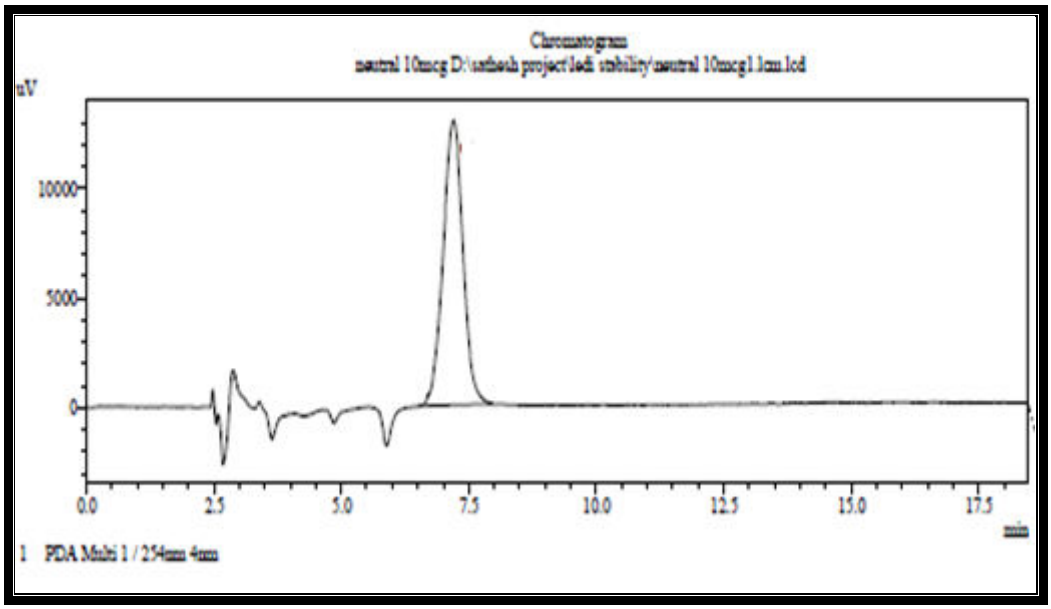
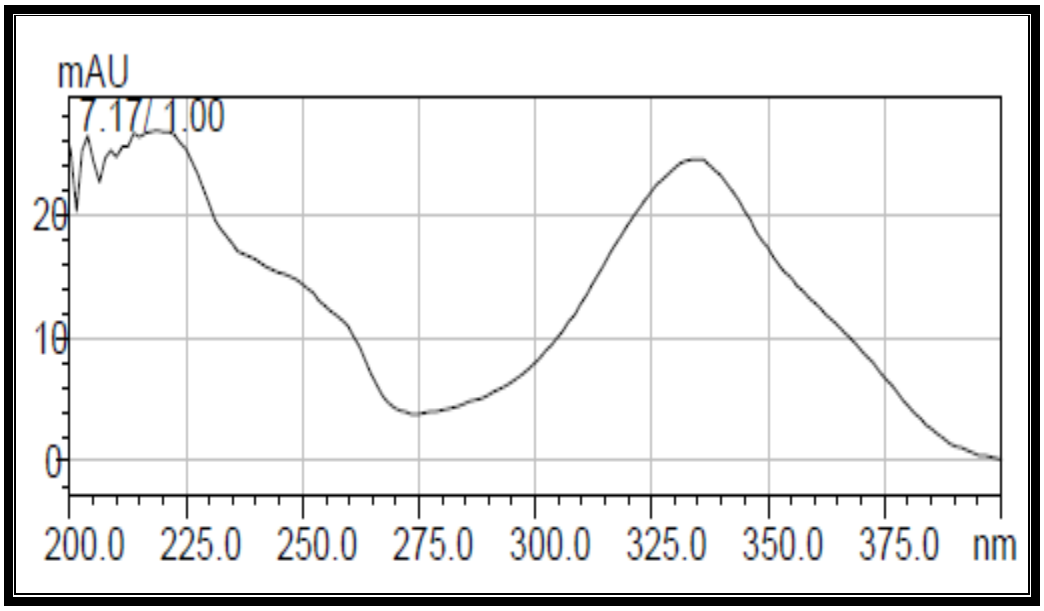


Figure 66b: Spectrum of Sofosbuvir



Oxidative degradation:

The drug was subjected to oxidation no additional peaks were observed, which was shown in figure 67a &67b.

Figure 67a: Chromatogram of oxidative degradation of Sofosbuvir

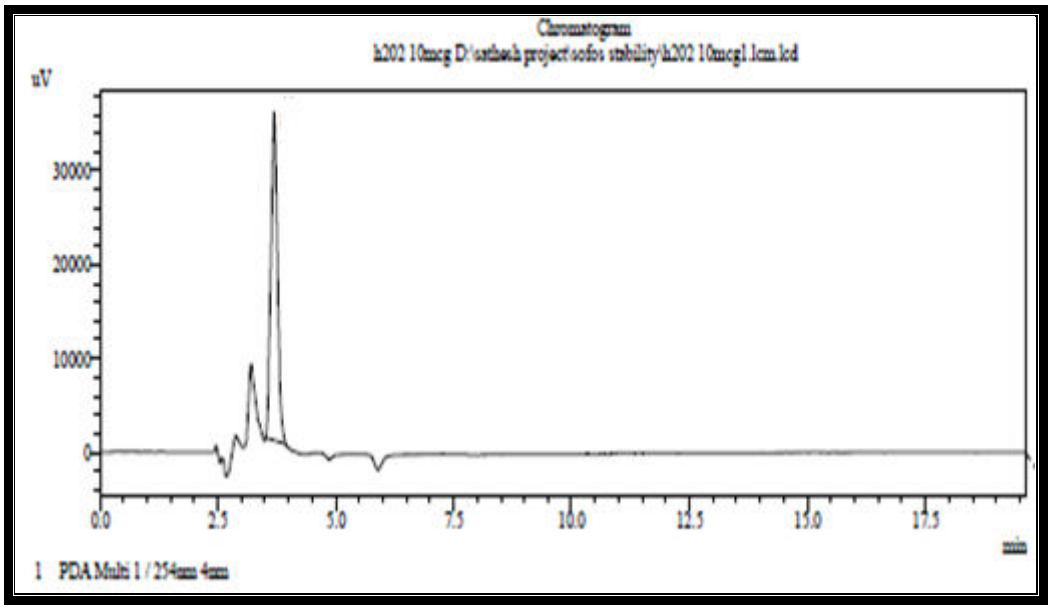
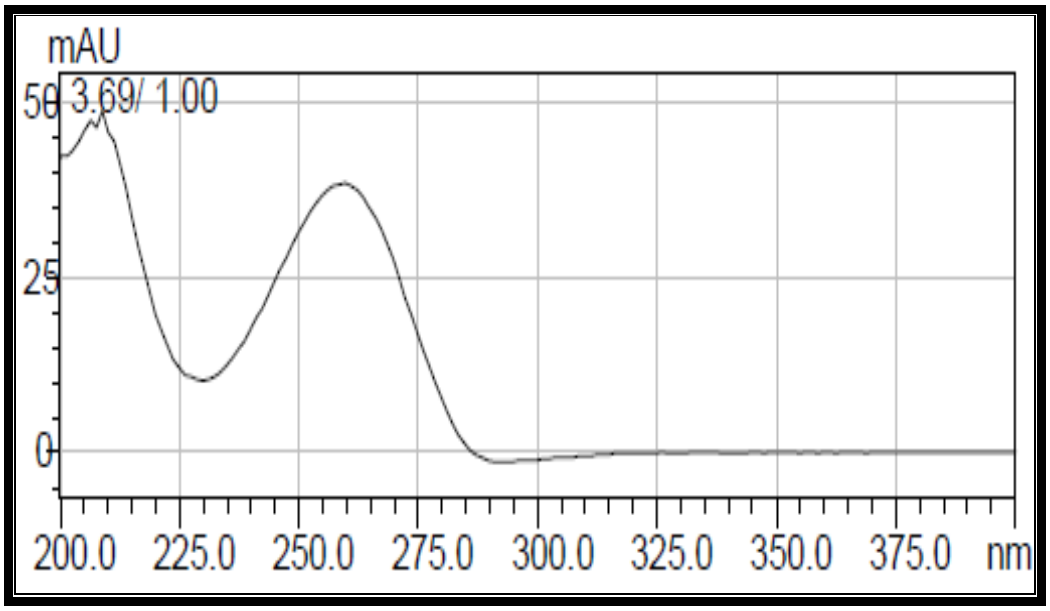


Figure 67b: Spectrum of Sofosbuvir



Photolytic degradation:

The drug was subjected to photolytic degradation no additional peaks were observed, which was shown in figure 68a.

Figure 36: Chromatogram of photolytic degradation of Sofosbuvir

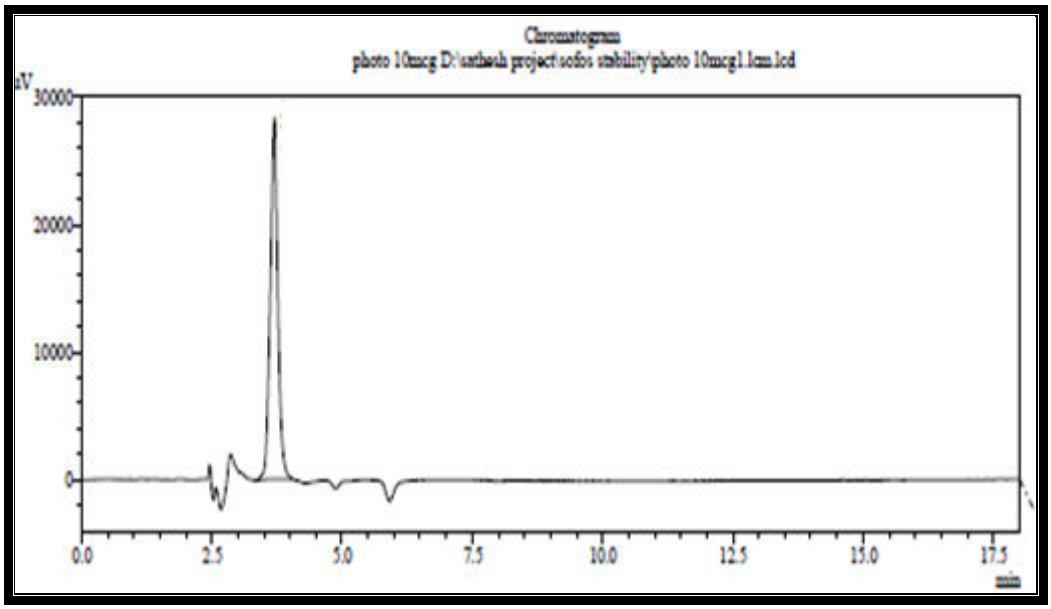
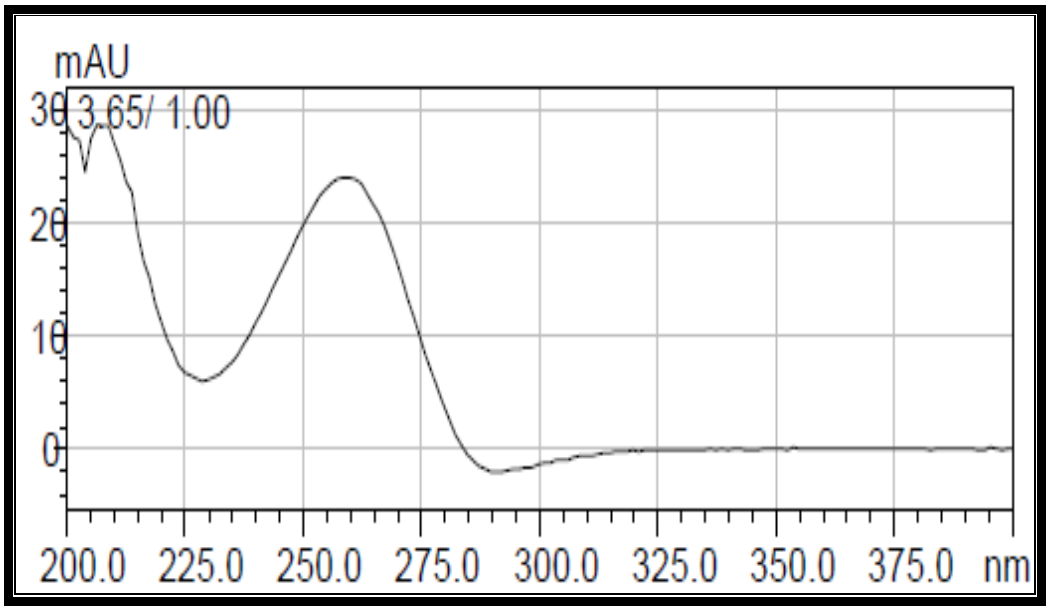


Figure 68b: Spectrum of Sofosbuvir



Thermal degradation

The drug was subjected to thermal degradation no additional peaks were observed, which was shown in figure 69a & 69b.

Figure 69a: Chromatogram of thermal degradation of Sofosbuvir

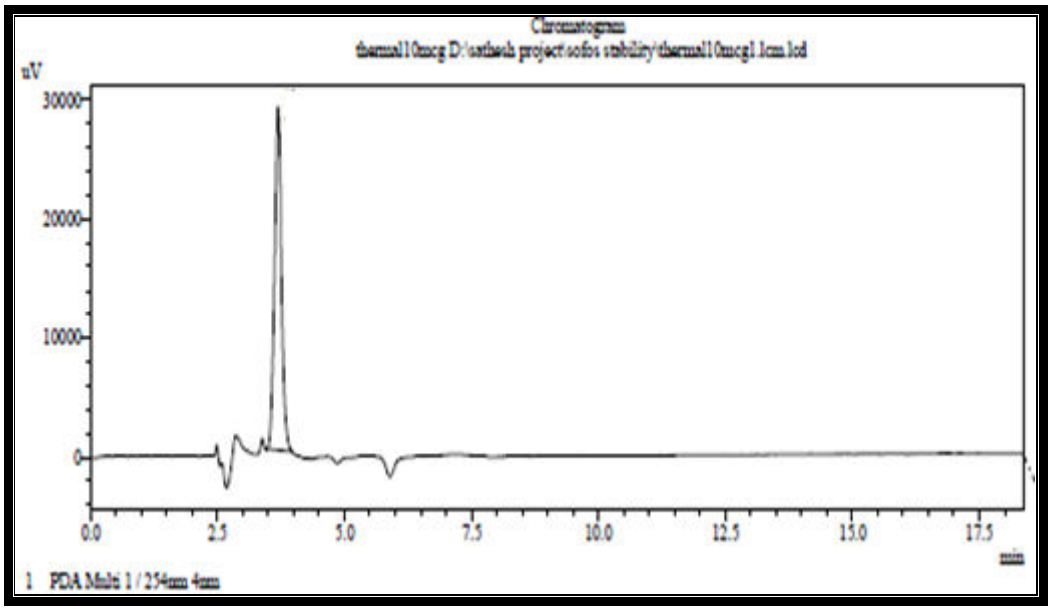
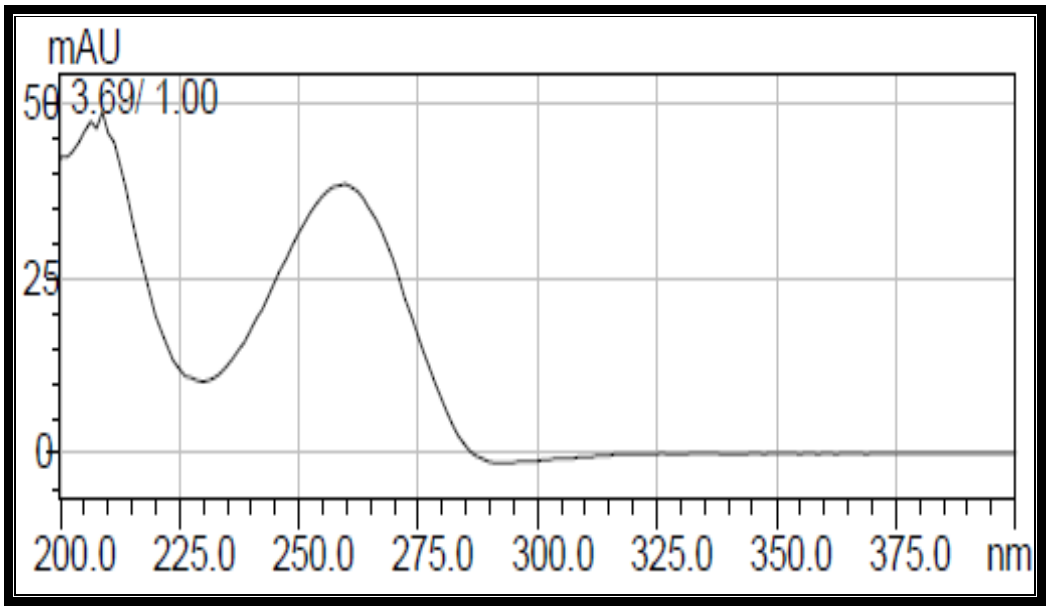


Figure 69b: Spectrum of Sofosbuvir



Summary of forced degradation was shown in the table 42.

Table 42 Summary of forced degradation studies for Sofosbuvir:

Type of stress	Stress condition	Retention time(min)	
		Drug	Degraded Product
Acid hydrolysis	Drug refluxed with 0.1M HCl for about 5 hours	3.7	No additional peak
Basic hydrolysis	Drug refluxed with 0.1m NaoH for about 5 hours	3.7	2.8±0.02 4.1±0.02
Neutral hydrolysis	Drug treated with water and refluxed for about 5 hours	3.7	No additional peak
Oxidative degradation	Drug treated with 6% hydrogen peroxide at normal room temperature	3.7	No additional peak
Photo degradation	Drug exposed to Sunlight for 5 hours	3.7	No additional peak
Thermal degradation	Drug introduced in Hot air oven for 5 hours	3.7	No additional peak

FORCED DEGRADATION STUDIES: LEDIPASVIR AND SOFOSBUVIR

Acid hydrolysis:

Ledipasvir and sofosbuvir were subjected to acid hydrolysis, no additional peak was observed, that is shown in figure 70a & 70b.

Figure 70a: Chromatogram of acid hydrolysis of Ledipasvir and Sofosbuvir

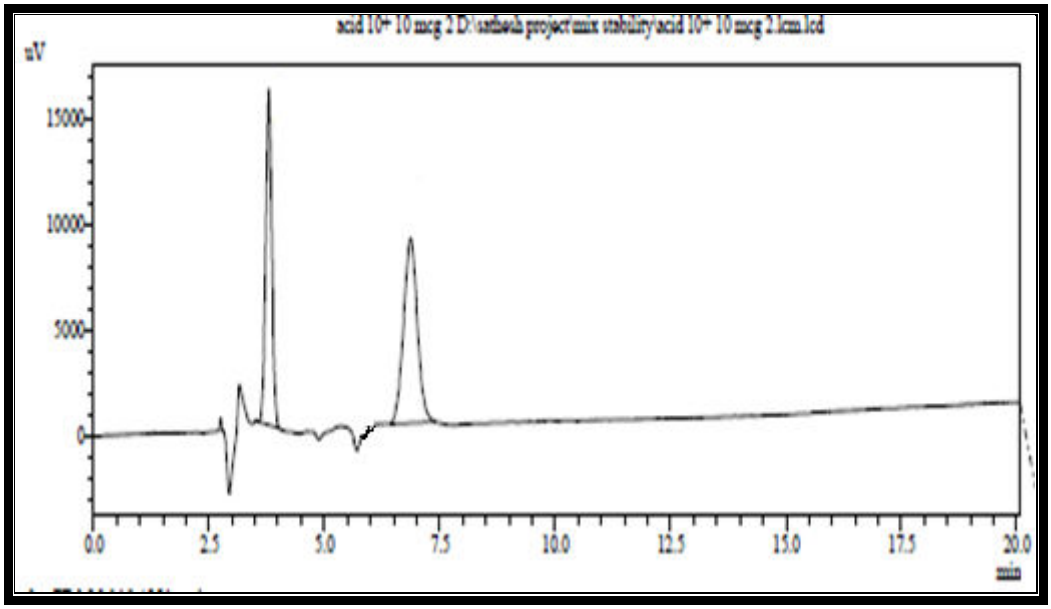
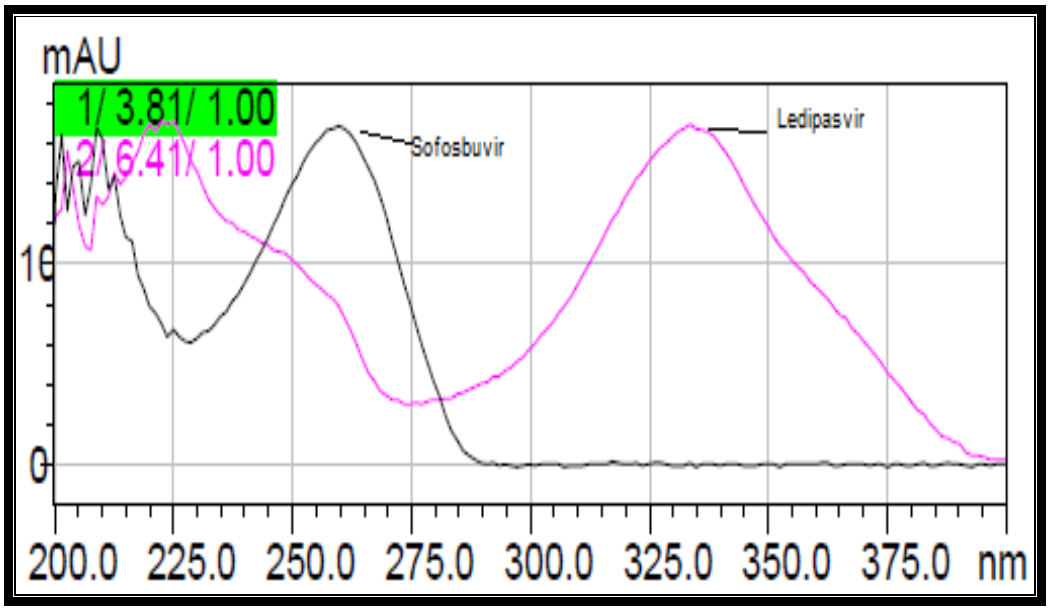


Figure 70b: Spectrum of Ledipasvir and Sofosbuvir



Alkaline hydrolysis:

The drug was subjected to alkaline hydrolysis two additional peaks were observed at R_t 3.1 and 4.2 value which was shown in figure 71a,71b,71c & 71d.

Figure 71a: Chromatogram of alkaline hydrolysis of Ledipasvir and Sofosbuvir

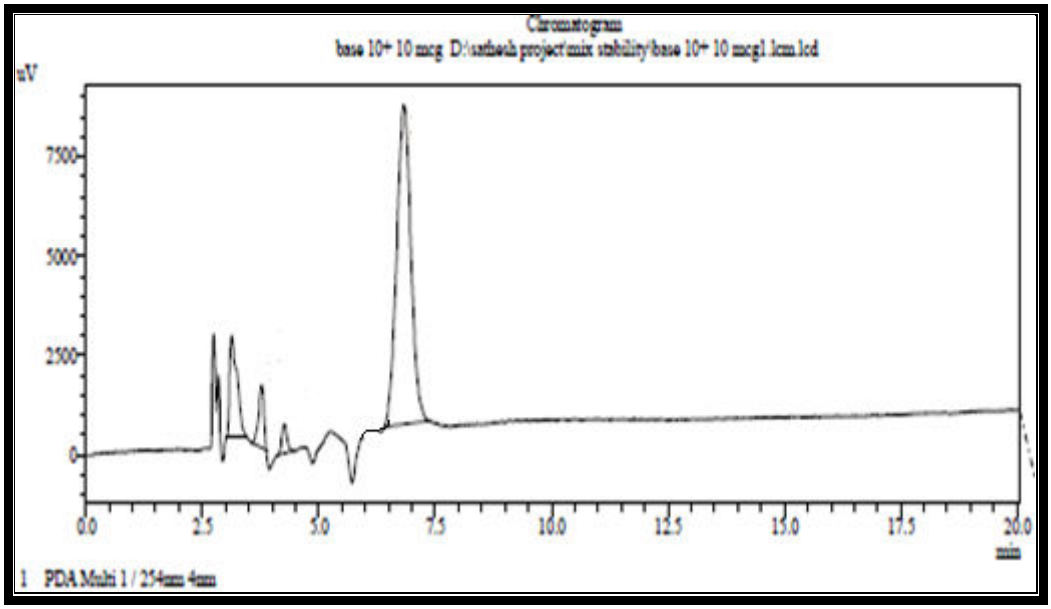


Figure 71b: Spectrum of Ledipasvir and Sofosbuvir

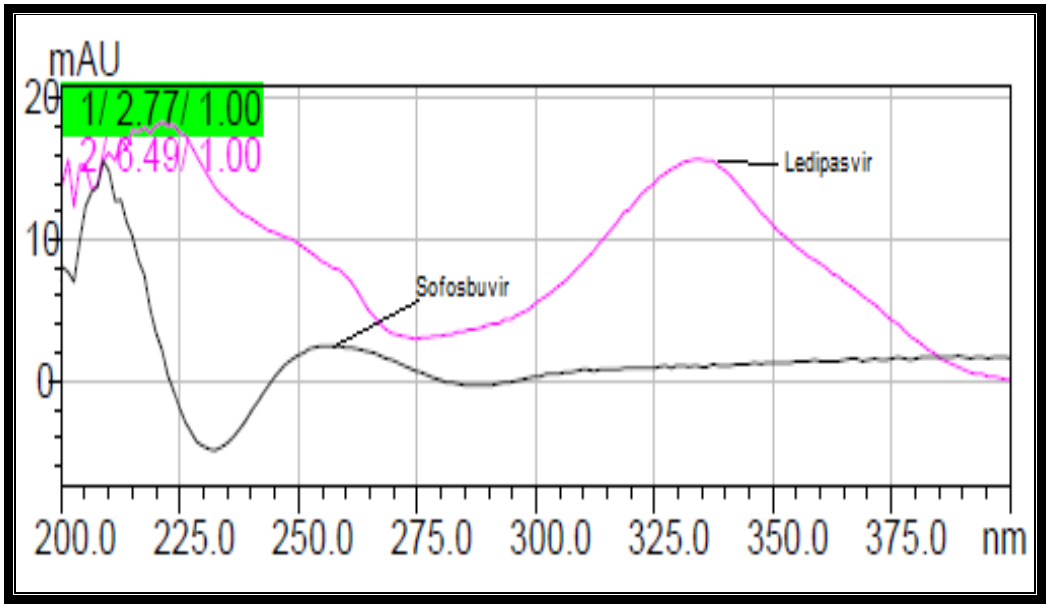


Figure 71c: Spectrum of Sofosbuvir degradants

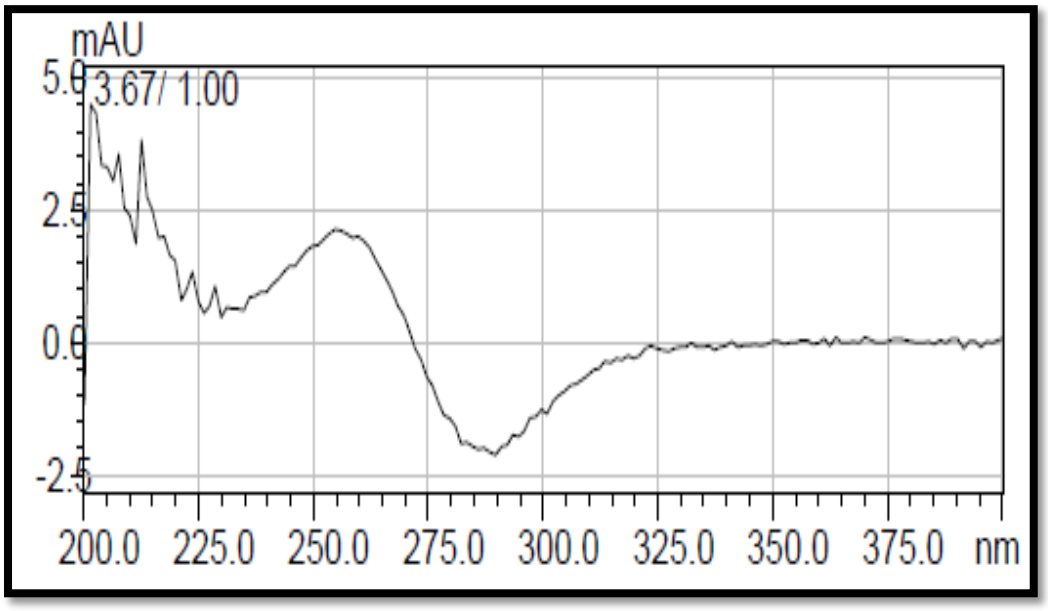
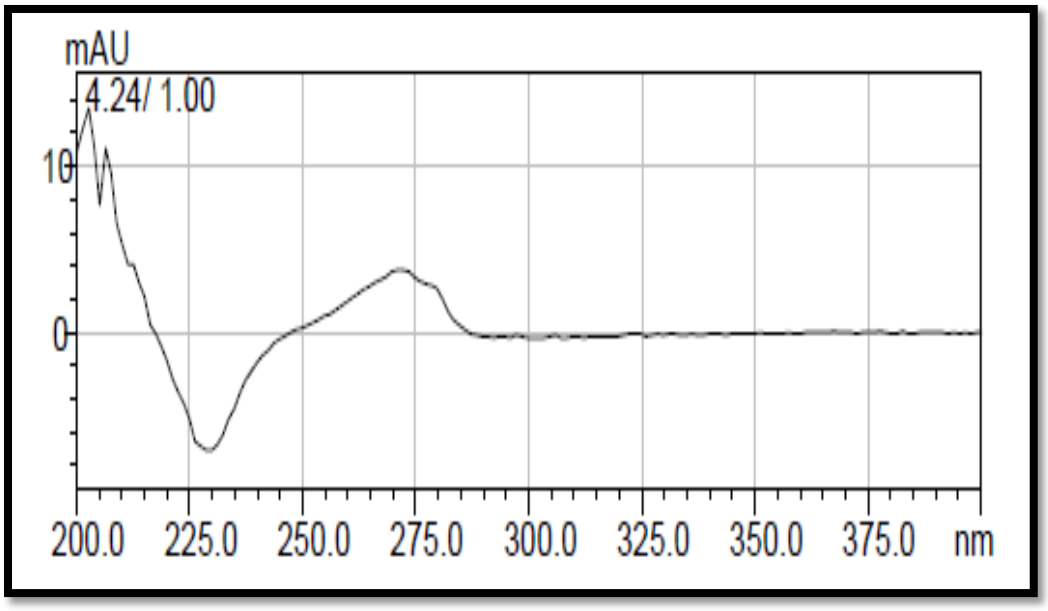


Figure 71d: Spectrum of Sofosbuvir degradants



Neutral hydrolysis:

Ledipasvir and sofosbuvir were subjected to neutral hydrolysis no additional peaks were observed, which was shown in figure 72a & 72b.

Figure 72a: Chromatogram of neutral hydrolysis of ledipasvir and sofosbuvir

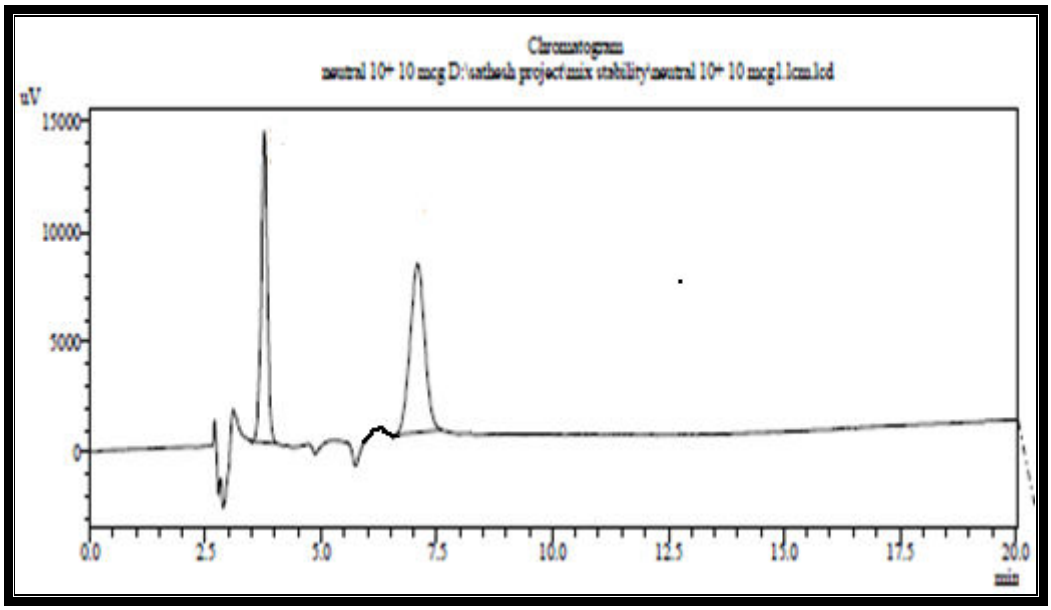
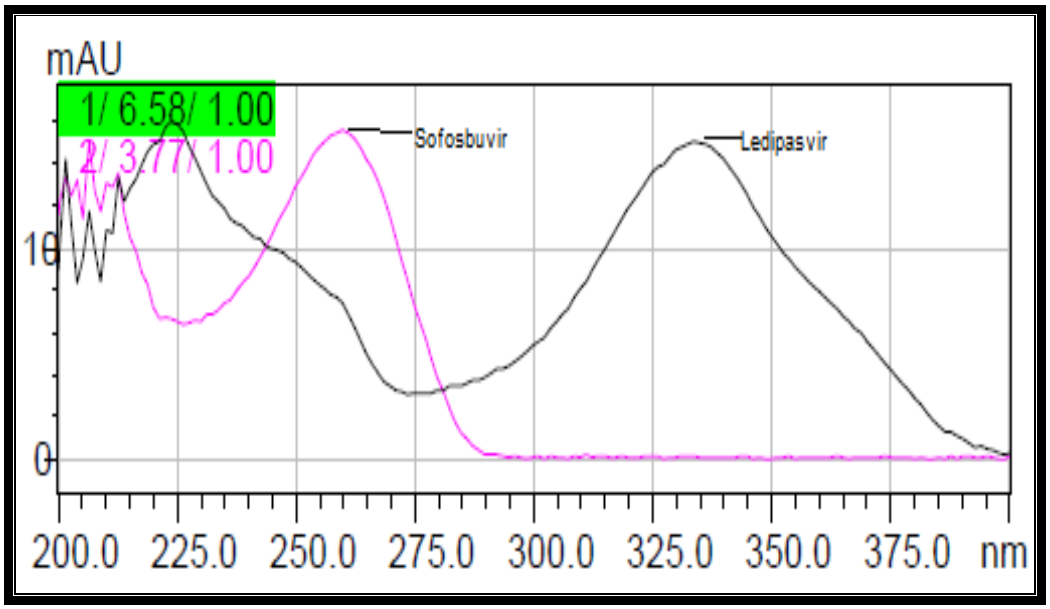


Figure 72b: Spectrum of Ledipasvir and Sofosbuvir



Oxidative degradation:

Ledipasvir and sofosbuvir were was subjected to oxidation no additional peaks were observed , which was shown in figure 73a &73b.

Figure 73a: Chromatogram of Oxidative degradation of Ledipasvir and Sofosbuvir

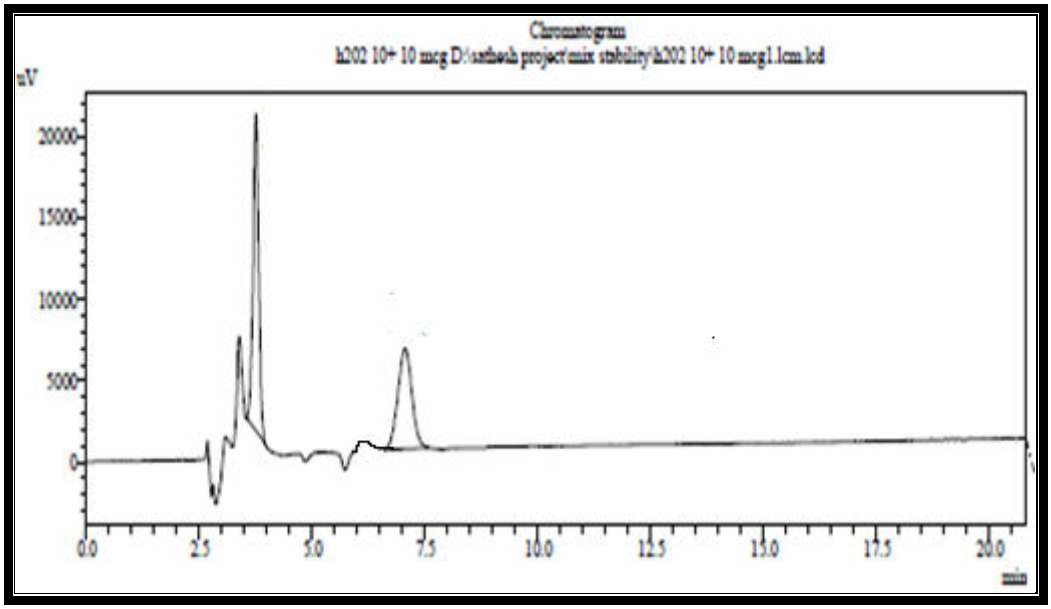
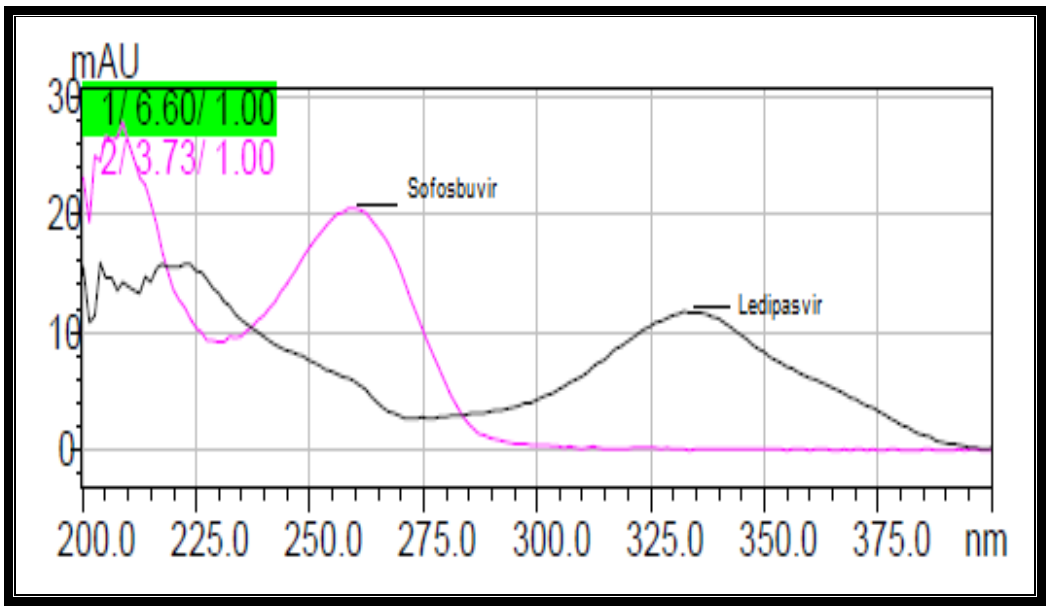


Figure 73b: Spectrum of Ledipasvir and Sofosbuvir



Photolytic degradation:

Ledipasvir and sofosbuvir were was subjected to photolytic degradation no additional peaks were observed at, which was shown in figure 74a & 74b.

Figure 74a: Chromatogram of neutral hydrolysis of Ledipasvir and Sofosbuvir

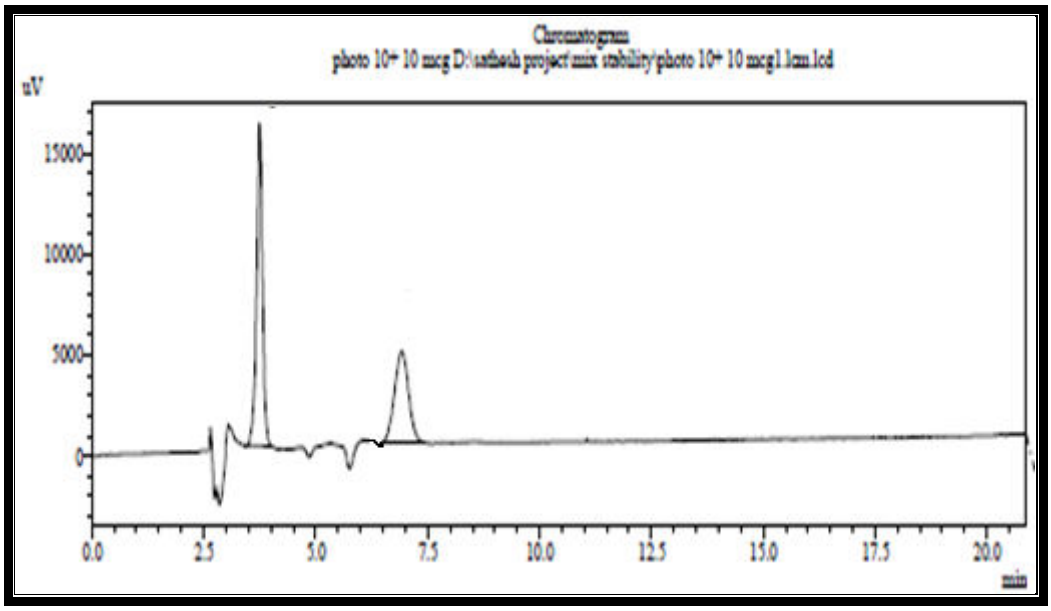
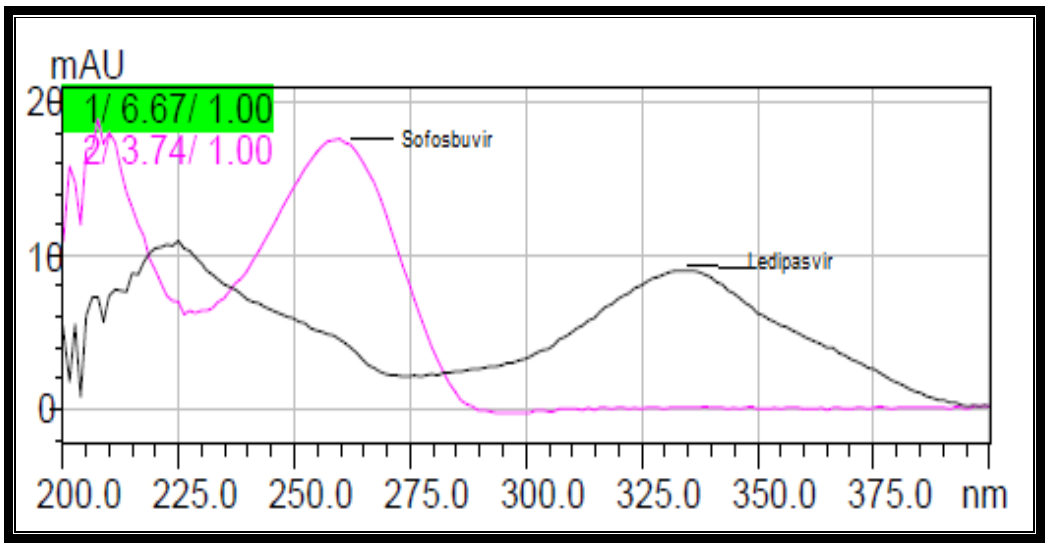


Figure 74b: Spectrum of Ledipasvir and Sofosbuvir



Thermal degradation

The drug was subjected to thermal degradation no additional peaks were observed, which was shown in figure 75a & 75b.

Figure 75a : Chromatogram of thermal degradation of ledipasvir and sofosbuvir

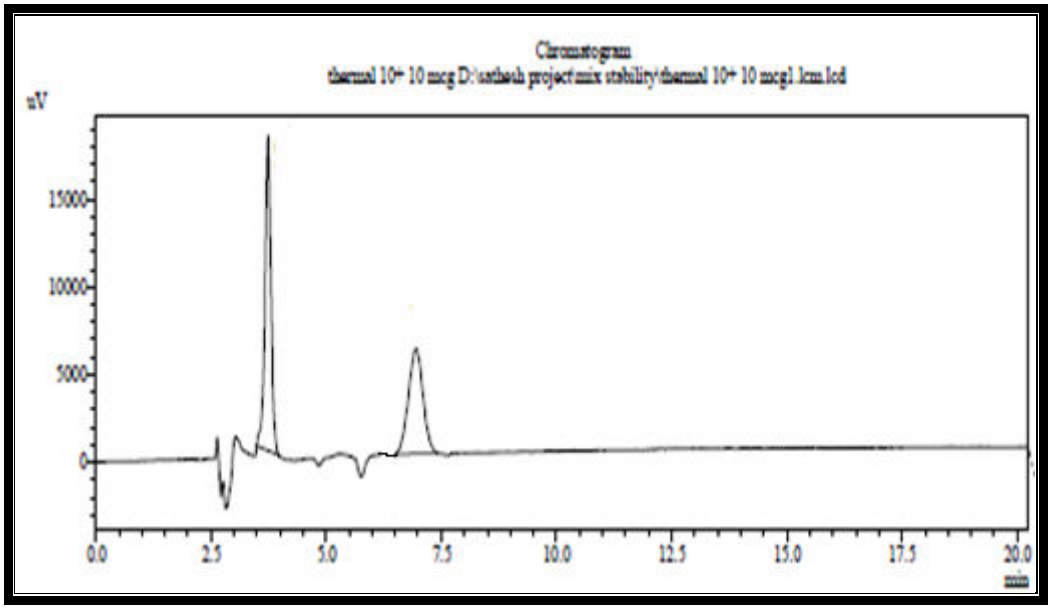


Figure 75b: Spectrum of Ledipasvir and Sofosbuvir

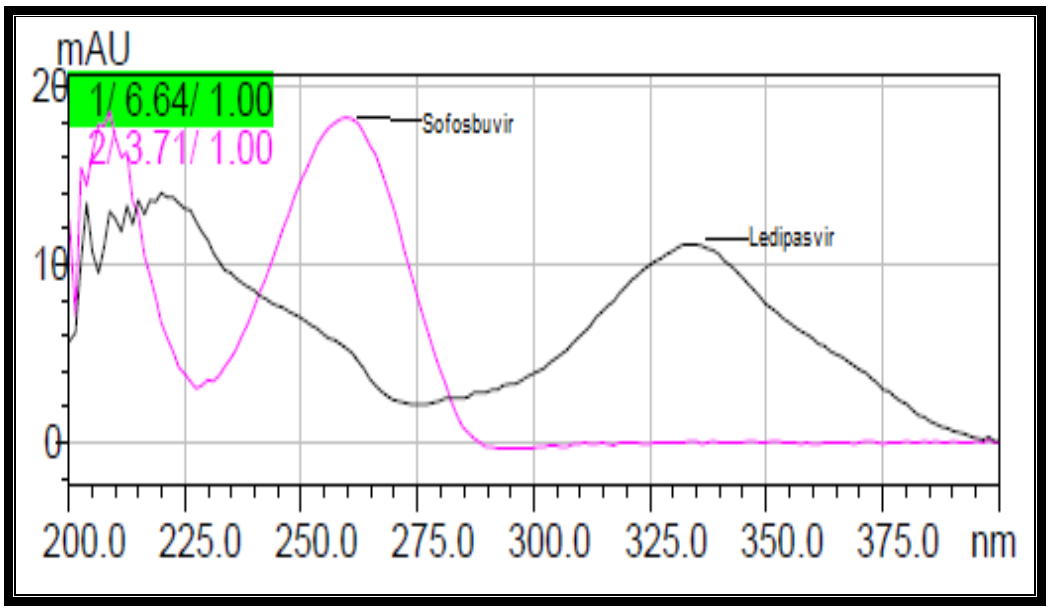


Table 43 Summary of forced degradation studies for Ledipasvir and Sofosbuvir:

Type of stress	Stress condition	Retention time(min)			
		Ledipasvir	Sofosbuvir	Ledipasvir Degradant	Sofosbuvir Degradant
Acid hydrolysis	Drugs refluxed with 0.1M HCl for about 5 hours	7.1	3.7	No additional peak	No additional peak
Basic hydrolysis	Drugs refluxed with 0.1m NaoH for about 5 hours	7.1	3.7	No additional peak	2.8±0.02 4.1±0.02
Neutral hydrolysis	Drugs treated with water and refluxed for about 5 hours	7.1	3.7	No additional peak	No additional peak
Oxidative degradation	Drugs treated with 6% hydrogen peroxide at normal room temperature	7.1	3.7	No additional peak	No additional peak
Photo degradation	Drugs exposed to Sunlight for 5 hours	7.1	3.7	No additional peak	No additional peak
Thermal degradation	Drugs introduced in Hot air oven for 5 hours	7.1	3.7	No additional peak	No additional peak

SUMMARY AND CONCLUSION

Each year 3,99,000 people were died for hepatitis C. Ledipasvir and Sofosbuvir are the two anti-viral lifesaving drugs commonly used in combination for treating hepatitis C.

The present study deals with development and validation of a two simple, economical UV Spectroscopic methods, and two sensitive, reliable stability indicating HPTLC and HPLC methods for simultaneous estimation of Ledipasvir and Sofosbuvir in pure and fixed dose combination.

The UV Spectroscopic methods involved by solving (i)simultaneous equation (ii)second order derivative methods. The two selected wavelengths were 333 nm (λ_{max} of LEDI) and 261 nm (λ_{max} of SOF) for both the methods. Linearity was observed in the concentration range of 10 – 40 mcg/ml for LEDI and 10 - 70 mcg/ml for SOF, respectively.

The stability indicating HPTLC method involved using TLC plates precoated with Silica gel $G_{60}F_{254}$ and the mobile phase comprising n-hexane: ethyl acetate: methanol in the ratio of 4: 5: 1 v/v/v. Ledipasvir and Sofosbuvir were well resolved with R_f 0.43 ± 0.02 and 0.21 ± 0.02, respectively. Wavelength selected for the quantification was 288 nm. Inherent stability of these drugs was studied by exposing both drugs to various stress conditions as per ICH guidelines viz. thermal, oxidative, photolysis and hydrolytic conditions under different pH values. Ledipasvir showed an additional peak (Rf = 0.34) in all stress condition except for neutral hydrolysis. Whereas Sofosbuvir showed three additional peaks at R_f = 0.12, 0.28 and 0.33, respectively. The specificity of the method was confirmed by peak purity profile of the resolved peaks which were found to be close to 1.

In the stability-indicating reverse phase high performance liquid chromatography method, the wavelength selected for quantitation was 254 nm. (The method has been validated for linearity, accuracy, precision, robustness, limit

of detection and limit of quantification. Linearity was observed in the concentration range of 1-10 µg/ml for both the drugs). In this method, the separation was achieved by Hibar C_{18} (250×4.6mm) 5µm column using 1% O-Phosphoric acid (pH 6.4):Acetonitrile (30:70 v/v) as mobile phase with flow rate 0.8 ml/min. The retention time of ledipasvir and sofosbuvir were found to be 7.1min and 3.7 min, respectively. During force degradation, drug product was exposed to hydrolysis (acid and base hydrolysis), oxidation, thermal degradation and photo degradation. Both the drugs were not degraded under thermal, oxidative, photolytic, acid and neutral hydrolytic conditions, but Sofosbuvir showed degradation under alkaine hydrolytic condition with a retention time 2.8 min and 4.1 min, respectively. The degraded products of Ledipasvir and Sofosbuvir were well resolved from the individual bulk drug response. The specificity of the method was confirmed by peak purity profile of the resolved peaks.

All the developed methods were validated according to ICH guidelines and were found to be simple, specific, sensitive, accurate, precise and economic, hence the proposed methods could be used for simultaneous estimation of Ledipasvir and Sofosbuvir in tablet dosage form. Among these methods UV spectroscopic methods are simple, reliable and less time consuming which could be an alternative for chromatographic analysis.

The summery of all the proposed methods are shown in table 44.

Table 44: Summery of the proposed methods

Parameters	UV-Spectroscopy methods				HPTLC		HPLC	
	Simultaneous equation method		Second order derivative					
	LEDI	SOFOS	LEDI	SOFO	LEDI	SOFO	LEDI	SOFO
Working wavelength (nm)	333	261	333	261	288	288	254	254
Linearity range	10-40 µg/ml	10-70 µg/ml	10-40 µg/ml	10-70 µg/ml	100-700 ng/spot	440-3080 ng/spot	1-10 µg/ml	1-10 µg/ml
Correlation co-efficent(r^2)	0.9987	0.9972	0.9978	0.9982	0.994	0.992	0.9987	0.9995
SD slope*	0.0211±0.00016	0.0350±0.0001	0.3571±0.0004	0.475±0.0008	4.847 ±0.0010	1.461 ±0.001	3.123 ± 2.608	3.123 ±2.611
SD intercept*	0.02528±0.12	0.0172±0.0001	0.7857±0.14	0.0015±0.52	349.9 ±0.1941	870.4 ±0.192	1112.6 ±2.436	870.4 ±0.192
Data point	6	6	6	6	6	6	6	6
Molar absorptivity (L mol^1 cm^1)	2640762	919303	-	-	-	-	-	-
LOD*	0.2424µg/ml	0.24246 µg/ml	0.0044µg/ml	0.004454µg/ml	0.13ng/spot	0.4345ng/spot	0.008 µg/ml	0.0006 µg/ml
LOQ*	0.7347µg/ml	0.7347µg/ml	0.0134µg/ml	0.0134µg/ml	0.40ng/spot	1.316ng/spot	0.27 µg/ml	0.0020 µg/ml
Assay %	99.12	99.60	102.24	99.74	101.61	99.76	99.30	99.54
Recovery								
(i) 50%	99.41	97.38	101.84	101.86	99.41	100.25	98.34	99.73
(ii) 100%	98.37	98.15	103.71	99.20	98.25	101.85	98.01	100.36
(iii) 150%	98.15	97.21	101.65	102.79	101.84	100.89	99.27	101.48
Precision	<2	<2	<2	<2	<2	<2	<2	<2

***Mean of six determination**

The statistical evaluation of the methods are summarized in table 45.

Table 45: Statistical comparison between the developed methods:

Parameters	Uv normal method		HPTLC		HPLC		Reported method[12]	
	LEDI	SOFO	LEDI	SOFO	LEDI	SOFO	LEDI	SOFO
Mean (assay)	99.39	99.53	101.56	99.52	99.40	99.44	99.37	99.46
Number of points	6	6	6	6	6	6	6	6
SD*	0.2779	0.2855	0.2556	0.3395	0.2526	0.2393	0.3883	0.2464
SEM	0.1135	0.1166	0.1043	0.1386	0.1031	0.0977	0.1585	0.1006
Medium	99.34	99.58	101.53	99.60	99.37	99.49	99.33	99.12
P	0.0425							
F	1.056							

***Mean of six determination**

This results shows that there is no significant difference among these methods. Hence, the developed methods can be used for routine analysis of ledipasvir and sofosbuvir from pharmaceutical dosage form.

BIBLIOGRAPHY

1. Beckett AH, Stenlake JB. Practical pharmaceutical chemistry. 4th ed. Vol II. New Delhi: CBS publishers and distributors; 2005. p. 1-3, 300 301.
2. Napoleon AA, pharmaceutical titrimetric analysis. 1st ed. Kalaimani publisers and distributers; 2006. P. 1-2.
3. Sethi PD. HPTLC: High performance thin layer Chromatography. Vol I. Quantitative Analysis of Pharmaceutical formulations. New Delhi: CBS publishers and distributers: 1997.p. 2-81.
4. Snyder LR, Kirkland JJ, Glajch JL. Practical HPLC method development. 2nd ed. New York: John Wiley & Sons, Inc; 1997. p.1-15, 205-13, 234-66.
5. Satinder A, Michael WD. Handbook of Pharmaceutical Analysis by HPLC. 6th ed. New Delhi: Elseveier; 2009. P. 22-39.
6. The United States Pharmacopoeia, 27th ed. Rockville: United state Pharmacopoeial Convention; 2004. p. 221-82
7. ICH harmonized tripartite guidelines. Stability testing of new drugs substance and products Q2(R_1) 2005 Nov. Geneva, Switzerland.
8. Bakshi M, Singh S, Development of validated stability-indicating assay methods critical review. J. Pharm Biomed Anal. 2002 Jun 15; 28(6): 1011-40.
9. ICH Q2B Validation of Analytical Procedure Methodology. International Conference on Harmonization. Geneva: 1996
10. Mohan Vikas P, Satyanarayana T, Vinod Kumar D, Mounika E, Sri Latha M.Development and validation of new rp-hplc method for the determination of sofosbuvir in pure form, World J Pharm Pharm Sci. 2016; 5: 775-81
11. Devila J, Durgaprasad B, Narottam Pal, Srinivasa Rao A. New method development and validation for the determination of ledipasvir in bulk drug form by using reverse phase hplc technique, World J Pharm Pharm Sci. 2016; 8: 1312-21.

12. Chenwei Pana, Yongping Chenb, Weilai Chenc, Guangyao Zhoua, ingxiang Jina. Simultaneous determination of ledipasvir, sofosbuvir and itsmetabolite in rat plasma by UPLC–MS/MS and its application to a pharmacokinetic study, J. Chromatogr. B. 2016; 1009: 255-59.

13. Nebsen M, Elzanfaly S. Stability-Indicating Method and LC–MS-MS Characterization of Forced Degradation Products of Sofosbuvir, J CHROMATOGR SCI. 2016; 1-10.

14. Ehab Elkady F, Ahmed Aboelwafa A. A Rapid and Optimized LC-MS/MS Method for the Simultaneous Extraction and Determination of Sofosbuvir and Ledipasvir in Human Plasma, J. AOAC Int. 2016; 99: 1252-59.

15. Debasish Swain, Gananadhamu Samanthula. Study on the forced degradation behaviour of ledipasvir: Identification of major degradation products using LC-QTOF-MS/MS and NMR, J Pharm Biomed Anal. 2017;

16. European Medicine Agency. Harvoni fact sheets [Internet]. 2017 August [cited 2014sep], London. Available from; http://www.ema.europa.eu/docs/en_GB/document_library.

17. World Health Organization. Hepatitis C fact sheets [Internet], 2017 august [cited 2017 august]. Geneva, Switzerland. Available from: http://www.who.int/mediacentre/factsheets/fs164/en/

18. Wikipedia. Hepatitis C [document on the internet]. [updated February 2017; cited 2017 august 5]. Available from https://en. Wikipedia.org/ wiki/ Hepatitis C.

19. Wikipedia. Ledipasvir [document on the internet]. [updated February 2017; cited 2017 august 5]. Available from https://en. Wikipedia.org/ wiki/ Ledipasvir.

20. Wikipedia. Sofosbuvir [document on the internet]. [updated February 2016; cited 2017 august 5]. Available from https://en. Wikipedia.org/ wiki/ Sofosbuvir.