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STRESS DEGRADATION STUDIES AND DEVELOPMENT OF A VALIDATED RP-UHPLC METHOD OF LEDIPASVIR

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ABSTRACT

The intent of the research work was to develop and validate a simple, selective and precise reversed phase ultra-high performance liquid chromatography (RP-UHPLC) method for the determination of ledipasvir. A forced degradation study was performed as per International Conference on Harmonisation (ICH) guidelines Q1A (R2) and Q1B. Ledipasvir was found to be well separated from degradation products using an analytical C18 column (150 mm × 4.6 mm i.d., 5 µm particle size) with a ratio of mobile phase (75:25 v/v) consisting of methanol and 0.1% trifluoroacetic acid (TFA) kept at ambient temperature. The average retention time of ledipasvir was found 4.45 min at 254 nm wavelength with 1.6 mL/min isocratic flow rate and 10 µL injection volume. Linearity, accuracy, precision, sensitivity, robustness, and ruggedness were studied according to ICH guideline Q2 (R1) to validate the method. Then, this validated method was applied for forced degradation studies of ledipasvir. In conclusion, the developed method has been successfully used to study degradation behaviour of ledipasvir and may be useful to quantify the drug in different pharmaceutical dosage forms.

***Keywords*:** Ledipasvir, RP-UHPLC, ICH, Validated method, Degradation

INTRODUCTION

Hepatitis C is one type of liver disorder caused through the infection of blood borne hepatitis C virus (Shepard, Finelli and Alter 2005). Ledipasvir is a novel medication which acts against the virus directly (Link *et al*. 2014). It inhibits the non-structural 5A (NS5A) protein function leading to blockade of the hyper phosphorylation process which is vital for viral replication (Kwon *et al*. 2015). The market product of ledipasvir is available as

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combination dosage form with another anti-viral agent sofosbuvir (Gane *et al*. 2014). Molecular formula and molecular weight of this drug are $C_{49}H_{54}F_2N_8O_6$ and 889.00 g/mol, correspondingly (Figure 1). The colour of ledipasvir powder ranges from white to greyish, tan, yellow, orange or pink, somewhat hygroscopic solid in physical state. It is not soluble practically (< 0.1 mg/mL) over the pH range of 3.0–7.5 and a little soluble below pH 2.3 (1.1 mg/mL) (German *et al*. 2016). According to solubility studies, ledipasvir is freely soluble in ethanol and methanol; slightly soluble in acetone and isopropanol; very slightly soluble in water (Kiran *et al*. 2017). The log *P* (partition coefficient) for ledipasvir is 3.8 while the value of pKa1 is 4.0 and pKa2 is 5.0 (European Medicines Agency 2014).

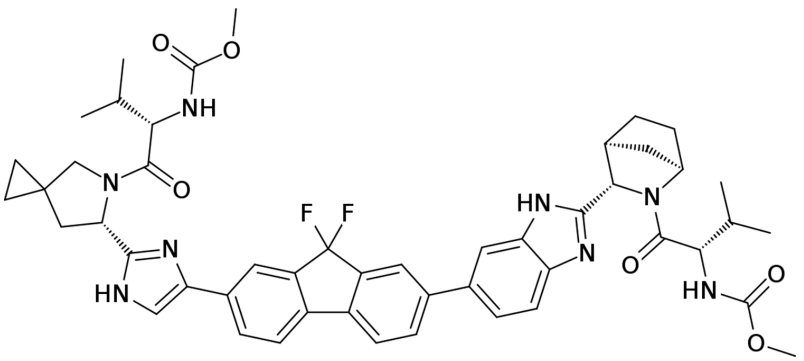

Figure 1: Chemical structure of ledipasvir.

Forced degradation studies are carried out as a fundamental experiment to comprehend about drug molecule stability issues, i.e., International Conference on Harmonisation (ICH) Q1A (R2). Several crucial changes are being observed within the chemical moiety due to their exposure to diverse environmental settings. Therefore, forced degradation studies help to understand the intrinsic stability of drug molecules and predict potential changes that are very important with respect to drug development process (Singh and Rehman 2012; Singh *et al*. 2013; Patel *et al*. 2015; Chiguru *et al.* 2016; Swain *et al*. 2016).

Literatures showed several reports on the development of reversed phase ultra-high performance liquid chromatography (RP-UHPLC) method for quantification of ledipasvir in pharmaceutical products, as well as forced degradation studies (Devilal *et al*. 2016; Hassouna, Abdelrahman and Mohamed 2017; Zaman, Siddique and Hassan 2016). However, many of the methods reported were found to exhibit either poor resolution, longer retention time or complicated mobile phase composition which rendered them inappropriate for routine analysis. Therefore, it is very much required to develop a suitable and uncomplicated procedure to quantitate the drug in dosage forms and the current work is continuation of our previous work in similar research area (Sultana *et al*. 2018). The current study aims to develop a precise, sensitive and accurate method validation technique of ledipasvir by RP-UHPLC with short retention time and high selectivity.

METHODS

Chemicals and Reagents

Ledipasvir powder was dispensed as an open-handed contribution by Incepta Pharmaceuticals Limited, Bangladesh. HPLC grade methanol was purchased from Merck (Germany) and trifluoroacetic acid (TFA) of analytical grade was procured from BDH Chemicals (England). Millipore Milli-Q water purification system from Bedford, MA, USA was used to prepare HPLC grade water. The rest of the materials and reagents were also of analytical grade.

Instrumentation

The study was conducted using a Perkin Elmer Flexar series (auto-sampler) UHPLC system (PerkinElmer Inc., USA) along with FX-15 binary pump, PDA plus detector, SIL/20A auto-sampler aligned with Chromera Manager Software. Other instruments included sonicator (Human lab Instrument Co., South Korea), CyberScan 500 pH meter (Eutech Instruments Pte Ltd., Singapore), electronic balance (Shimadzu, Japan) and photo stability chamber (ACMAS Technologies Pvt. Ltd., India).

Chromatographic Condition

The chromatographic separation was executed utilising a Brownlee Analytical C18 column (150 mm × 4.6 mm i.d., 5 µm particle size). The flow rate of the method was 1.6 mL/min with the detection wavelength of 254 nm. The oven temperature was kept at 25°C and an injection volume of 10 µL was adapted. Mobile phase was comprised of methanol: 0.1% TFA (75:25 v/v). Nylon membrane filter 0.45 µm (Restek, USA), was employed to filter the mobile phase prior to sonication and degassing. All samples were filtered before injection through syringe filter 0.22 µm.

Preparation of Ledipasvir Stock Solution

An amount of 50 mg of ledipasvir was precisely weighed and transferred into a clean and dry 50 mL volumetric flask. Around 20 mL of diluent (mixture of methanol and 0.1% TFA in the proportion of 50:50 v/v) was sonicated well for 5 min to dissolve the powder. Then, the solution volume was made up to mark with diluent. After proper mixing, the concentration of the standard stock solution of ledipasvir was 1,000 µg/mL (1 mg/mL).

Method Development

The method was developed after conducting many trials by changing different chromatographic conditions. An isocratic mode was adopted for chromatographic separation on Brownlee Analytical C18 column and the retention time of ledipasvir was found at 4.45 min using mobile phase (combination of methanol and 0.1% TFA in the ratio of 75:25 v/v) with flow rate of 1.6 mL/min.

Validation Parameters

According to ICH Q2 (R1) guideline (ICH Harmonised Tripartite Guideline 2005), in this study the considered validation parameters were system suitability, specificity, accuracy, precision, intermediate precision, linearity, limit of detection (LOD), limit of quantification (LOQ), robustness and ruggedness.

System Suitability

The percentage of relative standard deviation (% RSD) of peak area, theoretical plate number, tailing factor and retention time were considered to assess system suitability of the proposed method. Six replicates of ledipasvir standard solution of 100 µg/mL (prepared through dilution from stock solution of 1,000 µg/mL) were injected to calculate % RSD.

Specificity

The specificity was carried out by comparing the chromatogram of the mobile phase and ledipasvir solution to confirm the absence of any interfering peak at the retention time of the analyte.

Linearity

Five working standard solutions of ledipasvir over the 80% to 120% of nominal test concentration (100 µg/mL) ranging from 80–120 µg/mL were prepared in triplicate to evaluate linearity of the method. Analyte concentrations were plotted against peak area in the obtained calibration curve.

Accuracy (Recovery Test)

The accuracy of the method was tested by calculating mean percentage recoveries of ledipasvir standard solutions whose concentrations were of 80 µg/mL, 90 µg/mL, 100 µg/mL, 110 µg/mL and 120 µg/mL, each in three replicate injections.

Precision

In the precision study, intra-day precision (on the same day) and inter-day precision (daily for six times over a period of 3 days) were determined at nominal standard concentration (100 µg/mL) of ledipasvir in six replicates. The outcomes were expressed as the % RSD of the measurements.

LOD and LOQ

Two equations are applied with LOD and LOQ calculations which are: $LOD = 3.3 \times \sigma/S$ and $LOQ = 10 \times \sigma/S$, where σ denotes the standard deviation of y intercepts of regression lines and S is the slope of the calibration curve.

Robustness

Robustness of the method was performed through a variation of chromatographic conditions customising flow rate and mobile phase composition in six replicates. The flow rates were set at 1.5 and 1.7 mL/min instead of 1.6 mL/min and the mobile phase compositions were methanol: TFA = 73:27 v/v and methanol: TFA = 77 : 23 v/v, instead of methanol: TFA = 75: 25 v/v.

Ruggedness

Ruggedness was performed by varying two analysts in six replicates.

Methods of Stress Degradation Studies

The ICH guidelines Q1A (R2) (ICH Harmonised Tripartite Guideline 2003) and Q1B (ICH Harmonised Tripartite Guideline 1996) were followed during stress degradation studies and the detailed procedure of each of the degradation studies (Naazneen and Sridevi 2017) is described as follows:

Acid degradation study

For acid degradation study, 10 mg ledipasvir drug substance was transferred into a 100 mL volumetric flask and then 3 mL 0.1 N HCl solution was added to it and mixed well with a mild shake. After heating for 1 h at 40°C, it was cooled to room temperature and diluent was added to adjust the volume up to mark. From this solution, 10 µL was injected into the system filtered through hydrophobic polyvinylidene difluoride (PVDF) membrane disc filter (0.22 µm) and the chromatograms detected the stability in acidic condition.

Base degradation study

For base degradation study, 10 mg ledipasvir drug substance was transferred into a 100 mL volumetric flask and then 3 mL 0.1 N NaOH solution was added to it and mixed well with a mild shake. After heating for 1 h at 40°C, it was cooled to room temperature and diluent was added to adjust the volume up to mark. From this solution 10 µL was injected into the system filtered through hydrophobic PVDF membrane disc filter (0.22 µm) and the chromatograms were recorded to detect the stability in basic condition.

Oxidative degradation study

For oxidative degradation study, 10 mg ledipasvir drug substance was transferred into a 100 mL volumetric flask and then 3 mL 3% H_2O_2 solution was added to it and mixed with a mild shake. After heating for 1 h at 40°C, it was cooled to room temperature and diluent was added to adjust the volume up to mark. From the above solution 10 µL was injected into the system filtered through hydrophobic PVDF membrane disc filter (0.22 µm) and the recorded chromatograms detected the stability of sample.

Thermal degradation studies

Dry heat

An amount of 10 mg ledipasvir drug substance placed in an aluminium foil was exposed to 80°C in an oven system. After 1 day of exposure, a solution of concentration of 100 µg/mL was prepared with the exposed drug. From this solution 10 µL solution was injected into the HPLC system filtered through hydrophobic PVDF membrane disc filter (0.22 µm) and the chromatogram was recorded to detect the effect of dry heat condition.

Moist heat

An amount of 10 mg ledipasvir drug substance was exposed to 60°C with 75% RH for 1 h in a stability chamber. Then a drug solution with concentration of 100 µg/mL was prepared with the exposed drug substance. From this solution the injected volume was 10 µL and the recorded chromatograms detected the effect of moist heat condition.

Photo stability study

An amount of 10 mg ledipasvir drug substance was exposed to UV light of 315–400 nm for 24 h in a UV chamber and a solution of 100 µg/mL was prepared with it. From this solution, the injected volume was 10µL and the recorded chromatograms detected the photo stability.

RESULTS AND DISCUSSION

Once the method was developed, it was validated for linearity, accuracy, precision, sensitivity, robustness and ruggedness, and afterward applied for forced degradation studies as per the ICH guiding principle. The ardent objective of the study was to develop a precise and simple method for routine laboratory analysis of ledipasvir and ICH Q2 (R1) guideline (ICH Harmonised Tripartite Guideline 2005) was followed rigorously to ensure this. In the current study, a C18 analytical column was used for chromatographic separation and the retention time was found as 4.45 min while another work done by Zaman, Siddique and Hassan (2016) found higher retention time (8.242 min) using C8 column. The work done by Swain and Samanthula (2017) eluted ledipasvir at a higher retention time of about 11 min which is even longer than our developed method. This manifests the superiority of choosing C18 over C8 column for rapid isolation of ledipasvir with better resolution. Also, a non-cumbersome process of mobile phase preparation made the analytical method easier to perform in comparison with other procedures of ledipasvir analysis reported in literature. For example, Hassouna, Abdelrahman and Mohamed (2017) prepared a mobile phase made up of potassium dihydrogen phosphate and hexanesulfonic acid of sodium salt monohydrate in which pH was adjusted with orthophosphoric acid and acetonitrile which is inconvenient to prepare.

Method Validation

System suitability

The results of system suitability study (mean ± % RSD of 6 replicates) are given in Table 1 indicating the suitability of the system as all the obtained value (%RSD of area, tailing factor, retention time and minimum limit of theoretical plate number) were within acceptable limit (Center for Drug Evaluation and Research [CDER] 1994).

Table 1: System suitability parameters (*n* = 6)

Parameters	Value (mean ± % RSD)	Acceptable limit
Peak area	3322896.25 ± 0.55	% RSD ≤1
Tailing factor	1.262 ± 0.81	≤ 2.0
Theoretical plate	2051.84 ± 0.96	> 2000
Retention time	4.45 ± 0.42	% RSD ≤ 0.5

Specificity

Good resolution was obtained for ledipasvir and the recorded chromatogram of the mobile phase revealed that there was no interference within retention time (around 4.45 min) indicating the specificity of the developed method (Figures 2 and 3).

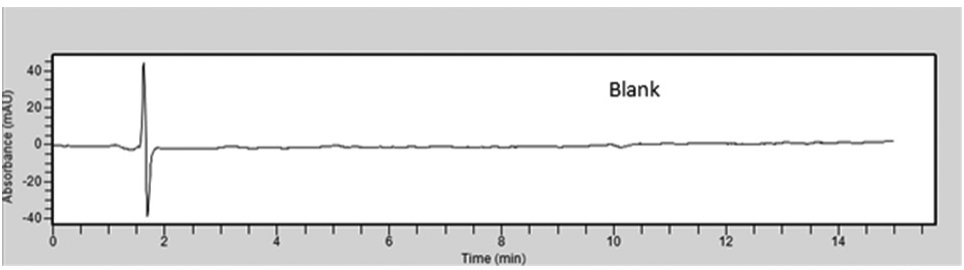

Figure 2: Chromatogram of mobile phase.

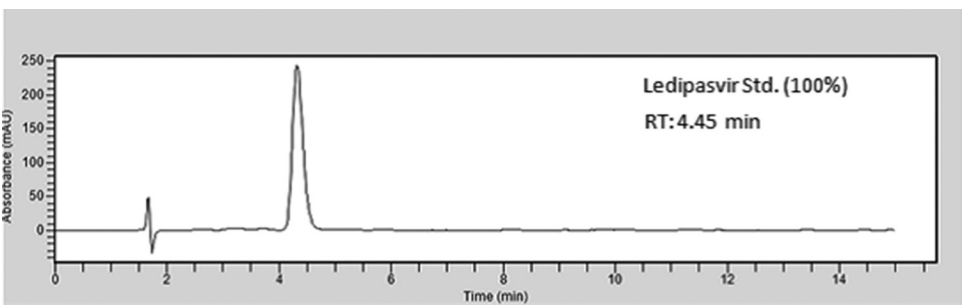

Figure 3: Chromatogram of ledipasvir.

Linearity

The calibration curve obtained with the data using the linear least square regression procedure is shown in Figure 4. The representative linear equation was

$$y = 34973x\text{-}182461$$

obtained by plotting peak area (y) versus concentration (x). The correlation co-efficient (R^2) value was 0.998.

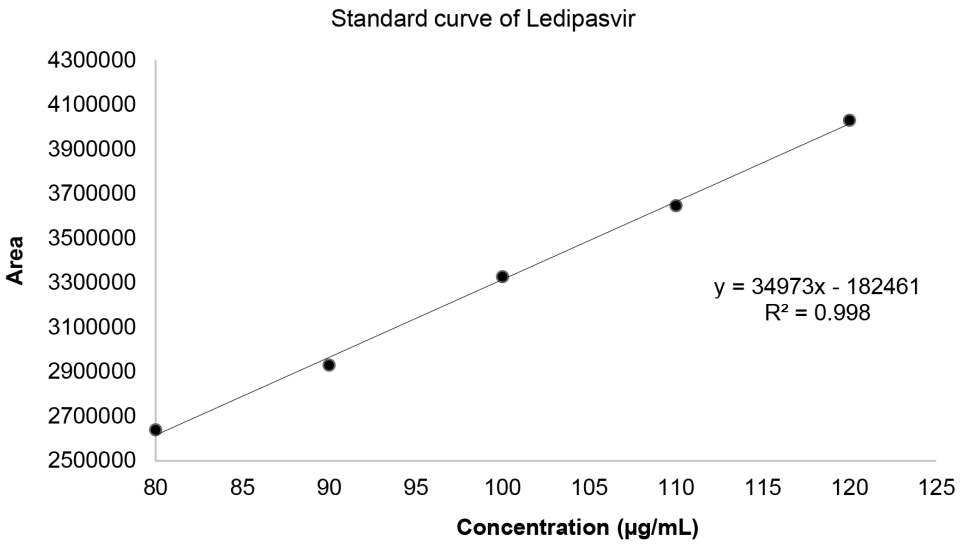

Figure 4: Standard curve of ledipasvir.

Accuracy

Accuracy of the method was studied by recovery experiments. The overall result of percent recoveries (mean ± % RSD of 3 replicates) of ledipasvir is presented in Table 2 indicating good accuracy of the proposed method. The calculated recovery values of ledipasvir ranged from 99.66 ± 0.28% to 100.67 ± 0.40% RSD.

Table 2: Result of accuracy study (n = 3).

Amount added (µg/mL)	% Recovery (mean ± % RSD)
80	100.34 ± 0.25
90	100.67 ± 0.40
100	99.66 ± 0.28
110	99.67 ± 0.22
120	100.05 ± 0.30

Precision

Intra-day precision and inter-day precision of the method were determined by using the solution of standard ledipasvir (100 µg/mL) and the solution was analysed in 6 replicates on the same day (intra-day precision) and daily for six times over a period of 3 days (inter-day precision). The results of precision study are given in Table 3 which indicate the method as precise since all the recovery limits were within acceptable range (98%–102%) and relative standard deviation was less than 2%.

Table 3: Result of precision study (*n* = 6).

Amount Added (µg/mL)	Intra-day % recovery (mean ± % RSD)	Inter-day (% recovery ± % RSD)			Inter-day % recovery (mean ± % RSD)
		Day 1	Day 2	Day 3	
100	99.78 ± 0.60	99.89 ± 0.20	100.09 ± 0.18	100.09 ± 0.31	100.02 ± 0.23

Sensitivity

The limit of detection (LOD) and limit of quantification (LOQ) of ledipasvir by the proposed method were found as 0.08 µg/mL and 0.24 µg/mL, respectively, through several trials with diluted solution of ledipasvir. Selectivity and sensitivity of the developed method were excellent (LOQ 0.24 µg/mL) compared to the work done by Devilal *et al*. (2016) (LOQ 3.224 µg/mL) and Hassouna, Abdelrahman and Mohamed (2017) (11.03 µg/mL), respectively. Moreover, in the research work executed by Swain and Samanthula (2017), no data regarding sensitivity of the developed method was mentioned.

Robustness

Mobile phase composition and flow rate were altered whose results are presented in Table 4. It depicts the proposed method as robust as the mean recoveries for all kinds of varied conditions were within acceptable limit.

Ruggedness

The results of ruggedness study (% recovery mean ± % RSD of 6 assay samples) are reported in Table 4. The method can be said as rugged since the mean recovery data for both analysts were within acceptable range.

Table 4: Result of robustness and ruggedness study.

Study type	Parameters	Variations (mL/min)	Amount added (µg/mL)	Peak area	Retention time (min)	% Recovery (mean ± % RSD)
Robustness study (*n* = 3)	Mobile phase flow rate (mL/min)	1.5	100	3372239.93	4.46	100 ± 0.33
		1.6	100	3318427.32	4.45	100 ± 0.28
		1.7	100	3318427.32	4.44	100.03 ± 0.05
	Mobile phase composition (% methanol: % TFA)	73:27	100	3318427.32	4.47	100.01 ± 0.17
		75:25	100	3318427.32	4.45	100 ± 0.32
		77:23	100	3318427.32	4.43	100 ± 0.34
Ruggedness study (*n* = 6)	Analyst variation	Analyst 1	100	3369178.72	4.45	100.07 ± 0.39
		Analyst 2	100	3375106.51	4.45	100.02 ± 0.33

Stress Degradation Studies

Results of the stability tests done following ICH guidelines Q1A (R2) and Q1B are shown in Table 5 which summarise all the stress degradation study results by percentage of recovery and degradation along with their required time for each of the studies. The results showed noteworthy degradation of ledipasvir in hydrolytic (acidic, alkali), oxidative, photolytic and thermal (moist heat) conditions while it was found relatively stable at dry heat condition as there was only 1.88% degradation after 24 h of time period.

Table 5: Results of stress degradation studies (*n* = 3).

Serial number	Stress conditions	Time	% Recovery (mean ± % RSD)	% Degradation (mean ± % RSD)
1	Acid degradation	1 h	82.85 ± 0.35	17.15 ± 0.35
2	Base degradation	1 h	83.51 ± 0.21	16.49 ± 0.21
3	Peroxide degradation	1 h	83.18 ± 0.43	16.82 ± 0.43
4	UV degradation	1 day	88.78 ± 0.62	11.22 ± 0.62
5	Thermal degradation (dry heat)	1 day	98.12 ± 0.48	1.88 ± 0.48
6	Thermal degradation (moist heat)	1 h	81.36 ± 0.54	18.64 ± 0.54

The results conform to previous degradation profile of ledipasvir found in literature to a remarkable extent (Hassouna, Abdelrahman and Mohamed 2017; Naazneen and Sridevi 2017; Swain and Samanthula 2017) and allow us to perform a more sensitive, selective, accurate and simple method for its analysis. However, in the current experiment, we found, ledipasvir was labile to photolytic degradation and moist heat while Swain and Samanthula (2017) found it stable in that stressed condition. The developed method showed non-interference between degradation products and the spectrum of ledipasvir. Hence the proposed analytical method can be valuable for the determination of ledipasvir stability both in bulk and pharmaceutical dosage forms.

CONCLUSION

A simple, precise, accurate, robust and economical method was established for the routine analytical investigations of ledipasvir. The method was effectively validated regarding linearity, precision and accuracy in accordance with ICH guidelines. This method can be proposed as a good quantification technique for quality control tests, degradation studies of pharmaceutical preparations and forensic pharmacy. Our studies exhibited that ledipasvir is highly sensitive to acid, alkali and in oxidative environment. It also degraded upon exposure to light and should not be kept in high humidity at higher temperature (above 40°C), which is an important exploration of this work. These findings should be useful for the design and fabrication of stable commercial product(s) and storage of ledipasvir thus assisting the production of stable and safe pharmaceutical products.

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