Original Article

Development of Validated Stability-indicating RP-HPLC Method for Determination of Novel Directly Acting Antiviral agent and Characterization of its Degradants by LC–ESI–MS

Babita Agarwal[1,*], Santosh Gandhi[2]

[1]Department of Pharmaceutical Chemistry, Marathwada Mitra Mandal's College of Pharmacy, Pune, Maharashtra, INDIA.
[2]Department of Pharmaceutical Chemistry, AISSMS College of Pharmacy, Shivaji Nagar, Pune, Maharashtra, INDIA.

ABSTRACT

Aim: The current study was performed to develop and validate stability indicating high performance liquid chromatography method (RP-HPLC) for determination of ledipasvir (LPR); to identify and characterize its major degradants by liquid chromatographic–tandem mass spectrometric method (LC-ESI-MS). **Materials and Methods:** The method was developed using reverse phase gradient elution and validated for standard ICH parameters. The optimized mobile phase comprised of acetonitrile:water with 0.2 % formic acid (70:30% v/v) at 1 ml/min flow rate with satisfactory retention time (tR), theoretical plates and good resolution of LPR and its degradants. Further, forced degradation under acid, base, thermal, photolytic and oxidative stress conditions was studied as per ICH guidelines. LC-ESI-MS with time of flight analyser was used to characterize the degradants. The degradation pathways for major degradants were proposed. **Results:** The developed method had retention time of 6 mins. The RSD for system was found to be less than 2% whereas mean recovery was obtained 97.2 – 102.5%. Linearity range of 5-30 μg/ml with 0.998 regression coefficient (R^2) was observed. Detection and quantification limits were obtained as 0.010 μg/mL and 0.032 μg/mL, respectively. LPR was stable in photolytic and thermal environments whereas degraded in acid, base and oxidative states. LC–ESI–MS was used effectively for characterization and structural elucidation of degradants. **Conclusion:** The results indicated that validated RP-HPLC technique can be employed for routine analysis of LPR in bulk and dosage formulas and also would be capable of separating degradants from analyte peak.

Key words: RP-HPLC, LC-ESI-MS, Ledipasvir, Stability indicating ICH method, Validation, Degradation pathway.

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Correspondence:
Mrs. Babita Agarwal
Department of Pharmaceutical Chemistry, Marathwada Mitra Mandal's College of Pharmacy, Pune, Maharashtra, INDIA.
Phone: +91 9822544505
E-mail: babita_a_agarwal@yahoo.co.in

INTRODUCTION

Viral hepatitis has become a serious public health concern as it affects more than 3% of world population. Out of this more than 1% of the population is infected by hepatitis C virus (HCV).[1,2] The prevalence of HCV infection doubled between 2010-2014 and till date. Annually almost 1.75 million infections are reported worldwide for HCV infection.[3] If HCV infection remains untreated; can progress to cirrhosis, fibrosis and hepatocellular carcinoma.[4] Such long-term problems are fatal, lethal and a reason for 96% of the deaths owing to viral hepatitis. The people infected with HCV are unaware about the infection, as they don't receive the well identified symptoms till complications emerge. The people may be infected for a period greater than 30 years before they develop clinical symptoms.

Till the development of directly acting antivirals (DAA), ribavirin in combination with the PEGlyated interferon was the only option available for the treatment. Nonetheless, it has been accompanied

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with the numerous side effects limiting its use.[5] Use of PEGlyated interferon is also associated with the neuropsychiatric side events, toxicities, lesser efficacy against the some of the genotypes of HCV.[6] The various studies have explored the life cycle of the HCV and the role of non-structured proteins in the viral replication. It has led to the complete transformation of the treatment line used for the HCV infections. The various DAAs are developed successfully and are widely used in the treatment of HCV infections. It includes sofosbuvir,[7] velpatasvir,[8] ledipasvir,[9] voxilaprevir,[10] boceprevir,[11] telaprevir,[12] vaniprevir,[13] filibuvir,[14] simeprevir,[15] ombitasvir,[16] grazoprevir,[17] etc.

Ledipasvir (LPR) is developed by Gilead Pharmaceuticals (Figure 1). LPR is a carbamic acid derivative with white solid crystals and chemical molecular formula as $C_{49}H_{54}F_2N_8O_6$. NS5A protein is essential for RNA replication in addition to assembly of HCV visions. It is also an inhibitor of the HCV.[18] Even though its exact mechanism of action is unfamiliar, it is proposed to inhibit hyper phosphorylation of NS5A that is vital for viral production.[19] Chemically, a carbamate ester, a L-valine derivative, a bridged compound, a carboxamide, a benzimidazole, a member of fluorenes, an organofluorine compound, a member of imidazoles, a N-acylpyrrolidine and an azaspiro compound. Ledipasvir is official by the U.S. Food and Drug Administration (FDA) on 2014 for treating patients of HCV either alone or in combination with sofosbuvir.[20]

Fewer bioanalytical methods for estimation of the LPR in plasma but not stability indicating[21-23] are available. These methods are specifically worked for the estimation of LPR in biological fluids. Few researchers have reported the analytical methods for simultaneous determination of the ledipasvir and sofosbuvir in bulk and tablet forms by RP- HPLC[24-27] and by UV.[28] Very few of these papers have discussed the forced degraded analysis of drug. Ghante MR *et al.* discussed the validation for the simultaneous estimation of ledipasvir in bulk and tablet dosage form.[29] In this study, C_{18} column was used for chromatographic analysis. The mobile phase used in the study consisted of acetonitrile: 1mM ammonium acetate buffer (90:10 v/v) at the flow rate of 1.0 mL/min. The work has been extended with the forced degradation in altered conditions but the methods were not appropriately validated as well as no characterization of degradants was performed. The identification and characterization of degradants by the suitable method is useful from the large scale production point of view. The alternative, validated stability indicating assay methods at the accelerated conditions with the identification and characterization of degradants are needed for the study of the LPR.

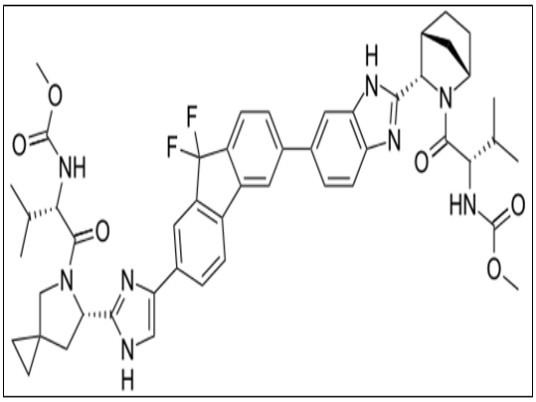

Figure 1: Structure of LPR.

So, the present analytical study has been performed as per the ICH guidelines, to develop and validate an effective stability indicating assay method which would be capable of detecting the small changes in the drug and its products with time under several conditions. The results obtained could find applications in method development for new drugs and their formulations, routine analysis, inclusive drug degradation studies, stability indicating assay methods. Moreover, the identification and characterization of degraded products were carried by the use of LC-ESI-MS with time of flight analyzer. With the accurate mass measurements; the degradation pathways were developed to provide the better insight into the degradation mechanism.

MATERIALS AND METHODS

Chemicals

LPR was gift sample from Dr. Reddy's Laboratory, Hyderabad, India. Analytical reagents like methanol, acetonitrile, formic acid, sodium hydroxide (NaOH), Hydrogen peroxide (H_2O_2) hydrochloric acid (HCl) were procured from Loba Chemicals, Mumbai, India. Methanol used for LC-MS study was purchased from Sigma Aldrich, Bangalore, India. HPLC grade water was distilled out using double distillation assembly.

Instrumentation

For HPLC

The gradient HPLC system was used in the study which consisted of the quaternary pump (model Agilent LC-1260) and the photo diode array detector (PDA) (Agilent model no. G1315D 1260 DAD VL with 220 VA). The analytical column of Agilent 5 TC-C_{18} column (150 mm: 4.6 mm id, 5 μm) was used to perform the separation. The chromatograms were recorded over a

computer and treated with the aid of LC Open Lab Software solution. The filtration was done by Millipore glass filter assembly fitted with a vacuum pump.

For LC-ESI-MS

LC-ESI-MS analysis was executed with the use of Nexera 2 LC system (Shimadzu Corporation, Kyoto, Japan) connected to a triple quadrupole mass spectrometer (LC-MS 8040; Shimadzu, Kyoto, Japan) equipped with an electrospray ionization (ESI) source. The LC system was connected to the mass spectrometer via electron spray ionizer operating in a positive mode. The mass spectra of degradants were obtained in ESI (Turbo Spray) positive ion mode and analysed in the triple quadrupole analyser. The columns used were SUPELCOSIL LC-18-S HPLC column (5-µm particle size, 25-cm length × 4.6-mm inner diameter; Sigma-Aldrich). The chromatographic run time was kept at 10 mins with the flow rate of 1 mL min^{-1}. The injection volume of sample was 20 µL. The effluent from the column was passed into the mass spectrometer which splitted the bulk of mobile phase and delivered the least quantity of mobile phase to the mass spectrometer. The obtained MS data was integrated using analyst software.

Preparation of Solution

Mobile Phase Optimisation

The mobile phase was optimised using different ratios of acetonitrile and water with 0.2 % formic acid. The symmetry as well as the plate count was found to vary drastically with variation of acetonitrile concentration in the mobile phase. Also, the peaks were not upto the mark and peak tailing was observed. Finally, the mobile phase was optimised with mixture of acetonitrile and water with 0.2 % formic acid in 70:30% v/v proportions at a flow rate of 1 ml/min. It resulted in satisfactory retention time (tR), theoretical plates and good resolution of the peaks of ledipasvir and degradants. With the aid of 0.45 µm membrane filter the mobile phase was filtered and sonicated on ultrasonicator for 10 mins before use.

Reference and Sample Stock Solutions

Ledipasvir (1000 µg/mL) stock solution in methanol was prepared and UV spectrum of 10 µg/mL solution of ledipasvir was taken for the determination of λ_{max}. The test samples of LPR were prepared by accurately weighed twenty tablets of LPR. Then weighed 100 mg of drug sample was transferred into a 100 mL volumetric flask which was previously added with 75 mL of diluent. For 15 min the samples were sonicated, then the diluted with the diluent to 100 mL graduation mark. The resultant solution was passed over 0.45 µm nylon membrane filter before injected for analysis.

Method Validation

The liquid chromatographic technique was validated with respect to ICH guidelines.

Linearity and Range

The six concentrations of LPR in the range of 05 - 30 µg/mL were prepared in the mobile phase. The study was performed in six replicates. The calibration graphs were plotted using the peak areas verses the concentrations using least-square linear regression equation as (Y = slope X + C intercept). The LOD and LOQ were computed with the use of slope and expressions (3.3δ/slope) and (10δ/slope), respectively.

Precision

The reproducibility of the results by the developed method was assured using precision studies at three different concentrations (5, 10, 15 µg/ml) in three replicates. The samples were analysed for intra-day and inter-day. The %RSD for intraday and inter day readings was calculated and compared against the acceptable limits (± 2% to) to define the method precision.

Robustness of the method

Robustness was evaluated by performing the analysis under conditions where organic phase ratio and flow rate were altered and their effects on the peak area were noted. Robustness was tested at three different levels at (-1, 0 and 1). The effect of these modifications over the results was obtained using the values of (% RSD) compared to acceptable limits of ± 2% of peak response and retention time.

Specificity

The method specificity was confirmed by the study of resolution factor of the LPR peak from the nearest resolving peak. By determination of purity for individual degradation peak using PDA detector the selectivity was established.

Accuracy and recovery studies

Accuracy was worked by employing the method to known amount of ledipasvir standard equivalent to 50%, 100% and 150% of claim was added (standard addition method). The recovery study was based on ICH guidelines Q3B (R^2). The recovery was determined by relating the peak response against the individual reference solution. From the calibration curve data, the recoveries were calculated. The values of % RSD

were evaluated against the acceptance limits of ± 2% for LPR.

Forced Degradation Study of LPR

Forced degradation or stress testing is important for evaluation of stability of drug substances or drug products by the influence of environment which is above the accelerated stability studies environment. The various forced degradation conditions used in this study include acidic and alkaline conditions, hydrogen peroxide induced degradation, photochemical and thermal degradation.[30-32] Forced degradation studies were accomplished using 20 µg/mL of LPR solutions. Samples were analysed over HPLC at a range of 200 to 400nm with the use of PDA detector.

Acid and base degradation studies were accomplished by refluxing the solution of LPR in 0.1 N HCl and 0.1 N NaOH respectively at 70°C for 7 hr. The obtained solutions were neutralized and diluted with acetonitrile to get 50 µg/mL solutions and 20 µL was injected in the system. The oxidative degradation induced by hydrogen peroxide was achieved by exposing LPR solution to 6 % H_2O_2 at 70°C for 24hr and then heating on a water bath for 10 min.

The photochemical stability of the LPR was studied by keeping the stock solution of LPR (1000 µg/mL) in direct sunlight for 21 days according to ICH Q1 B guidelines.[33] Further, the thermal stability was evaluated by exposing the stock solution to a temperature of 50°C for a period of 21 days.

Structural characterization of degradants of LPR

The degradants obtained under different forced degradation conditions were characterized using LC-ESI-MS method. The determination of exact mass was carried out in the experimentation. Based upon mass determination, fragmentation pattern was determined. It was further used to propose the possible mechanism of the degradation and to deduce the degradation pathway.

RESULT AND DISCUSSION

Optimization of Chromatographic Method

The HPLC method was developed with an objective of making it compatible for the efficient separation of LPR and its degraded products. Inertsil ODS C_{18} (150 mm × 4.6 mm) HPLC column was selected for the study due to better shapes of peak and better resolution between the peaks of LPR and its degraded products. The mobile phase composition in chromatographic method development studies significantly affects the sensitivity and types of peaks obtained. Initially acetonitrile and water was tried in combination in different proportions. With variation in acetonitrile concentration; the symmetry and plate count was observed to be changed drastically yet not found up to the mark and tailing of the peaks was observed. Formic acid is widely preferred as a solvent of choice in LC-MS studies because of its less interference in mass spectrum due to lower molecular weight. Later on mobile phase was set by adding varying concentrations of formic acid. Finally, the mobile phase was optimised as acetonitrile and water with 0.2 % formic acid (70:30% v/v) at 1 ml/min flow rate. It gave satisfactory retention time (tR), theoretical plates and good resolution of the LPR and its degradants. This liquid chromatographic method was optimized successfully for the efficient separation of degradants from the LPR. 219nm was selected as the detection wavelength due to the higher sensitivity of the degradants at this wavelength when full range scan was performed between 200 to 400 nm (Figure 2). The typical sharp chromatogram was obtained at this wavelength at tR 6 mins with no interference which may allow the rapid determination of drug in different samples under the study. Figure 3A showed typical chromatogram obtained by the serial dilutions of standard stock solution of LPR.

The system suitability parameters for the LPR were evaluated and were found to comply as per USP standards. The retention time of LPR was 6 ± 0.1 min using optimum conditions. The peak symmetries of all the chromatograms were less than 2. The theoretical plate counts were greater than 3000. The %RSD values were less than 2 for all the peak areas studied. All these above mentioned results were within acceptable limits which ascertained the correctness of the developed chromatographic conditions for the accurate and precise analysis of LPR. The developed HPLC method followed linearity over the concentration range of 5-30 µg/ml. Beer's law was very well followed in this analysis

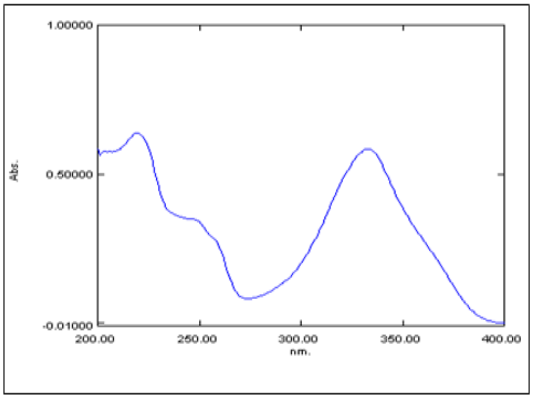

Figure 2: UV spectrum reflecting λmax of LPR.

at the obtained linearity range concentrations.[34] The linearity equation and regression coefficient was found to be y = 15762x + 6477.2 and 0.9988 respectively. In addition to this, the detection limit depends upon the sensitivity of the instrument to analyse lowest concentration with greater accuracy. The LOD and LOQ in the analysis were found to be 0.010 µg/mL and 0.032 µg/mL respectively. These outcomes asserted that the established and validated HPLC technique was linear according to least square regression analysis. The results obtained were statistically significant with % RSD value less than 2; indicating the uniform reproducibility in the replicates of different test concentrations. The insignificant variation was observed in intraday and interday studies proving the reproducibility and repeatability of the developed HPLC method to perform the accurate and precise measurement of the serial concentrations of the LPR (Table 1). The results for accuracy exhibited better reproducibility with %RSD values below 2. It seemed to be accurate as % recovery was observed high within range of 97.2 – 102.5%. Table 2 proved that the developed method exhibited good agreement between the standard and the observed values and demonstrated adequate accuracy within the specified limits. The robustness was demonstrated by altering the two selected factors i.e. flow rate (ml/min) and acetonitrile in the mobile phase (v/v) at three levels (-1, 0 and 1). Minor differences in peak areas and less

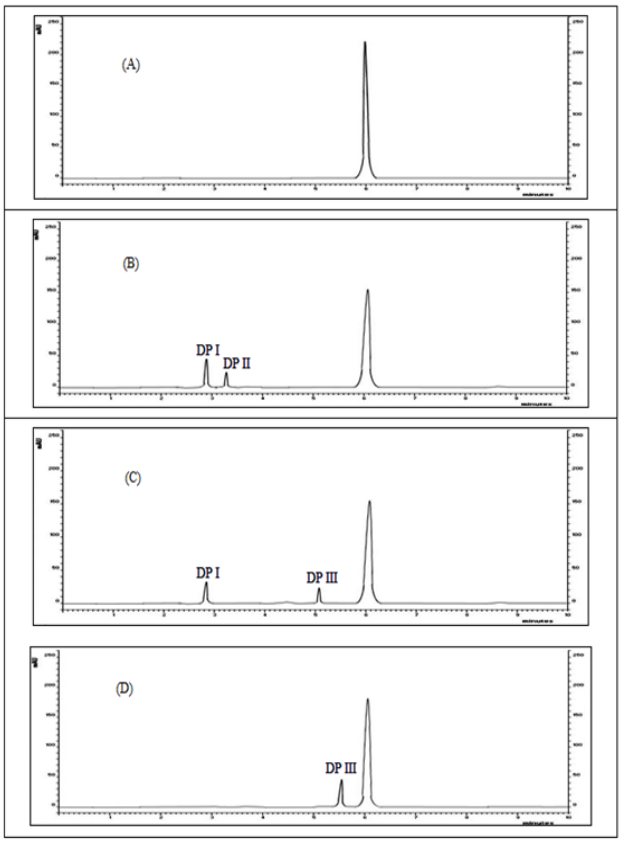

Figure 3: Forced degradation studies LPR under various stress conditions

(a) Chromatogram of standard stock solution of LPR (b) Acid degradation studies (0.1N HCl at 70oC, 7 hrs) (c) Base degradation studies (0.1N NaOH at 70oC, 7hrs) (d) Oxidative degradation (6% H_2O_2 at 70oC, 24 hrs).

Table 1: Intraday and interday precision of HPLC method developed for LPR.

Intraday precision						
Conc. (µg/ml)	Morning		Afternoon		Evening	
	Peak	SD	Peak	SD	Peak	SD
10	167015	0.003	167012	0.003	167015	0.003
15	239910	0.241	239909	0.243	239910	0.241
20	328195	0.020	328185	0.019	328195	0.020
Interday precision						
Conc. (µg/ml)	Day 1		Day 2		Day 3	
	Peak	SD	Peak	SD	Peak	SD
10	165572	0.431	164571	0.451	164275	0.432
15	235122	0.305	236120	0.325	236520	0.305
20	321083	0.080	321282	0.080	321581	0.050

Table 2: The accuracy of developed HPLC method.

Nominal concentration (µg/ml)	Level of addition (%)	Concentration prepared (µg/ml)	Amount recovered	% RSD	% Recovery
10	50	15	14.61	0.004	97.42
10	100	20	20.51	0.004	102.56
10	150	25	24.79	0.005	99.18

Acceptable recovery of 90-110 % was obtained as per ICH guidelines proving the accuracy of the method

variation in retention time were witnessed proving that the developed HPLC method is rugged in nature and can be engaged for the routine assay of the LPR in its tablet dosage forms and its degradants (Table 3).

The analysis of the LPR in its marketed tablets was performed using RP HPLC analysis. The results showed 99.16% assay value with a %RSD value less than 2 (0.31 %). The developed analytical method warranted better agreement for the analysis of the labelled statement of the LPR in the tablet dosage form. It proved selectivity of the developed analytical method without any peak interference in the retention time of the drug.

Thus, the results detailed that the developed HPLC technique was accurate, precise as well as linear. Cost effective solvents and reagents which can be used on routine basis for the analysis in research and industries were employed. The overall results of the method development and validation demonstrated that the current technique can be adopted for the assays, repetitive analysis and quality purposes. Table 4 summarizes all the validation parameters that were studied by RP-HPLC analytical method.

Forced Degradation Study of LPR

Generally the forced degradation studies are performed to recognize possible degradants and to comprehend the stability of the drug molecules under diverse conditions. It is also performed to figure out the possible degradation paths for respective degradants. For this reason, the validated stability indicating assay technique is needed for drug substances and their products. Such studies help to obtain the degradants within lesser time period. LPR was exposed to the different forced conditions like acid, base, oxidative stress, thermal and photolytic conditions. No particular guidelines are mentioned for the conditions like pH, temperature and oxidising agent to perform forced degradation.

The percentage degradation of LPR under these forced conditions was found out by relating the area under the peak with area of the peak of control sample (Table 5). The LPR degradation was ranged between 18.98% to 26%. The LPR showed stability under forced thermal and photolytic conditions as no degradation of LPR was observed. The maximum degradation was observed under H_2O_2 induced oxidative stress conditions. The study was further extended for the characterization of these degradants using mass spectrometric studies deducing m/z ratios. The observation of the individual chromatograms of forced degradation studies reflected that the formation of two impurities each in forced acidic and basic conditions whereas one impurity is formed in the forced oxidative conditions. One same impurity was found in acid and base stressed conditions. On the same line second impurity of base degradation study was observed under stressed oxidative conditions. During the acid hydrolysis study, two degradants were formed and were designated as DP I and DP II (Figure 3B). Their retention time was observed as 2.8 min and 3.2 min respectively. In forced basic degradation studies two degradants were obtained and were denoted as DP I and DP III. Their retention time was observed as 2.8 min and 5.1 min respectively (Figure 3C). Maximum degradation was practical under forced oxidative environments (Figure 3D). The degradant was denoted as DP III with retention time of 5.5 min. Almost

Table 3: Robustness testing for LPR [a] (*n*=3).

Factor[b]	Level	[c]t_R	[d]K	[e]T
Flow Rate (mL/min)				
0.9	-1	6	3.80	0.15
1.0	0	6.1	3.13	0.21
1.1	+1	6	2.85	0.20
Mean ± S.D.		6.03 +/- 0.05	3.26 +/- 0.49	0.19 +/- 0.03
% Acetonitrile in the mobile phase (v/v)				
49	-1	6	3.58	0.39
50	0	6.2	3.63	0.48
51	+1	6	3.43	0.53
Mean ± S.D.		6.06 +/- 0.11	3.55 +/- 0.08	0.47 +/- 0.06

[a]Average of three concentrations 20 µg/mL
[b]Two factors were slightly changed at three levels (-1, 0 and +1)
[c]t_R= retention time (min)
[d]K= retention factor
[e]T= tailing factor

Table 4: Summary of validation parameters of LPR.

Sr. No.	Validation Parameter	Results
		Ledipasvir
1.	Linearity	y = 15762 x + 6477.2 R^2 = 0.998
2.	Range	05-30 µg/ml
3.	Precision	(% RSD)
	Intraday precision	1.923
	Interday precision	1.838
4.	Accuracy	% Recovery (Mean ± % RSD)
	50 %	97.42 +/- 0.004
	100 %	102.56 +/- 0.004
	150 %	99.18 +/- 0.005
5.	LOD	0.010 µg/ml
6.	LOQ	0.032 µg/ml
7.	Specificity	Specific
8.	Robustness	Robust

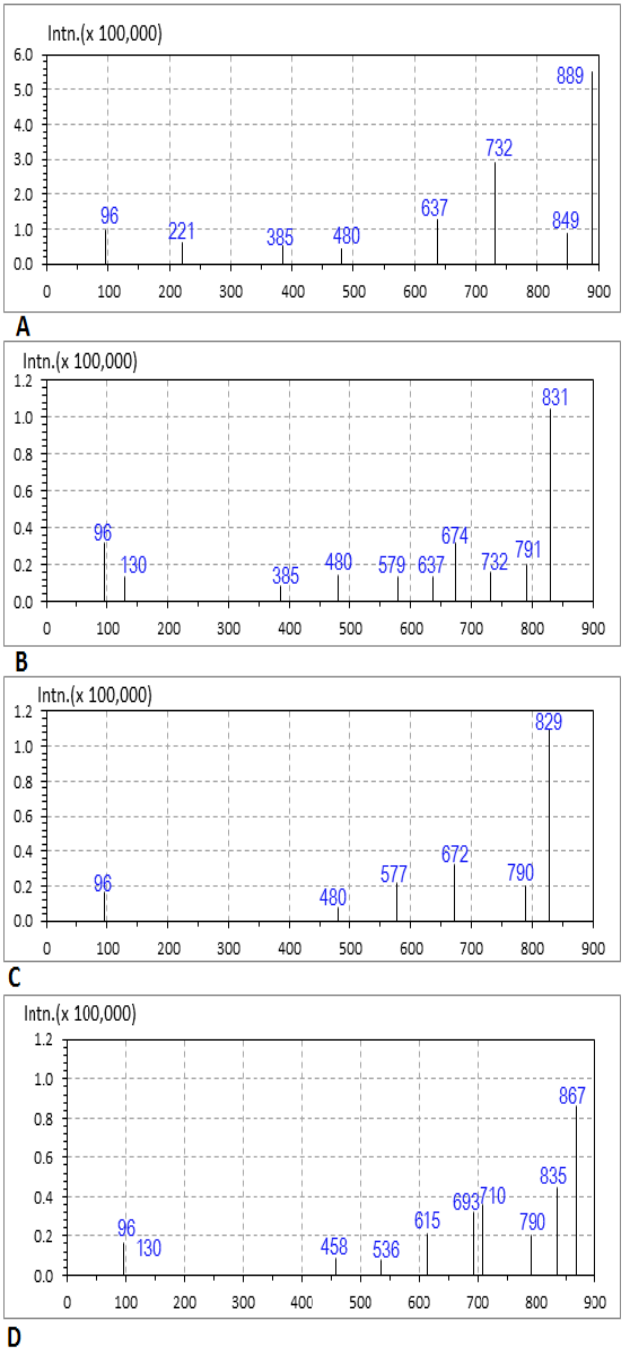

Figure 4: The positive ion mass spectra of (A) LPR (6 min; m/z 890) (B) Degradation product DP – I (2.8 min; m/z 831 amu) (C) Degradation product DP - II (3.2 min; m/z 829 amu) (D) Degradation product DP III (5.1 min; m/z 867 amu) by ESI scanning m/z 0 to 900 amu.

22.43% degradation was practically seen under oxidative environments. No degradation of LPR was practical in thermal and photolytic environments.

LC-ESI-MS Characterization of LPR and its degradants

The analysis of LPR and its degradants was carried out using LC-ESI-MS. The formed degradants under various forced conditions were identified and characterized using mass spectrometry. The structural characterization of these identified degradants was performed based upon obtained mass data and the possible pathway elucidating the mechanism of degradation was sketched out to provide the detailed insight into the degradation mechanism. All the obtained mass spectra are shown in Figure 4.

MS/MS spectra of LPR

LC–ESI–MS positive ion mode was used to perform the analysis. The structure of LPR is shown in Figure 1. The mass spectrum of LPR displayed an abundant $[M+H]^+$ ion at m/z 889 (Figure 4A). The MS/MS spectrum

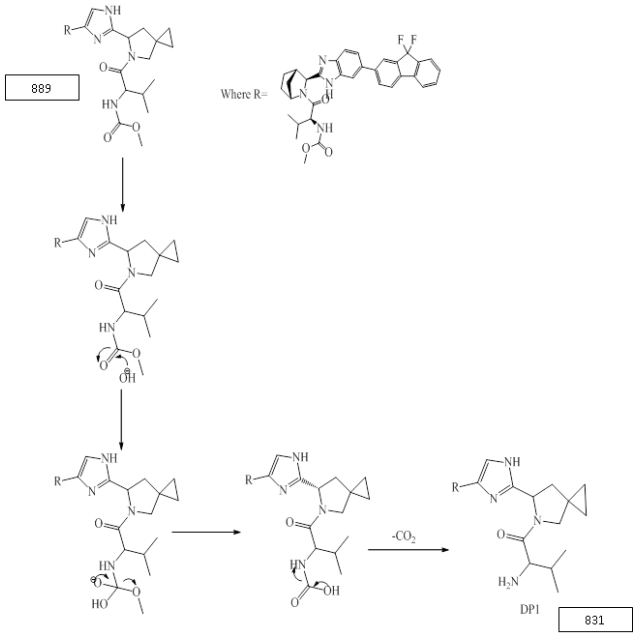

Figure 5: Possible degradation pathway and structure of acid and base degradation product DP I.

Table 5: Summary of forced degradation study results of LPR.

Sr. No.	Condition	Degradation Agent	Percent Degradation	Result
1.	Acid	0.1N HCl, 7 hrs	18.98	Degradation
2.	Alkali	0.1 N NaOH, 7 hrs	26	Degradation
3.	Oxidative	6 % H_2O_2, 24 hrs	22.43	Degradation
4.	Thermal	50°C, 21 days	0	No Degradation
5.	Photolytic	21 days	0	No Degradation

displayed fragment ion peak at *m/z* 849 (loss of two HF molecules), *m/z* 732 (loss of $C_7H_{11}NO$ from *m/z* 849), *m/z* 637 (loss of $C_{13}H_{20}N_2O_3$ from *m/z* 732), *m/z* 480 (loss of $C_{13}H_{20}N_2O_3$ from *m/z* 637), *m/z* 385 (loss of C_6H_9N from *m/z* 480), *m/z* 221 (loss of $C_{24}H_{18}N_4O$ from *m/z* 637*), m/z 96* (obtained from m/z 221).

Structural Interpretation of Degradation Product DP I, DP II and DP III

The mass spectrum of DP I was obtained as degradant when LPR was subjected to the acid and base induced forced degradation (Figure 4B). The $[M + H]^+$ ion at *m/z* 831 which was 59 Da lesser than of LPR was shown in the mass spectrum. It is assumed that the - 59 amu and m/z value of 831 could be due to loss of methyl formate and formation of an amide bond which was observed in both acidic and basic degradation. The degradation pathway is shown in Figure 5. DP I was identified as 9-fluoro-8-(4-hydroxypipe ridin-1-yl)- 5-methyl-6,7-dihydropyrido[3,2,1-ij]quinolin-1(5 H)-one as per the fragmentation pathway.

The mass spectrum of DP II degradant exhibited $[M+H]^+$ ionic peak at *m/z* 829 which was 60Da lesser than the drug when the LPR was treated under forced acidic conditions (Figure 4C). It also showed the peaks at m/z 480 and 96 as exhibited by the drug and DP I in their respective mass spectra. Thus, it indicates the structural similarity between DPI and DPII (Figure 6).

The degradant DP III was obtained as a result of forced base hydrolysis and H_2O_2 induced oxidative degradation. It showed $[M+H]^+$ m/z ionic peak at 867 (Figure 4D). The degradant DP III was formed due to – 22 amu because of loss of H_2O. According to literature this reaction seemed as basis of Reimer Teimer reaction. The discolouration observed to pink might be due to extended conjugation in presence of H_2O_2. Carbamoyl group might also have occurred in the first step as shown in the proposed pathway (Figure 7).

Degradation mechanism of LPR

From the forced degradation studies under the impact of various environments as per ICH guidelines, a degradation mechanism for the LPR has been suggested. LPR was found to form three degradation products under the forced acidic, basic and oxidative conditions. DP I was formed by hydrolysis reaction under the acidic and basic conditions. DP II was formed by the forced acidic conditions as like that of the DP I. DP III was formed by base hydrolysis and oxidative degradation by the mechanism similar to that of Reimer Teimer reaction. The mechanisms and pathways for the formation of DPI, DP II and DP III are sketched out in Figure 5, Figure 6 and Figure 7 respectively.

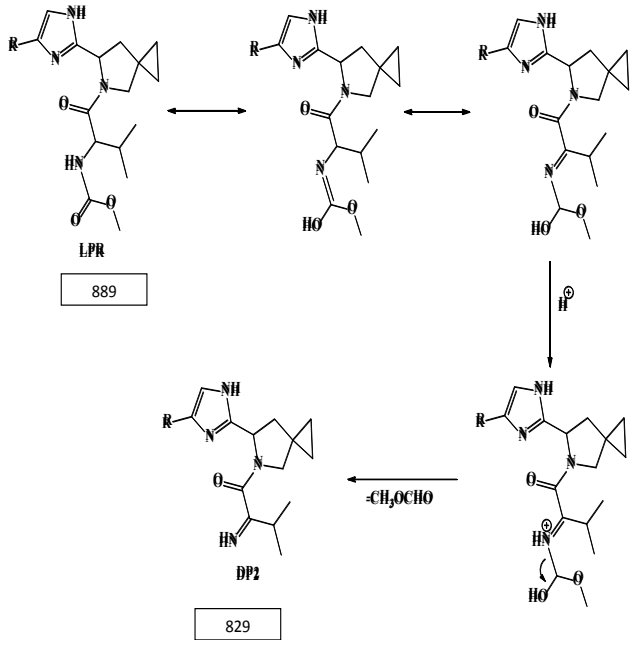

Figure 6: Possible degradation pathway and structure of acid and base degradation product DP II.

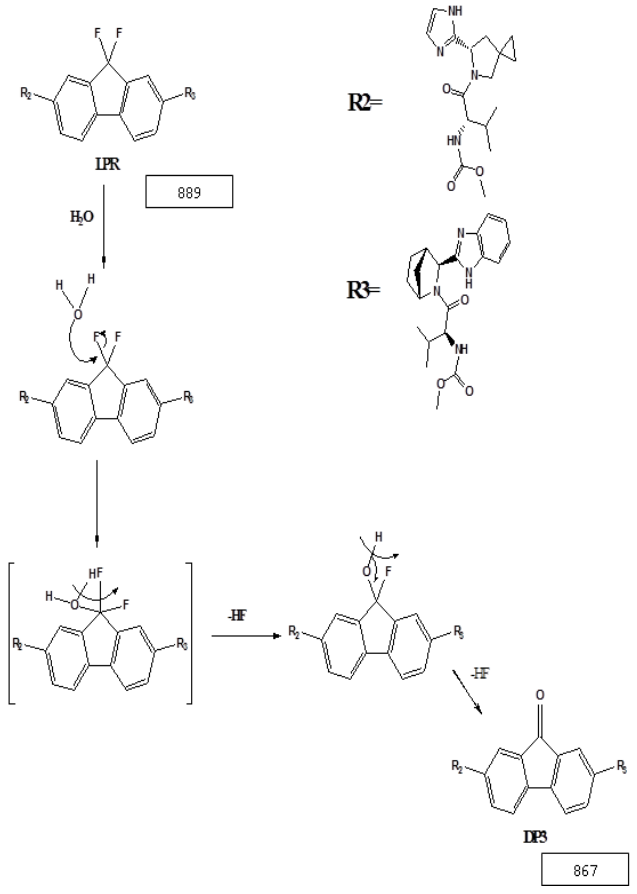

Figure 7: Possible degradation pathway and structure of base and oxidation degradation product DP III.

CONCLUSION

The determination of LPR in bulk and its dosage form was developed and validated for the stability indicating HPLC method. All the standard method validation parameters were determined and were found to be within the standards. Furthermore, this method was utilized for determination and interpretation of products of LPR degradation. The degradation studies were performed under the influence of various forced degradation conditions. The LPR was found to be liable to the acid, basic and oxidative degradation resulting into the formation of three degradants. The drug was observed to be stable in the photolytic and thermal conditions. The developed method was found to be accurate, sensitive and robust exploring its application in qualitative and quantitative determination in variety of analytical purposes. The degradants formed as DP I, DP II and DP III were characterized using LC-ESI-MS technique. With the use of this information, the possible degradation mechanism pathways were proposed for the formation of these degradants.

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CONFLICT OF INTEREST

Authors declare that there is no any conflict of interest.

ABBREVIATIONS

RP-HPLC: high performance liquid chromatography method; **(LC-ESI-MS):** liquid chromatographic–electrospray ionization mass spectrometric method; **LPR:** Ledipasvir; **tR:** retention time; **HCV:** Hepatitis C virus; **DAA:** directly acting antivirals; **ICH:** International Conference on Harmonization

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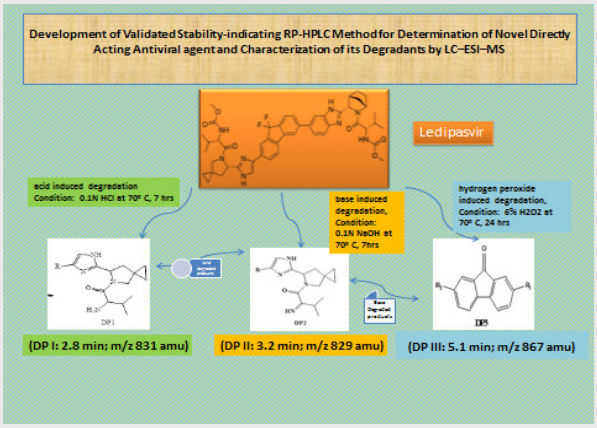
PICTORIAL ABSTRACT



SUMMARY

The accurate and precise determination of the ledipasvir was developed and validated for stability indicating RP-HPLC method. The results of the forced degradation studies identified the stressed conditions in which drug is unstable. Further, Mass Spectrophotometer was used for the identification and characterization of the degradants. The possible structures of the degradants were proposed and the possible degradation pathways were sketched out. Therefore, the current method can be successfully applied for the determination of the drug in bulk and dosage forms. As well as it will find the applications in routine analysis, quality control and research and development.

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