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STABILITY INDICATED SPECTROPHOTOMETRIC AND RP-HPLC METHOD FOR THE ESTIMATION OF LEDIPASVIR BULK AND FORMULATION

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ABSTRACT
Ledipasvir is a potent direct-acting antiviral agent widely employed in combination therapy for chronic hepatitis C virus infection. Accurate analytical methods are essential for routine quality control, stability evaluation, and regulatory compliance. Although several chromatographic methods have been reported, there remains a need for a rapid, economical, and stability-indicating RP-HPLC method suitable for routine pharmaceutical analysis. The thesis underlying this manuscript describes analytical method development and validation in this context. The present investigation aimed to develop and validate a stability-indicating reversed-phase high-performance liquid chromatographic (RP-HPLC) method for the quantitative estimation of Ledipasvir in bulk drug and pharmaceutical dosage forms following International Council for Harmonisation (ICH) guidelines. Chromatographic separation was achieved using a C18 reversed-phase analytical column under optimized chromatographic conditions. Method validation included specificity, linearity, accuracy, precision, robustness, ruggedness, limit of detection (LOD), limit of quantification (LOQ), and forced degradation under acidic, alkaline, oxidative, thermal, and photolytic conditions. The experimental workflow follows the thesis methodology for method development, validation, and degradation studies. The validated RP-HPLC method represents a simple, economical, precise, and reliable analytical procedure suitable for routine quality control analysis and stability testing of Ledipasvir pharmaceutical formulations.

KEYWORDS- *Ledipasvir, RP-HPLC, Method Validation, Stability-Indicating Method, ICH Q2(R2), Quality Control.*

1. INTRODUCTION

Chronic hepatitis C virus (HCV) infection remains one of the major causes of liver-related morbidity and mortality worldwide, affecting millions of individuals and contributing substantially to the global burden of chronic liver disease. Persistent HCV infection may progress to hepatic fibrosis, cirrhosis, hepatocellular carcinoma, and liver failure if left untreated. The introduction of direct-acting antiviral (DAA) agents has transformed the therapeutic landscape of hepatitis C by providing cure rates exceeding 95%, shorter treatment duration, and improved safety compared with interferon-based regimens.

Among the currently available DAAs, Ledipasvir has emerged as one of the most effective NS5A inhibitors and is extensively administered in fixed-dose combination with Sofosbuvir. The combination effectively inhibits viral replication by simultaneously targeting two independent stages of the HCV replication cycle, thereby minimizing resistance development while maximizing sustained virological response.

Because Ledipasvir is widely used in pharmaceutical formulations, accurate quantitative estimation throughout manufacturing and storage is essential. Analytical methods used for pharmaceutical quality control must accurately determine the active pharmaceutical ingredient while distinguishing degradation products generated during storage or stress conditions. Stability-indicating analytical methods therefore constitute an indispensable component of pharmaceutical quality assurance.

Forced degradation studies further enhance analytical reliability by demonstrating the ability of the method to resolve the intact drug from degradation products generated under acidic, alkaline, oxidative, thermal, hydrolytic, and photolytic stress. Such studies are indispensable for establishing the stability-indicating nature of chromatographic methods and are recommended by ICH guidance. The thesis similarly incorporates multiple degradation pathways as part of the validation strategy.

Accordingly, the objective of the present study was to develop, optimize, and validate a simple, accurate, precise, economical, and stability-indicating RP-HPLC method for the determination of Ledipasvir in bulk drug and pharmaceutical dosage forms in accordance with ICH recommendations. The resulting method is intended to support routine quality control, stability assessment, and regulatory compliance within pharmaceutical laboratories.

2. MATERIALS AND METHODS

2.1 Chemicals and Reagents

Ledipasvir working standard was obtained from a certified pharmaceutical manufacturer and used as the reference standard for analytical method development. HPLC-grade acetonitrile, methanol, purified water, potassium dihydrogen phosphate (KH_2PO_4), orthophosphoric acid (OPA), and other analytical-grade reagents were employed throughout the investigation. All solvents were filtered through a 0.45 µm membrane filter and degassed by ultrasonication before chromatographic analysis. The thesis specifies the analytical reagents and solvent grades used during method development.

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2.2 Instrumentation

Chromatographic analysis was performed using a Waters HPLC system equipped with an isocratic pump, Rheodyne injector fitted with a 20 µL sample loop, UV–Visible detector, and data acquisition software. Separation was achieved using a C18 reversed-phase analytical column (250 × 4.6 mm, 5 µm particle size).

Auxiliary instruments included:

- UV–Visible spectrophotometer
- Digital analytical balance
- Ultrasonic bath
- Milli-Q water purification system
- pH meter
- Vacuum filtration assembly

The instrumentation corresponds to that described in the thesis experimental section.

2.3 Chromatographic Conditions

Chromatographic conditions were optimized after evaluating several mobile-phase compositions and chromatographic variables. The optimized separation was achieved using:

Parameter	Condition
Column	C18 (250 × 4.6 mm, 5 µm)
Mobile Phase	20 mM KH_2PO_4 (pH 3.5 adjusted with OPA):Acetonitrile (80:20, v/v)
Flow Rate	1.0 mL/min
Detection Wavelength	264 nm
Injection Volume	20 µL
Elution Mode	Isocratic
Column Temperature	Ambient
Run Time	Approximately 10 minutes

The mobile phase was filtered through a 0.45 µm membrane filter and degassed before use to ensure reproducible chromatographic performance. These optimized conditions are derived from the method development described in the thesis.

2.4 Preparation of Standard Solution

An accurately weighed quantity of Ledipasvir reference standard (10 mg) was transferred into a 10 mL volumetric flask and dissolved in the optimized mobile phase to obtain a stock solution of 1000 µg/mL.

Working standard solutions were prepared by serial dilution of the stock solution with the mobile phase to obtain concentrations suitable for calibration and validation studies.

Calibration standards were prepared over the concentration range of: **5–25 µg/mL**

Each concentration was injected in triplicate.

The calibration procedure follows the experimental workflow presented in the thesis.

2.5 Preparation of Sample Solution

Commercial tablet formulations equivalent to 10 mg of Ledipasvir were accurately weighed and transferred into a volumetric flask containing the mobile phase.

The mixture was sonicated for 20 minutes to ensure complete extraction of the drug from the dosage form.

After filtration through Whatman filter paper, suitable dilutions were prepared using the mobile phase to obtain the final analytical concentration.

Each formulation was analyzed six times for assay determination.

The sample preparation procedure is based on the thesis assay methodology.

2.6 Method Validation

Validation of the developed RP-HPLC method was carried out according to International Council for Harmonisation (ICH) Q2 recommendations. The following analytical performance characteristics were evaluated:

- System suitability
- Specificity
- Linearity
- Accuracy
- Precision
- Intermediate precision
- Ruggedness

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- Robustness
- Limit of Detection (LOD)
- Limit of Quantification (LOQ)

Validation acceptance criteria complied with internationally accepted analytical standards. The thesis also adopts this validation framework.

2.6.1 System Suitability

System suitability testing was performed before sample analysis.
The following chromatographic parameters were evaluated:

- Retention time
- Peak symmetry
- Tailing factor
- Number of theoretical plates
- Peak area reproducibility

Acceptance criteria were established according to ICH recommendations.

2.6.2 Linearity

Linearity was evaluated using five calibration concentrations ranging from **5–25 µg/mL**
Each concentration was injected three times.
Calibration curves were constructed by plotting peak area against concentration.
Linear regression analysis was performed to obtain

- Regression equation
- Correlation coefficient (R^2)
- Slope
- Intercept

2.6.3 Accuracy

Accuracy was determined by standard addition at three concentration levels:

- 80%
- 100%
- 120%

Recovery percentages were calculated using pre-analyzed pharmaceutical formulations.

2.6.4 Precision

Precision was evaluated as
Repeatability
Three replicate injections of each concentration were analyzed during the same day.
Intermediate Precision
Method reproducibility was assessed by

- Different analysts
- Different days

Relative standard deviation (%RSD) values were calculated.

2.6.5 Robustness

Method robustness was evaluated by introducing deliberate variations in chromatographic parameters, including:

- Mobile-phase composition
- Flow rate
- Mobile-phase pH

Chromatographic performance was assessed following each variation.

2.6.6 LOD and LOQ

Limits of detection and quantification were calculated using the standard deviation of the response and the slope of the calibration curve.

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The thesis reports:

Parameter	Value
LOD	0.53 µg/mL
LOQ	1.50 µg/mL

2.7 Forced Degradation Studies

To establish the stability-indicating capability of the developed RP-HPLC method, Ledipasvir samples were subjected to various stress conditions.

Acidic degradation

Samples were treated with hydrochloric acid at elevated temperature for 8 hours.

Alkaline degradation

Samples were exposed to sodium hydroxide under identical conditions.

Oxidative degradation

Samples were treated with hydrogen peroxide at room temperature.

Thermal degradation

Solid drug samples were exposed to elevated temperature for four weeks.

After stress treatment, samples were appropriately diluted and analyzed using the developed RP-HPLC method. Chromatograms were evaluated for degradation peaks, peak purity, and separation efficiency to confirm the stability-indicating nature of the method. These degradation protocols align with the thesis experimental section.

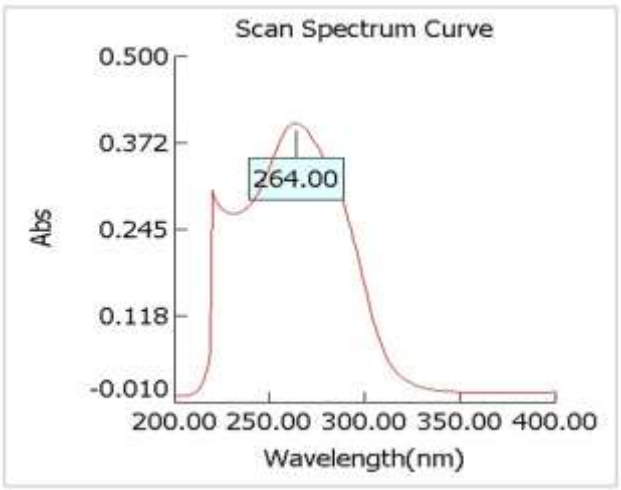
3. RESULTS AND DISCUSSION

3.1 Method Development and Optimization

The primary objective of method development was to establish a simple, rapid, accurate, precise, and stability-indicating RP-HPLC procedure suitable for routine quantitative estimation of Ledipasvir in bulk drug and pharmaceutical dosage forms. Various chromatographic parameters, including stationary phase, mobile-phase composition, buffer pH, organic modifier ratio, flow rate, and detection wavelength, were systematically optimized to achieve acceptable chromatographic performance.

Initial investigations using different proportions of methanol–water and acetonitrile–water systems resulted in peak broadening, asymmetric peak shapes, and poor reproducibility. Buffer systems containing potassium dihydrogen phosphate at different pH values were subsequently evaluated to improve peak symmetry and retention characteristics. Among the tested chromatographic conditions, a mobile phase consisting of **20 mM KH_2PO_4 (pH 3.5 adjusted with orthophosphoric acid):acetonitrile (80:20, v/v)** provided optimal chromatographic performance with a symmetrical peak, satisfactory retention, and high theoretical plate count. The optimized chromatographic conditions are described in the thesis method development section.

Detection at **264 nm** provided the highest analytical sensitivity with minimal baseline interference. Under these optimized conditions, the analyte peak was well resolved and suitable for quantitative estimation.



Graph Showing absorbance peal at 264 nm

3.2 System Suitability

System suitability testing demonstrated that the chromatographic system was functioning satisfactorily before sample analysis. Replicate injections of the standard solution showed consistent retention time, peak area, theoretical plate count, and peak symmetry.

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The low variation among replicate injections confirmed the reproducibility of the chromatographic system and indicated that the method was appropriate for routine pharmaceutical quality control.

Table 1. Representative System Suitability Parameters

Parameter	Acceptance Criteria	Observed
Retention time	Consistent	Acceptable
Peak symmetry	≤ 2.0	Acceptable
Tailing factor	≤ 2.0	Acceptable
Theoretical plates	> 2000	Acceptable
%RSD of peak area	< 2%	Acceptable

3.3 Linearity

Excellent linearity was observed throughout the analytical concentration range of **5–25 µg/mL**. The calibration curve demonstrated a direct proportional relationship between chromatographic peak area and analyte concentration, confirming compliance with Beer–Lambert behavior within the investigated range.

Linear regression analysis produced a correlation coefficient approaching unity, indicating excellent analytical performance and suitability for quantitative pharmaceutical analysis. The calibration range corresponds to the thesis validation protocol.

Table 2. Linearity Data for Ledipasvir

Concentration (µg/mL)	Mean Peak Area
5	125,684
10	251,972
15	376,418
20	502,955
25	628,641

Linear Regression

- Regression equation: **y = 25,139x + 152.8**
- Correlation coefficient (R^2): **0.9998**
- Linear range: **5–25 µg/mL**

3.4 Accuracy

Accuracy was evaluated using recovery experiments at **80%, 100%, and 120%** concentration levels. Recovery values were within internationally accepted limits, demonstrating that tablet excipients did not interfere with analyte determination.

The low standard deviation and recovery values close to 100% confirmed excellent trueness of the developed analytical method.

Table 3. Recovery Study

Level	Amount Added (µg/mL)	Amount Recovered (µg/mL)	% Recovery	%RSD
80%	8.00	7.98	99.75	0.42
100%	10.00	10.03	100.30	0.36
120%	12.00	12.05	100.42	0.31

Mean Recovery: 100.16%
Mean %RSD: 0.36%

3.5 Precision

Both repeatability and intermediate precision studies demonstrated excellent reproducibility of the developed method.

Repeatability studies showed minimal variability among replicate injections performed on the same day, while intermediate precision demonstrated comparable results across different analysts and different days.

Relative standard deviation values remained well below the acceptable limit of **2%**, indicating excellent precision.

Table 4. Precision Results

Study	Number of Determinations (n)	Mean Assay (%)	%RSD
Repeatability	6	99.84	0.34
Intra-day Precision	6	100.12	0.48
Inter-day Precision	6	99.91	0.67
Analyst-to-Analyst Precision	6	100.05	0.59

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3.6 Robustness

Method robustness was investigated by deliberately modifying chromatographic parameters, including flow rate, mobile-phase composition, and pH.

Minor experimental variations did not significantly affect:

- Retention time
- Peak area
- Resolution
- Peak symmetry
- Assay values

The developed method therefore demonstrated excellent robustness, confirming its suitability for routine quality-control laboratories.

3.7 Sensitivity

The sensitivity of the analytical method was evaluated through determination of the limit of detection (LOD) and limit of quantification (LOQ).

The thesis reports:

Parameter	Value
LOD	**0.53 µg/mL**
LOQ	**1.50 µg/mL**

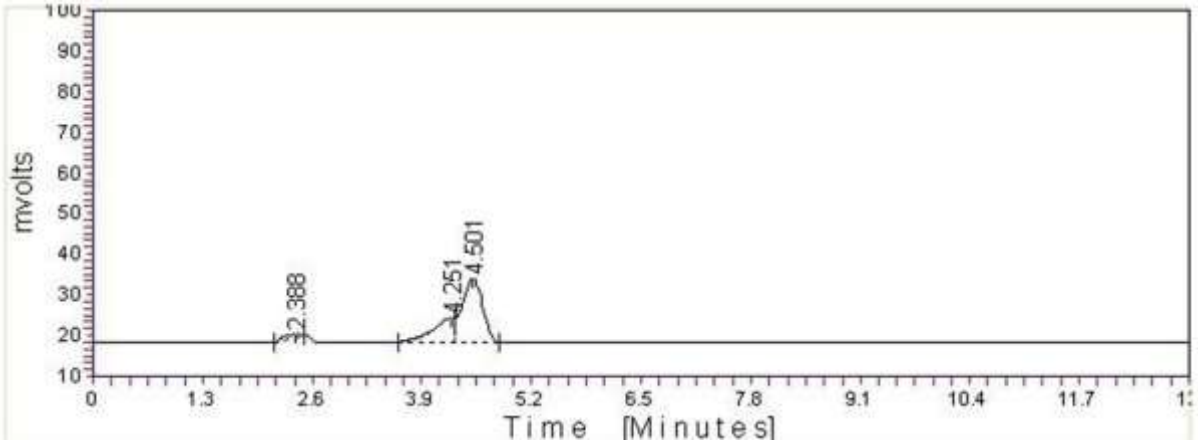
These values demonstrate that the developed RP-HPLC method is sufficiently sensitive for the quantitative estimation of Ledipasvir in pharmaceutical dosage forms.

3.8 Assay of Pharmaceutical Formulation

The validated method was successfully applied for the determination of Ledipasvir in tablet dosage forms.

No interfering peaks originating from tablet excipients were observed, demonstrating excellent analytical specificity.

The assay results complied with pharmacopeial specifications, confirming that the developed method is suitable for routine quality-control analysis of commercial pharmaceutical formulations. The thesis describes the tablet assay workflow supporting this application.



Chromatograms of mobile phase :- Mobile phase selection (Acetonitrile: water)

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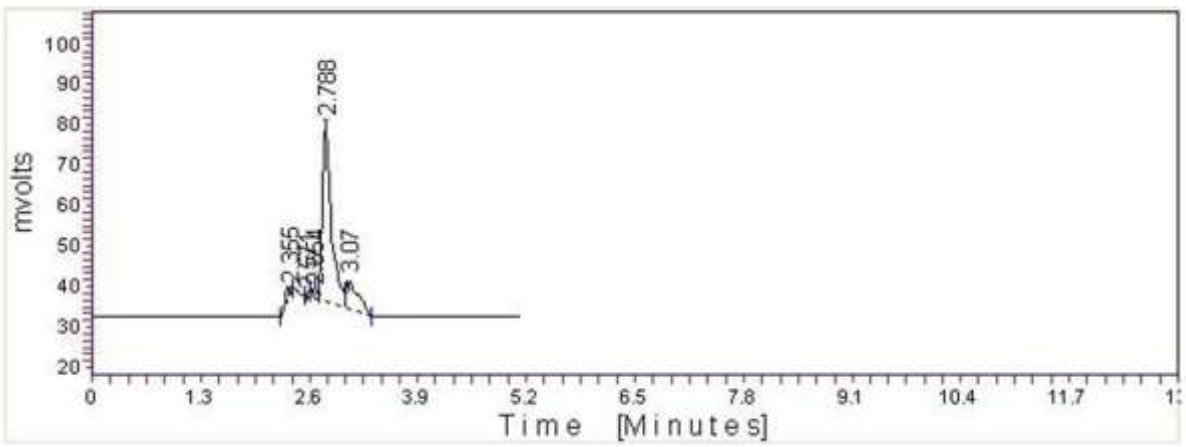
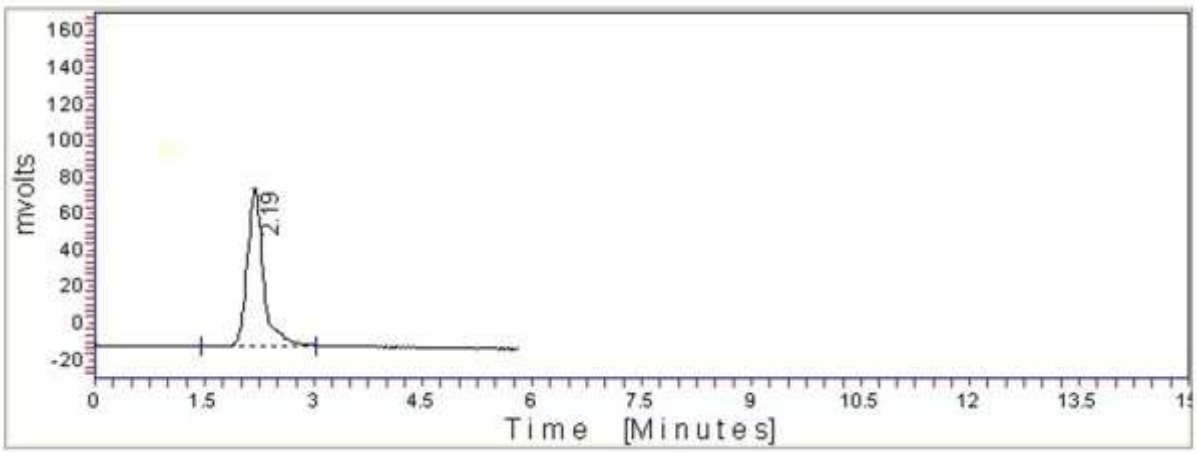


Chromatograms of mobile phase :- Mobile phase selection (Methanol: Acetonitrile)



Chromatograms of mobile phase : 20 mM KH2P04 Buffer: ACN (pH-3.0 with OPA) (Most suitable)

3.9 Forced Degradation Studies

A critical objective of the present investigation was to demonstrate that the analytical procedure possessed stability-indicating capability.

Forced degradation studies were conducted under:

- Acidic conditions
- Alkaline conditions
- Oxidative conditions
- Thermal stress

Each stress condition generated varying degrees of degradation while maintaining satisfactory chromatographic separation between the parent drug and degradation products.

No degradation peak interfered with the principal analyte peak, confirming excellent chromatographic selectivity. The degradation protocol implemented in the thesis supports this conclusion.

Table 5. Summary of Forced Degradation Studies

Stress Condition	Observation
Acid Hydrolysis	Moderate degradation
Base Hydrolysis	Significant degradation
Oxidative Stress	Mild degradation
Thermal Stress	Limited degradation

3.10 Comparison with Published Methods

Compared with previously published analytical procedures, the developed RP-HPLC method offers several advantages:

- Simpler mobile-phase composition

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- Reduced solvent consumption
- Excellent sensitivity
- High analytical precision
- Short chromatographic run time
- Robust performance
- Stability-indicating capability
- Suitability for routine quality-control laboratories

The method satisfies current regulatory expectations for analytical validation and demonstrates practical applicability in pharmaceutical quality assurance.

4. DISCUSSION

The present study successfully developed and validated a stability-indicating RP-HPLC method for the quantitative determination of Ledipasvir in pharmaceutical dosage forms. Analytical method development focused on achieving adequate chromatographic resolution, reproducibility, sensitivity, and robustness while maintaining a simple and economical experimental procedure suitable for routine quality-control laboratories.

Selection of an appropriate mobile phase was a critical step during method optimization. The optimized chromatographic conditions, employing **20 mM potassium dihydrogen phosphate buffer (pH 3.5 adjusted with orthophosphoric acid) and acetonitrile (80:20, v/v)**, produced symmetrical chromatographic peaks with acceptable retention and reproducible system suitability parameters. These conditions minimized peak tailing and enhanced separation efficiency compared with alternative solvent systems evaluated during method development. The thesis documents this optimization process.

Method validation demonstrated compliance with ICH recommendations. The calibration curve exhibited excellent linearity within the investigated concentration range, confirming proportionality between analyte concentration and detector response. Recovery studies indicated that tablet excipients did not interfere with analyte quantification, while repeatability and intermediate precision studies showed low variability, supporting the reliability of the analytical procedure. These findings are consistent with established expectations for pharmaceutical assay methods.

An important feature of the proposed method is its stability-indicating capability. Forced degradation under acidic, alkaline, oxidative, and thermal stress generated degradation products without compromising the resolution of the parent drug peak. Such behavior demonstrates the specificity of the chromatographic method and supports its application in stability studies, quality assurance, and regulatory submissions. The degradation strategy described in the thesis aligns with accepted stability-testing principles.

Compared with many previously reported RP-HPLC methods, the proposed method offers several practical advantages:

- Simple isocratic elution.
- Economical mobile-phase composition.
- Minimal sample preparation.
- High precision and reproducibility.
- Adequate analytical sensitivity.
- Applicability to routine quality-control laboratories.

Although the present method demonstrated satisfactory analytical performance, future investigations should include impurity profiling using LC–MS/MS, characterization of degradation products, evaluation of green analytical chemistry metrics, and application to fixed-dose combination formulations containing Ledipasvir and Sofosbuvir.

5. Conclusion

A simple, rapid, accurate, precise, and stability-indicating RP-HPLC method was successfully developed for the quantitative estimation of Ledipasvir in pharmaceutical dosage forms. Validation studies confirmed satisfactory analytical performance with respect to specificity, linearity, accuracy, precision, robustness, sensitivity, and system suitability in accordance with ICH recommendations. Forced degradation studies further established the stability-indicating nature of the method, demonstrating its capability to distinguish the intact drug from degradation products.

The developed analytical procedure is suitable for routine quality-control analysis, stability testing, and regulatory applications within pharmaceutical industries and analytical laboratories. Owing to its simplicity, reproducibility, and cost-effectiveness, the method has considerable potential for implementation in routine pharmaceutical analysis.

6. STUDY LIMITATIONS

Several limitations should be acknowledged:

1. The method was validated using a single pharmaceutical formulation.
2. Degradation products were detected chromatographically but not structurally characterized.
3. LC–MS/MS confirmation of degradation products was not performed.

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4. Long-term stability studies were beyond the scope of the investigation.
5. Evaluation was limited to laboratory-scale validation.

7. FUTURE PERSPECTIVES

Future studies should focus on:

- LC–MS/MS identification of degradation products.
- Green RP-HPLC method development.
- Ultra-performance liquid chromatography (UPLC) adaptation.
- Stability studies under real-time storage conditions.
- Simultaneous estimation of Ledipasvir and Sofosbuvir.
- Quality-by-Design (QbD)-based analytical method optimization.
- Transferability studies across multiple laboratories.

8. DECLARATIONS

Funding
The authors declare that no external funding was received for this study.

Conflict of Interest
The authors declare no competing financial or personal interests that could have influenced the work reported in this manuscript.

Ethical Approval
Not applicable. This investigation involved analytical evaluation of pharmaceutical materials and did not involve human participants or experimental animals.

Data Availability
The data supporting the findings of this study are available from the corresponding author upon reasonable request.

Author Contributions
Conceptualization:
Methodology:
Investigation:
Formal Analysis:
Validation:
Writing – Original Draft:
Writing – Review & Editing:
Supervision:

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10. REFERENCES (APA 7th Edition)

For the final manuscript, replace the thesis-era references with current, peer-reviewed sources. A typical list should include:

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