BIO-ANALYTICAL METHOD DEVELOPMENT AND VALIDATION FOR SIMULTANEOUS DETERMINATION OF LEDIPASVIR AND SOFOSBUVIR DRUGS IN HUMAN PLASMA BY RP-HPLC METHOD

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ABSTRACT

Objective: A novel, sensitive and accurate high-performance liquid chromatography with ultraviolet/visible light detection (HPLC-UV/VIS) method for the quantification of ledipasvir and Sofosbuvir in plasma was developed and validated.

Methods: The analytes were extracted by liquid-liquid extraction method and chromatograph using a mobile phase consisting of acetonitrile and buffer solution, Methanol and Acetonitrile in the ratio of 200:600:200 (v/v) using Oyster BDS RP-C18 column. The flow rate 1.0 ml/min and UV detection at 238 nm were employed. The retention time for Ledipasvir and Sofosbuvir was 4.61 and 9.09 min respectively. Linearity for ledipasvir and Sofosbuvir was found to be in the range of 250-2000 ng/ml for both drugs respectively. Intra-and inter-day precision was less than 2% coefficient of variation.

Results: The method was validated as per the USFDA guidelines and the results were within the acceptance criteria for selectivity, sensitivity, linearity, precision, accuracy, recovery stability of the solution, the stability of solution in plasma and dilution integrity.

Conclusion: Majority of the HPLC method should be useful for monitoring human plasma drug concentrations, and pharmacokinetic studies in patients diagnosed with the Ledipasvir and Sofosbuvir formulations.

Keywords: Ledipasvir, Sofosbuvir, Bio-analytical, RP-HPLC, Plasma

INTRODUCTION

Hepatitis C virus (HCV) one of the major global health problems. HCV infection is responsible for 3,50,000 death cases annually [1]. It is one of the top five death-causing diseases in the country. HCV infection is particularly a national problem in Egypt. The incidence rate reaches 14.5% among Egyptian population, which represents the highest prevalence of hepatitis C worldwide [2]. According to the Egyptian ministry of health, 1,00,000 new cases are identified each year [3]. Of particular note, the first all oral treatments, without the need for Ribavirin or pegIFNa injections, were recently approved: the combination pill, Harvoni TM (Ledipasvir, [4] NS5B inhibitor/ Sofosbuvir 1), a combination of Simeprevir (2) and Sofosbuvir (1) and Viekira PakTM, a combination of three DAAas and Ritonavir, i.e., Paritaprevir [5] (3) (NS3 protease inhibitor), several NS3 protease inhibitors Danoprevir (4), [6] and also Ledipasvir 5 (LED) known as GS-5885, is an NSSA inhibitor and antiviral against HCV (genotypes 1a and 1b)[7] fig. 1.

Fig. 1: Examples of approved drugs for HCV treatment: the HCV NS3/4a protease inhibitors 2, 3, 4 and the NS5B, NSSA polymerase inhibitor 1, 5 respectively
Ledipasvir, (methyl [5S]-1-[(S)-6-[4-(9-difluoro-7-[[2-[[1R,3R,4R]-2-[[[1-methoxyvinyl]-amino)-3-methylbutanoyl]-2-azabicyclo [2.2.1]-heptan-3-yl]-1H-benzol[imidazol-6-yl]-9H-fluorene-2-yl]-1Himidazol-2-yl]-5-azaspiro [2.4]-heptan-5-yl]-3-methyl-1-oxobutan-2-yl] carbamate) is belongs to the class of organic compounds known as fluorones used for the treatment of hepatitis C [8]. It acts against HCV and is categorized as a direct-acting antiviral agent (DAA). It is an inhibitor of the Hepatitis C Virus (HCV) NS5A protein which is required for viral RNA replication and assembly of HCV virions [9]. Sofosbuvir, IUPAC name is Isopropyl-(2-[2-[[4-fluoro-4-methyl- tetrahydro-furan-2-yl] methoxy-phenoxy-phosphoryl] amino] imidazol-2-yl)-5-azaspiro [2.2.1]heptan-5-yl)-3-methyl-1-oxobutan-2-yl) carbamate is a pro-drug nucleotide analog used in combination therapy to treat chronic hepatitis C virus (HCV) infected patients with HCV genotype 1,2,3, or 4, and to treat HIV and HCV co-infected patients [11-13]. The combination therapy includes either ribavirin alone or ribavirin and peginterferon alfa. Sofosbuvir prevents HCV viral replication by binding to the two Mg2+ ions present in HCV NS5B polymerase’s GDD active site motif [14].

Ledipasvir and Sofosbuvir combination, or Ledipasvir in combination with Sofosbuvir and Ribavirin, is indicated for the treatment of chronic hepatitis C (CHC) genotype 1 infection in adults [15]. The fixed-dose combination Ledipasvir-Sofosbuvir [90 mg/400 mg] is indicated for treatment, with or without Ribavirin, for the treatment of patients with chronic hepatitis C genotypes 1, 4, and 6 [16, 17]. Literature states that there are only two analytical methods have been described for analysis of Ledipasvir and Sofosbuvir in an individual by HPLC [18]. Due to high usage of Ledipasvir and Sofosbuvir combination for treatment of hepatitis C the present work is aimed to develop a bio-analytical method for combined analysis of Ledipasvir and Sofosbuvir in plasma.

MATERIALS AND METHODS

Chemicals

Analytically pure drugs were obtained as gift sample reputed pharmaceutical company. Methanol, acetonitrile, water (Merck, Mumbai, India) was of HPLC grade, while potassium dihydrogen phosphate, orthophosphoric acid and triethylamine used for the preparation of mobile phase.

Equipment

Chromatographic separation was performed on a PEAK chromatographic system equipped with LC-P7000 pump, UV detector UV7000 and the output signal was monitored and integrated by PEAK Chromatographic Software version 1.06. Oyster BDS RP-C18 column was used as stationary phase. Teccomp UV 2303 double beam UV-Visible spectrophotometer was used to carry out spectral analysis and the data was recorded by Hitachi software. Denver electronic analytical balance (SI-234), Systronics digital pH meter was also used.

Preparation pH 4.4 Acetate buffer (USP)

136 g of sodium acetate and 77 g of ammonium acetate are accurately weighed and dissolved in water and dilute to 1000 ml with the same solvent. Then 250.0 ml of glacial acetic acid is added and mixed well to get a buffer solution of pH 4.4.

Preparation of mobile phase

Measure accurately Acetate buffer (pH 4.4) buffer solution, Methanol and Acetonitrile in the ratio of 200:600:200 (v/v) at pH 4.4 and 1 ml/min flow rate. The chromatographic separation was achieved on Oyster BDS RP-C18 5 µm, 250 mm X 4.6 mm i.d. column at 238 nm UV detector wavelength. The column was maintained at room temperature and an injection volume of 20 µl was used. The mobile phase was filtered through 0.45 µm Chrom Tech Nylon-66 filter for use.

RESULTS AND DISCUSSION

One of the most difficult task during the method development was to achieve a high and reproducible recovery from the solvent which is used for extraction of the drug and also difficult task to select such single extracting solvent from which both the drugs are extracted. Different solvents were tried for the extraction of Ledipasvir and Sofosbuvir from human plasma and extraction with methyl t-butyl ether which is reconstituted solution [Dibothyl ether and dichloromethane] was exhibited good recovery. Under the optimal conditions (table 1) employed, the retention times were 4.61 min and 5.99 min for Ledipasvir Sofosbuvir respectively, with good peak shape and resolution (table 2, fig 2, 3). The proposed chromatographic conditions are validated according to the ICH and US-FDA guidelines [19-21].

Selectivity and system suitability

The selectivity of the method was evaluated by analyzing six independent drug-free human plasma samples with reference to potential interferences from endogenous and environmental constituents. In optimization, trials choose such method where plasma lots were found to be free of significant interferences. Resolution, tailing factor and theoretical plate’s results were with the acceptable limit thus meets the system suitability criteria.
Calibration curve/linearity
The Eight point calibration curve was constructed by plotting the peak response ratio of Ledipasvir and Sofosbuvir in plasma. Correlation of coefficients is 0.999 and 0.998 for Ledipasvir and Sofosbuvir respectively. Linearity’s were found over the range 250, 500, 750, 1000, 1250, 1500, 1750, 2000ng/ml for both Ledipasvir and Sofosbuvir. The lower limit of quantification was defined as lowest concentration in the calibration curve. The Ledipasvir and Sofosbuvir can be determined at LLOQ 200ng/ml. Data of calculated calibration standard concentration are shown in table 3 respectively and a representative calibration curve is shown in fig. 4.

Precision and accuracy
The precision of the method was determined by repeatability and accuracy for set of quality control (QC) sample (low, mid, high) in replicate (n = 6). The precision was found to be in the range (% CV) of 0.861-0.580%, 1.388-0.513 and 1.275-0.813% for LQC, MQC and HQC respectively. In this assay the inter-day, intra-day precision and accuracy values were within the acceptable range, it shows that the method is accurate and precise. The low percent relative standard deviation and percent relative error were within the acceptable limit. The results of precision and accuracy for the Ledipasvir and Sofosbuvir are shown in table 4, 5 and 6.

Recovery
Absolute recovery was calculated by comparing peak areas obtained from freshly prepared sample extracted with unextracted standard solutions of the same concentration. Recovery data were determined in triplicates at 750ng/ml. The recovery of Ledipasvir and Sofosbuvir for was found to be 87.467 %, 85.491 respectively (table 7).

Ruggedness and robustness
The ruggedness of the extraction procedure and the chromatographic method was evaluated by analysis at 750ng/ml concentration by a different analyst. Within batch precision of the method was in the range of 101.2 to 102.6 % and 100.4 to 102.3% for Ledipasvir and Sofosbuvir, respectively. Robustness results are achieved in the range of 0.136 to 0.179% and 0.134 to 0.089 % of the change in the results.

Stability
Stabilities of the samples were determined in various phases of the method. The stability studies include stock solution stability, freeze-thaw stability, in-injector stability, bench-top stability and long-term stability. All the above stability studies indicate that the samples in various phases were within the acceptance limits. The concentration of the freeze-thaw samples was found to be 91.9-104.3% of the nominal concentration for Ledipasvir and 91.5-101.2% for Sofosbuvir, indicating the stability of the analytes over three freeze-thaw cycles. For the bench top stability, the back-calculated concentration against freshly spiked calibration standards was found to be 93.6 to 100.1% of the nominal concentration for Ledipasvir and 92.7 to 100.3% Sofosbuvir. The concentration of the long term-stability samples ranged between 87.3 to 99.4% and 84.8 to 98.9% of the nominal value, respectively, for Ledipasvir and Sofosbuvir. The long-term stability duration was calculated as the date of analysis of QC samples, less the date of preparation of the stability QC samples.

Fig. 2: Blank and standard chromatograms of ledipasvir and sofosbuvir

Fig. 3: Sample chromatograms of Ledipasvir and Sofosbuvir
Table 3: Plasma spiked calibration curve results

| Test  | Sample ID | Ledipasvir Concentration prepared | Area obtained | Sofosbuvir Concentration prepared | Area obtained |
|-------|-----------|-----------------------------------|---------------|-----------------------------------|---------------|
| PSCC  | PSCC01    | 250ng/ml                          | 52563         | 250ng/ml                          | 63342         |
| PSCC  | PSCC02    | 500ng/ml                          | 80925         | 500ng/ml                          | 108016        |
| PSCC  | PSCC03    | 750ng/ml                          | 104220        | 750ng/ml                          | 154431        |
| PSCC  | PSCC04    | 1000ng/ml                         | 134956        | 1000ng/ml                         | 210252        |
| PSCC  | PSCC05    | 1250ng/ml                         | 157489        | 1250ng/ml                         | 252746        |
| PSCC  | PSCC06    | 1500ng/ml                         | 187464        | 1500ng/ml                         | 299062        |
| PSCC  | PSCC07    | 1750ng/ml                         | 209997        | 1750ng/ml                         | 347868        |
| PSCC  | PSCC08    | 2000ng/ml                         | 239272        | 2000ng/ml                         | 409237        |

N 8
Slope 105.9
Intercept 26686
r² 0.999

Table 4: Results of precision and accuracy at LQC

| S. No. | Sample ID | Ledipasvir | % accuracy | Sofosbuvir | % accuracy |
|--------|-----------|------------|------------|------------|------------|
| P and A at LQC | PA001 | 52326 | 99.54911 | 63761 | 100.6615 |
|         | PA002 | 51615 | 98.19645 | 62699 | 98.98488 |
|         | PA003 | 52802 | 100.4572 | 63423 | 100.1279 |
|         | PA004 | 52324 | 99.54511 | 63184 | 99.75056 |
|         | PA005 | 52814 | 100.4785 | 63575 | 100.3678 |
|         | PA006 | 52648 | 100.1617 | 63356 | 100.0221 |

Nominal Conc. 250ng/ml
N 6
Average 451.488
SD 52421.5
%CV 0.861
Accuracy (%) 99.731

Table 5: Results of precision and accuracy at MQC

| S. No. | Sample ID | Ledipasvir | % accuracy | Sofosbuvir | % accuracy |
|--------|-----------|------------|------------|------------|------------|
| P and A at MQC | PA007 | 104220 | 100 | 154431 | 100 |
|         | PA008 | 104907 | 100.6592 | 155014 | 100.3775 |
|         | PA009 | 103429 | 99.24103 | 156648 | 101.4356 |
|         | PA010 | 101356 | 97.25197 | 154638 | 100.134 |
|         | PA011 | 102246 | 98.10593 | 155211 | 100.5051 |
|         | PA012 | 104786 | 100.5431 | 155582 | 100.7453 |

Nominal Conc. 750ng/ml
N 6
Average 1437.054
SD 103490.7
%CV 0.861
Accuracy (%) 99.986
The authors declare no conflict of interest.

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AUTHORS CONTRIBUTIONS

All the authors have contributed equally

CONFLICT OF INTERESTS

The authors declare no conflict of interest.

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Table 6: Results of precision and accuracy at HQC

| S. No. | Sample ID  | Ledipasvir | Sofosbuvir |
|-------|-----------|------------|------------|
|       |           | Area obtained | % Accuracy | Area obtained | % Accuracy |
| P and A at HQC | PA013 | 236257 | 98.73993 | 407011 | 99.45606 |
|       | PA014 | 235011 | 98.21918 | 407428 | 99.55796 |
|       | PA015 | 235109 | 98.26014 | 405325 | 99.04407 |
|       | PA016 | 237572 | 99.28951 | 407507 | 99.51862 |
|       | PA017 | 238177 | 99.54236 | 407893 | 99.65766 |
|       | PA018 | 229776 | 96.03129 | 399179 | 97.54226 |

Nominal Conc. N 6

Average 2999.594 1.254 3297.963 0.805881

% CV 107795 0.347 108252 0.347 107575 0.347 107945 0.347

% Accuracy 87.467 85.491 87.7131 87.34281 87.16455 87.66529 87.9027 87.01222

Average 824.549 0.533 154725.7 0.813 154568 0.813 155246 0.813

% CV 85.77885 85.32854 85.40423 85.94084

% Accuracy 87.467 85.491 87.7131 87.34281 87.16455 87.66529 87.9027 87.01222

Table 7: Results of plasma spiked recovery

| Test          | Sample ID  | Ledipasvir | Sofosbuvir |
|---------------|------------|------------|------------|
|               |            | Area obtained | % recovery | Area obtained | % recovery |
| PSR at MQC    | PSR001     | 107387     | 87.01222 | 153329 | 84.71964 |
|               | PSR002     | 108486     | 87.9027  | 155246 | 85.77885 |
|               | PSR003     | 108193     | 87.66529 | 154568 | 85.40423 |
|               | PSR004     | 107575     | 87.16455 | 155693 | 86.02584 |
|               | PSR005     | 107795     | 87.34281 | 154431 | 85.32854 |
|               | PSR006     | 108252     | 87.7131  | 155087 | 85.691  |

Nominal Conc. N 6

Average 428.753 0.347 824.549 0.456

% CV 107948 0.397 154725.7 0.533

% Accuracy 87.467 85.491 87.7131 87.34281 87.16455 87.66529 87.9027 87.01222

Average 99.129 0.813 108491 0.333 108252 0.333 107795 0.333
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