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DEVELOPMENT AND VALIDATION OF STABILITY INDICATING HPTLC METHOD FOR ESTIMATION OF LEDIPASVIR

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ABSTRACT

The present work describes development and validation of a new simple, accurate, precise and selective stability-indicating high performance thin layer chromatographic (HPTLC) method for determination of Ledipasvir as bulk drug and in synthetic mixture. As stability testing is major step with the development of new drug formulation, stress degradation studies were carried as per ICH guidelines. Chromatographic resolution of Ledipasvir and its degradation products was achieved by using precoated, silica gel 60 F-254 aluminium plates as stationary phase and Ethyl acetate: Methanol (9.5:0.5, v/v) as mobile phase. Densitometric scanning was carried out at 334 nm. The retention factor was found to be 0.31 ± 0.02. The developed method was validated with reference to linearity, accuracy, precision, limit of detection, limit of quantitation and robustness as per ICH guidelines. Results found to be linear within the concentration range of 200-1200 ng/band. Ledipasvir was mainly found susceptible to acid, alkali hydrolysis as well as oxidation. The developed method has been successfully applied for the estimation of drug in synthetic mixture.

KEYWORDS: Ledipasvir, HPTLC, Stability indicating, forced degradation, Method Development and Validation.

INTRODUCTION

Ledipasvir (LPV),chemically methyl N[(2S)-1-[(6S)-6-[5-[9,9-difluoro-7-[2[(1S,2S,4R) -3 -[(2S) -2(methoxycarbonylamino)-3methylbutanoyl]-3-azabicyclo[2.2.1]heptan-2-yl]-3H benzimidazol-5-yl]fluoren-2-yl]-1H-Imidazol-2yl]-5-azaspiro[2.4]heptan-5-yl]-3-methyl-1oxobutan-2yl]carbamate[1] (Figure 1) is an orally available inhibitor of the hepatitis C virus

(HCV)-non-structural protein 5A (NS5A) replication complex, with potential activity against HCV. Upon oral administration, and after intracellular uptake, Ledipasvir binds to and blocks the activity of the NS5A protein. This results disruption of the viral RNA replication complex, blockage of HCV RNA production, and inhibition of viral replication. NS5A, a zinc-binding and proline-rich hydrophilic phosphoprotein, plays an important role in HCV RNA replication. HCV could be a small, enveloped, single-stranded RNA virus belonging to the family, Flaviviridae; HCV infection is related to the event of hepatocellular carcinoma.[1] Various methods reported for determination of ledipasvir in combination with other drugs include HPTLC[1], HPLC[2-7], LC-MS/MS[8], chemometric method[9] and Spectrophotometric methods.[10,11] To better of our information, no reports were found for determination of Ledipasvir in synthetic mixture by stability-indicating high performance thin layer chromatographic (HPTLC) method. This paper describes development and validation of easy, precise, accurate and selective stability indicating HPTLC method for determination of ledipasvir in accordance with ICH Guidelines.

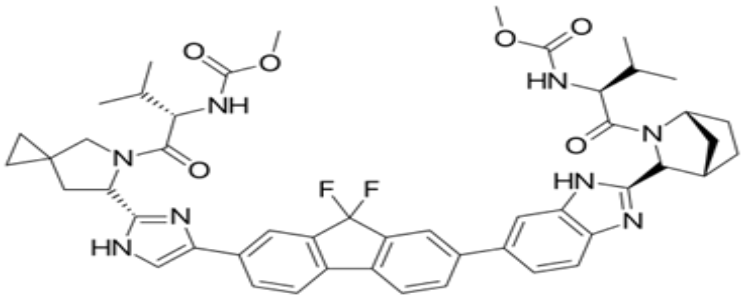

Fig. 1: Chemical structure of Ledipasvir.

MATERIALS AND METHODS

Chemicals and Reagents

Methanol (AR grade), Ethyl acetate (AR grade) was purchased from MERCK, Mumbai. Triethylamine (AR grade), Hydrochloric acid (HCL), Hydrogen peroxide (H_2O_2) and Sodium hydroxide (NaOH); all AR grade were purchased from LobaChemie Pvt, Ltd., Mumbai.

Chromatographic conditions

Chromatographic separation of drug was performed on precoated silica 60 F_{254} (10 cm × 10 cm with 250 µm layer thickness) Merck TLC plates as stationary phase employing a Camag Linomat V sample applicator (Switzerland). Samples were applied on the plate as a band of 6 mm width using Camag 100 µl sample syringe (Hamilton, Switzerland). Linear ascending development was administered in 10 cm x 10 cm twin trough glass chamber

(CAMAG, Muttenz, Switzerland). The mobile phase used was Ethyl acetate: Methanol (9.5:0.5, v/v). The mobile phase was saturated in chamber for 15 min. After development, TLC plates were dried in a current of air with the help of a drier. Densitometric scanning was performed on Camag thin layer chromatography scanner at 333 nm for all developments operated by winCATS software (version 1.4.3). The source of radiation utilized was deuterium lamp emitting a continous UV spectrum between 200 to 400 nm.

Selection of detection wavelength

After chromatographic development bands were scanned over the range of 200-400 nm. It was observed that drug showed considerable absorbance at 334 nm. So, 334 nm was selected as the wavelength for detection of Ledipasvir shown in Figure 2.

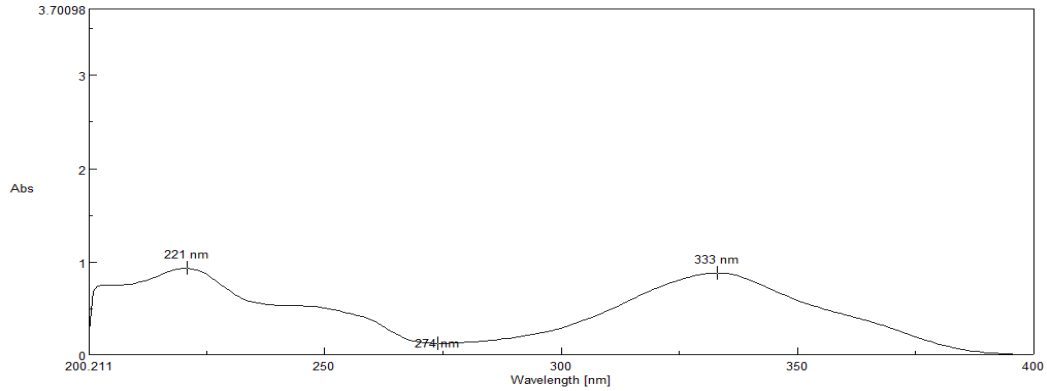

Fig. 2: UV spectrum of Ledipasvir.

Preparation of standard stock solution

Standard stock solution was prepared by dissolving 10 mg of drug in 10 ml of methanol to get working standard solution of concentration 1000 µg/ml. From which 1 ml was further diluted to 10 ml to make solution of 100 µg/ml. 10 µl of this solution was applied and densitgram was developed (Figure 3).

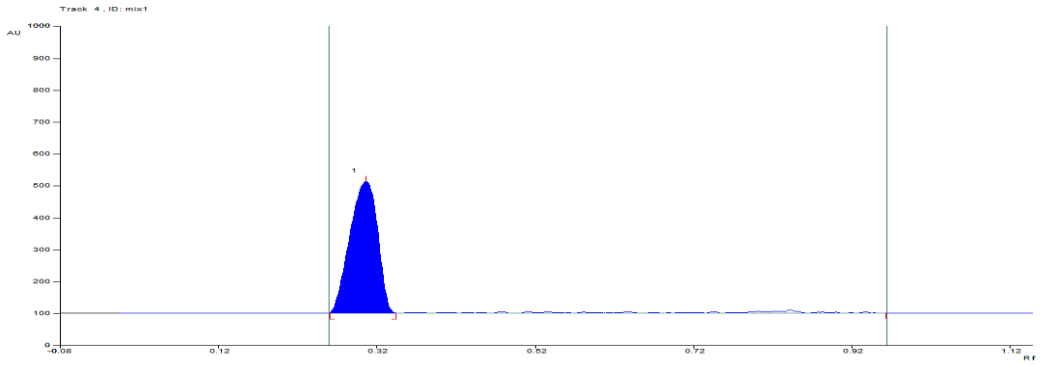

Fig. 3: Represntative densitogram of standard solution of Ledipasvir (Rf = 0.31 ± 0.02).

Force degradation studies[12]

The forced degradation studies on drug Ledipasvir was as per ICH guidelines. Ledipasvir was subjected to acid and base hydrolysis, oxidation, thermal, photo-degradation studies. Ledipasvir solution containing 10 mg/10 ml of methanol was prepared as the stock solution stock (A), which was further used for stress degradation studies in Table 1.

Table 1: Results of Forced Degradation Study.

Stress conditions/ duration	% Recovered	% Degradation
Acidic / 2 N HCl/ Heat at 80°C for 10 min	28.50	71.49
Alkaline / 0.1 N NaOH/ Heat at 80°C for 10 min	29.40	70.60
Oxidative / 30 % H_2O_2 / Heat at 80°C for 1 h	51.74	48.26
Dry heat/ 80ºC/ 4 h	88.09	11.91
UV light 200 watt h/m^2	81.11	18.89
Fluorescence (1.2 million lux hours/square meter)	88.68	11.32

Acid degradation studies

1 ml of stock (A) together with 1 ml of 2N HCl was heated for 10min at 80°C, neutralized with 2N NaOH then volume was made up with methanol to 10 ml to give (100 µg/ml). 10 µl of the resultant solution was then applied at TLC plate. Average 28.50 % of Ledipasvir was recovered.

Alkaline degradation studies

1 ml of stock (A) together with 1 ml of 0.1N NaOH was heated for 10 min at 80°C, neutralized with 0.1 N HCl then was made up with methanol to 10 ml to give (100 µg/ml). 10 µl of the resultant solution was then applied at TLC plate and Average 29.40 % of Ledipasvir was recovered.

Oxidative degradation studies

1 ml of stock (A) along with 1 ml of 30% H_2O_2 was heated for 1 h at 80°C, cool then volume was made up with methanol to 10 ml to give (100 µg/ml). 10 µl of the resultant solution was then applied at TLC plate and Average 51.74 % of Ledipasvir was recovered.

Dry heat degradation studies

Dry heat studies were performed by keeping drug sample in oven 80°C for a period of 4 h. Sample was withdrawn, dissolved in methanol and diluted to get 100 µg/ml. 10 µl of the resultant solution was then applied at TLC plate and Average 88.09 % of Ledipasvir was recovered.

Photo-degradation studies

The photo degradation stability study of the drug was studied by exposing the drug to fluorescence (1.2 million lux hours/square meter and 200 watt hours/square meter) and UV (200 watt h/m^2). After exposure accurately weighed 10 mg of drug was transferred to 10 ml of volumetric flask; the amount was made up with methanol to acquire 1000 µg/ml solution. 1ml of the resultant solution was then diluted with methanol to acquire the concentration of 100 µg/ml. 10 µl of the resultant solution was then applied at TLC plate and Average for photolytic UV 81.11 % while photolytic fluorescence 88.68 % was recovered.

RESULTS AND DISCUSSION

Method Development and Optimization

Experimental parameters, like mobile phase composition, detection wavelength and scan mode were optimized so as to get precise, accurate and reproducible results. Various solvent systems in varying proportions were tried as mobile phase and Ethyl acetate: Methanol (9.5:0.5, v/v) showed improved resolution of Ledipasvir (R_f: 0.31). Wavelength was selected based on maximum absorbance for optimum sensitivity.

Validation of Analytical Method[13]

The method was validated as per ICH guidelines.

Specificity

The specificity of the method was confirmed by peak purity profile studies. The peak purity values were found to be more than 0.998, indicating that no interference of the other peak of degradation product, impurity or matrix.

Linearity and Range

A solution containing (100 µg/ml) was prepared from standard stock solution 1000 µg/ml of Ledipasvir. This solution was further used for spotting. Six replicates per concentration were spotted. The linearity (relationship between peak area and concentration) was calculated through the study of six concentrations over the 200-1200 ng/band concentration range. The result was found to linear with regression equation of Y= 10.591 x + 5220.6 and R^2 = 0.994. The obtained calibration curve shown in Figure 4.

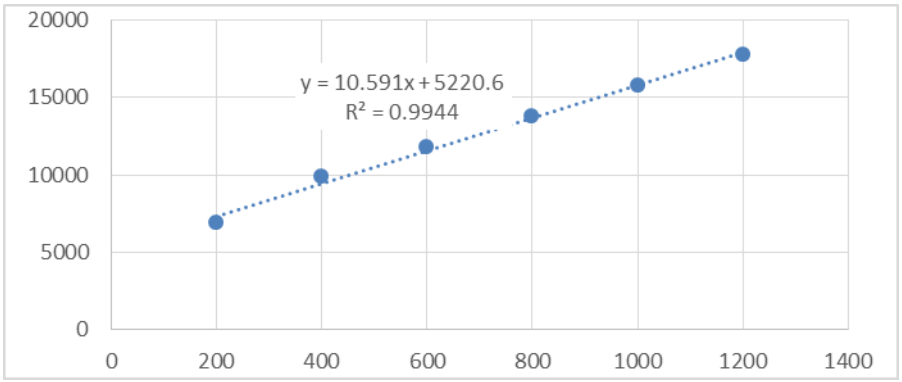

Fig. 4: Calibration curve of Ledipasvir (200-1200 ng/band) reference standard.

Precision

The precision of the process was demonstrated by intra-day and inter-day variation studies. Within the Intra-day studies 3 replicates of 3 concentrations were analyzed on the same day, and percentage RSD was calculated. For the inter-day variation studies, 3 concentrations were analyzed on 3 consecutive days and percentage RSD were calculated. For Intra-day and Inter-day precision results obtained are shown in Table 2.

Table 2: Intraday and Interday Variation Studies data for Ledipasvir.

Concentration (µg/ml)	Intra-day precision			Inter-day precision		
	Area	% recovery	% RSD	Area	% recovery	% RSD
600	11534.9	99.36	1.88	11467.9	98.31	1.75
	11765.9	102.01		11689.0	101.80	
	11589.7	100.22		11564.5	99.83	
800	13953.8	102.73	0.99	13781.6	101.04	1.09
	13866.6	102.04		13855.7	101.91	
	13781.6	101.04		13968.8	102.25	
1000	15688.5	98.83	1.34	15899.9	100.83	0.71
	15961.8	101.41		15752.6	99.44	
	15899.9	100.83		15855.0	100.40	

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

The LOD and LOQ of the method were developed using regression equation. A series of standard preparation containing 200-1200 ng/band were prepared over different levels. Calibration graphs were plotted for the obtained area under curve of each level against the concentration. The LOD and LOQ were calculated using equations LOD=3.3xσ/S, LOQ= 10xσ/S, where σ is the standard deviation of y-intercept and S is the slope of calibration curve. The LOD and LOQ were found to be 40.42 ng/band and 122.51 ng/band, respectively.

Assay

Synthetic mixture was prepared by mixing 100 mg of Ledipasvir with 200 mg lactose monohydrate and 200 mg cellulose microcrystalline. Blended and mixed in motor pestle. From this 50 mg of blend that was equivalent to 10 mg of drug was diluted to 10 ml with methanol (1000 µg/ml). Sonicated, filter and 1 ml of filtrate diluted to 10 ml with methanol (100 µg/ml). 4 µl volume of sample solution was applied and area was recorded. Concentration and % recovery determined from linear equation. Assay results obtained are presented in Table 3.

Table 3: Assay of Ledipasvir through Synthetic Mixture.

Sr. No.	Peak area	Amount recovered (µg/ml)	% Recovery	Mean ± % RSD
1	5329.8	411.42	102.86	101.72 ± 0.86
2	5288.8	407.77	101.94	
3	5282.3	407.19	101.80	
4	5256.8	404.92	101.23	
5	5214.9	401.20	100.30	
6	5301.5	408.90	102.23	

Accuracy (Recovery study)

To check accuracy of the method, recovery studies were carried by spiking the standard drug to the blend (synthetic mixture), at three different levels 50, 100 and 150 %. Basic concentration of sample chosen was 400 ng/band. Percentage recovery was determined from linearity equation. Accuracy results obtained are shown in Table 4.

Table 4: Results of Accuracy (Recovery Study)

Level	Amount taken (ng/band)	Amount added (ng/band)	Amount recovered (µg/ml)	% Recovery ± R.S.D*
50	400	200	609.679	101.61 ± 0.83
100	400	400	785.708	98.21 ± 0.86
150	400	600	998.479	99.84 ± 0.71

*Average of three determinations.

Robustnes

Robustness of the method was determined by carrying out the analysis under conditions during which detection wavelength (± 1 nm), chamber saturation time (± 2 min) were altered, mobile phase composition (± 0.2 ml), time from application to development (0,30,60,90 min), time from development to scanning (0,30,60,90 min) was also altered and the effect on the area were noted. As the results are within limit (%RSD <2); method found to be robust.

CONCLUSION

A simple, precise, accurate, reproducible and stability indicating HPTLC method was developed. The method did not show interference from the excipients or from degradation products and validated for the determination of Ledipasvir in synthetic mixture. This method can be used for quantitative analysis of Ledipasvir in pharmaceutical dosage form. The method was developed by using easily available solvents for analysis of drug hence can be considered as economic. Although no degradation product observed but Ledipasvir was mainly found susceptible to acid, alkali hydrolysis as well as oxidation.

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