DEVELOPMENT AND VALIDATION OF STABILITY-INDICATING REVERSE-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY METHOD FOR THE ESTIMATION OF LEDIPASVIR IN BULK AND TABLET DOSAGE FORM

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ABSTRACT

Objective: The objective of this study was to develop a stability-indicating reverse-phase high-performance liquid chromatography (RP-HPLC) method for the estimation of ledipasvir (LDP) in bulk and tablet formulation.

Methods: Stability-indicating RP-HPLC method was developed and validated for the estimation of LDP in bulk and tablet formulation. RP-HPLC was carried out on HiQ Sil C18 columns (250 mm × 4.6 mm, 5 µ particle size) using mobile phase acetonitrile:1 mM ammonium acetate buffer in the ratio of 90:10 v/v at a flow rate of 1 ml/min. The analytes were monitored using MD 2010 PDA detector at 333 nm.

Results: The retention time was found to be 3.843 min. The proposed method was found to be having linearity in the concentration range of 5–30 µg/ml. The number of theoretical plates obtained was 4236.50 which indicate the efficient performance of the column. The limit of detection was 0.305 µg/ml and limit of quantification was 0.923 µg/ml, which indicate the sensitivity of the method; the high percentage recovery indicates that the proposed method is highly accurate. The developed method has been validated according to the ICH guidelines and found to be simple, specific, precise, and accurate.

Conclusion: The proposed method is precise, accurate, and stability indicating. Therefore, the proposed method can be used for routine quality control and analysis of LDP during stability studies in bulk samples and tablet dosage forms.

Keywords: Method development, Stability indicating, Reverse-phase high-performance liquid chromatography, Ledipasvir, ICH.

INTRODUCTION

Ledipasvir (LDP) is chemically methyl N-[(2S)-1-[(6S)-6-[(9,9-difuoro-7-[(2S,2S,AR)-3-[(methoxy carbonylamino)-3-methylbutan-2-y])]-3-aza bicyclo[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. It is a potent inhibitor of HCV NS5A, a viral phosphoprotein that plays an important role in viral replication, assembly, and secretion. LDP in combination with sofosbuvir is used for the treatment of chronic hepatitis C, genotypes 1-6, usually in combination with other medications depending on the specific genotype [1,2]. A literature review revealed few spectrophotometric, reverse-phase high-performance liquid chromatography (RP-HPLC), stability-indicating RP-HPLC, and UPLC-ESI MS/MS [3-13] methods for the estimation of LDP. The purpose of this work was to develop a simple basic rapid and economic stability-indicating RP-HPLC method for the determination of LDP in its bulk and pharmaceutical dosage form so as to provide better scope for further research on the drug (Fig. 1).

METHODS

Chemical and reagents

The reagents used in this work were methanol (HPLC grade), ammonium acetate (AR) Grade, HCl (AR), NaOH (AR), and hydrogen peroxide (3%, v/v) (AR), which were procured from Merck, India. Distilled water (HPLC grade) was also used. LDP API was procured from Cipla.

Equipment

The instruments used in the study were HPLC (Jasco, PU 2080 Plus pump) integrated using Empower software, Photostability Chamber (Newtronic), Hot Air Oven (Kumar Laboratory Oven), electronic balance (Shimadzu balance), sonicator, digital pH meter, and calibrated glasswares.

Preparation of standard stock solution

Standard stock solution of LDP was prepared by dissolving 10 mg of drug in 10 ml of acetonitrile to get a concentration of 1000 µg/ml. From the standard stock solution, 1 ml was further diluted to 10 ml with mobile phase to get 100 µg/ml solution of LDP.

Analysis of formulations

Blend equivalent to 90 mg of LDP was prepared to satisfy dose/tablet of marketed formulation. Blend containing 90 mg LDP was prepared by spiking drug into excipients (20 mg starch, 4 mg magnesium stearate and lactose [qs upto 200 mg]). Mixing was done by geometric addition method.

Preparation of test solutions

Blend equivalent to 10 mg of LDP was weighed and transferred to 10 ml volumetric flask and was diluted with methanol. It was sonicated for 10 min and filtered so as to get solution having a concentration of 1000 µg/ml. 1 ml of filtrate was further diluted with methanol, of which 1 ml was diluted with mobile phase to get the final concentration of 10 µg/ml LDP. Six determinations were carried out from homogenous sample to determine percentage assay.

Preparation of mobile phase

Mobile phase was prepared by mixing acetonitrile and 1 mM ammonium acetate buffer in the ratio of 90:10 v/v. It was then filtered through 0.45-µm membrane filter paper using filtration assembly and then sonicated on ultrasonic water bath for 10 min.
HPLC method development
After trying several permutation and combinations, it was found that mixture of acetonitrile and 1 mM ammonium acetate buffer in the ratio of 90:10 v/v gave a sharp peak of LDP and this system was optimized. It was observed that the developed chromatographic condition provides better separation of LDP (3.843 min) as shown in Fig. 2.

Stress degradation studies of bulk drug [14]
Degradation studies of the drug substance can help to identify the likely degradation products which can assist to establish the degradation pathways and the stability of the molecule. LDP was subjected under different condition of acid, base, neutral hydrolysis, oxidation, dry heat, and photolysis. For each study, two samples were prepared (blank and of LDP drug solution). The blank subjected to stress in the same manner as the drug solution. Dry heat and photolytic degradation were carried out in a solid state.

API degradation
Alkaline hydrolysis
About 1 ml working standard solution of LDP (1000 µg/ml) was mixed with 1 ml of 1 N methanolic NaOH and 8 ml of methanol. The solution was kept for 24 h in dark place. The resulting solution was neutralised and 2 ml was diluted with mobile phase to 10 ml and was injected (20 µg/ml). The chromatogram of LDP after alkaline hydrolysis shows 68.49% recovery, RT 3.849, and RT of degradant 5.91 and 6.58 (Fig. 3).

Acidic hydrolysis
About 1 ml working standard solution of LDP (1000 µg/ml) was mixed with 1 ml of 1 N methanolic HCl and 8 ml of methanol. The solution was kept for 24 h in the dark place. The resulting solution was neutralised, and 2 ml was diluted with mobile phase to 10 ml and was injected (20 µg/ml). The chromatogram of LDP after acid degradation shows 94.175% recovery, RT 3.84, and RT of degradant 6.54 (Fig. 4).

Neutral hydrolysis
About 1 ml working standard solution of LDP (1000 µg/ml) was mixed with 1 ml of distilled water and 8 ml of methanol. The solution was kept for 24 h in the dark place. 2 ml of the resulting solution was diluted with mobile phase to 10 ml and was injected (20 µg/ml). The chromatogram of LDP after neutral hydrolysis shows 87.38% recovery, RT 3.84, and RT of degradant 2.52 and 3.04 (Fig. 5).

Fig. 1: Structure of ledipasvir

Fig. 2: Optimized chromatogram of standard ledipasvir (10 µg/ml)

Fig. 3: Chromatogram of blank NaOH and ledipasvir (20 µg/ml) after alkaline hydrolysis
Oxidation
About 1 ml standard solution of LDP (1000 µg/ml) was mixed with 1 ml of 30% H₂O₂ solution and 8 ml of methanol. The solution was kept for 24 h in the dark place. 2 ml of the resulting solution was diluted with mobile phase to 10 ml and was injected (20 µg/ml). The chromatogram of LDP after oxidation shows 97.07% recovery, RT 3.84, and RT of degradant 5.93 (Fig. 6).

Degradation under dry heat
Dry heat studies were performed by keeping drug sample in oven (80°C) for 24 h. Sample was withdrawn after 24 h and processed as per standard solution preparation procedure mentioned under 1.5 to get 20 µg/ml as final concentration and was injected. The chromatogram of LDP after exposing to dry heat shows 99.781% recovery and RT 3.84 (Fig. 7).

Fig. 4: Chromatogram of blank HCl and ledipasvir (20 µg/ml) after acid degradation

Fig. 5: Chromatogram of blank and ledipasvir (20 µg/ml) after neutral hydrolysis

Fig. 6: Chromatogram of blank H₂O₂ and ledipasvir (20 µg/ml) after oxidation
Photodegradation studies
Photolytic studies were carried out by exposure of drug to UV light up to 200 watt-h/m² and subsequently to cool fluorescent light to achieve an illumination of 1.2 million Lux-Hr. A sample was withdrawn after exposure and processed as per standard solution preparation procedure mentioned under 1.5 to get 20 µg/ml as final concentration and was injected. The chromatogram of LDP after photodegradation shows 98.71% recovery (Fig. 8).

RESULTS AND DISCUSSION

Linearity and range
From the standard stock solution (100 µg/ml) of LDP further dilutions were made with mobile phase to obtain a range of solution containing six different concentrations. Five replicates per concentration were injected. The linearity (relationship between peak area and concentration) was determined over the concentration range of 5–30 µg/ml as shown in Table 1.

Precision
The precision of the method was demonstrated by intra- and interday variation studies. In the intraday studies, three replicates of three different concentrations [10, 20, and 30 µg/ml] of LDP were analyzed in a day, and percentage RSD was calculated. For the interday variation studies, three replicates of different concentrations were analyzed on 3 consecutive days and percentage RSD was calculated as shown in Table 2.

Accuracy
To check the accuracy of the method, recovery studies were carried out by adding standard drug to sample at three different levels 50,

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**Table 1: Linearity study of LDP**

<table>
<thead>
<tr>
<th>Replicates</th>
<th>Concentrations of LDP</th>
<th>Peak area</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>5 µg/ml</td>
<td>10 µg/ml</td>
</tr>
<tr>
<td>1</td>
<td>151972.1</td>
<td>255900.9</td>
</tr>
<tr>
<td>2</td>
<td>149580.2</td>
<td>255500.5</td>
</tr>
<tr>
<td>3</td>
<td>150306.9</td>
<td>253866.2</td>
</tr>
<tr>
<td>4</td>
<td>151639.5</td>
<td>256245.6</td>
</tr>
<tr>
<td>5</td>
<td>150123.1</td>
<td>253683.1</td>
</tr>
<tr>
<td>Mean</td>
<td>150724.4</td>
<td>255457.3</td>
</tr>
<tr>
<td>SD</td>
<td>1029.481</td>
<td>1027.496</td>
</tr>
<tr>
<td>%RSD</td>
<td>0.683</td>
<td>0.402</td>
</tr>
</tbody>
</table>
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100, and 150%. Basic concentration of sample chosen was 10 µg/ml of LDP from tablet solution. These solutions were injected in stabilized chromatographic conditions in triplicate to obtain the chromatograms. The drug concentrations of LDP were calculated using linearity equation of LDP as shown in Table 3.

Limit of detection (LOD)
LOD is calculated from the following formula:

\[
LOD = 3.3 \sigma/S
\]

Where,
- \(\sigma\) = standard deviation of response for the lowest concentration in the range.
- \(S\) = slope of the calibration curve.

Limit of quantification (LOQ)
The quantitation limit is expressed as follows:

\[
LOQ = 10 \sigma/S
\]

Where,
- \(\sigma\) = standard deviation of response for the lowest concentration in the range.
- \(S\) = slope of the calibration curve.

Robustness
Robustness of the method was determined by carrying out the analysis under conditions during which flow rate and wavelengths were altered and the effects on the area were noted as indicated in Table 4. The summary of validation parameters is included in Table 5.

DISCUSSION
Stability-indicating RP-HPLC method for the determination of LDP was developed. Linearity for LDP was found in the range of 5–30 µg/ml with regression coefficient \((r^2) = 0.999\). LOD and LOQ values were 0.305 µg/ml and 0.923 µg/ml, respectively. The RSD values for intra- and inter-day precision studies were found to be <2%.

Degradation of LDP was found to occur under acidic condition (1N HCl, 24 h), alkaline condition (1N NaOH, 24 h), oxidative condition (30% \(H_2O_2\), 24 h), and neutral (24 h) stress. LDP was considerably stable in dry heat and photostability stress testing.

### Table 2: Precision study of LDP

<table>
<thead>
<tr>
<th>Concentration(µg/ml)</th>
<th>% Recovery</th>
<th>SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>98.611</td>
<td>0.236</td>
</tr>
<tr>
<td>20</td>
<td>98.544</td>
<td>0.257</td>
</tr>
<tr>
<td>30</td>
<td>101.023</td>
<td>1.273</td>
</tr>
</tbody>
</table>

### Table 3: Recovery study of LDP

<table>
<thead>
<tr>
<th>Level (%)</th>
<th>Concentration (µg/ml)</th>
<th>Area</th>
<th>% Recovery</th>
<th>Mean</th>
<th>% RSD</th>
</tr>
</thead>
<tbody>
<tr>
<td>50</td>
<td>10</td>
<td>369546</td>
<td>100.043</td>
<td>99.591</td>
<td>0.401</td>
</tr>
<tr>
<td>100</td>
<td>10</td>
<td>366987</td>
<td>99.287</td>
<td>98.977</td>
<td>0.469</td>
</tr>
<tr>
<td>150</td>
<td>10</td>
<td>589254</td>
<td>98.969</td>
<td>98.945</td>
<td>0.461</td>
</tr>
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</table>

### Table 4: Robustness study of LDP

<table>
<thead>
<tr>
<th>Drug</th>
<th>% RSD found for robustness study</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Flow rate (1 ml/min)</td>
</tr>
<tr>
<td>LDP</td>
<td>0.9</td>
</tr>
<tr>
<td></td>
<td>0.269</td>
</tr>
<tr>
<td></td>
<td>0.314</td>
</tr>
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</table>

### Table 5: Summary of validation study

<table>
<thead>
<tr>
<th>Validation parameter</th>
<th>LDP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Linearity equation</td>
<td>(y = 22567x + 30895)</td>
</tr>
<tr>
<td>Range</td>
<td>5-30 µg/ml</td>
</tr>
<tr>
<td>Assay (mean ± % RSD)</td>
<td>98.58±0.628</td>
</tr>
<tr>
<td>Accuracy (%)</td>
<td>50 99.59±0.401</td>
</tr>
<tr>
<td>100</td>
<td>98.97±0.469</td>
</tr>
<tr>
<td>150</td>
<td>98.94±0.461</td>
</tr>
<tr>
<td>LOD</td>
<td>0.305</td>
</tr>
<tr>
<td>LOQ</td>
<td>0.923</td>
</tr>
</tbody>
</table>

CONCLUSION
In the present work, stability-indicating RP-HPLC method for the estimation of LDP was developed and validated as per the ICH guidelines. The standard deviation and % RSD (<2%) are within limit, indicating a high degree of precision of the methods.

From the above discussion, it can be concluded that the proposed method is precise, accurate, and stability indicating. Therefore, the proposed method can be used for routine quality control and analysis of LDP during stability studies in bulk samples and in tablet dosage forms.

ACKNOWLEDGMENT
The authors are thankful to the management of STES and faculty of SKNCOP for support and M/s Cipla Ltd., Mumbai, for providing gift sample of LDP.

AUTHORS’ CONTRIBUTIONS
The work was carried out by Amoldeep Shinde, under the guidance of Dr. Mrs. Minal Ghante and Dr. Sanjay Sawant. Both, Dr. Vandana Nikam...
and Mrs. Shital Godse have contributed in writing the manuscript and proofreading of the same.

CONFLICTS OF INTEREST
The authors do not have any conflicts with any organization.

REFERENCES