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Development of Validated Bioanalytical RP-HPLC Method for Determination of Sofosbuvir In Human Plasma

S. Satheshkumar^{1*}, V. Muruganatham²

1. Ph.D Scholar, Department of Pharmaceutics, College of pharmacy, Vinayaka mission's college of pharmacy, Salem - 636008, Tamilnadu, India.

2. Professor, Department of Pharmaceutics, College of pharmacy, Vinayaka mission's college of pharmacy, Salem - 636008, Tamilnadu, India.

ABSTRACT

A linear, precise, accurate and simple reversed-phase high performance liquid chromatography (RP-HPLC) bioanalytical method was developed and validated for determination of sofosbuvir in human plasma (K₂EDTA) using ledipasvir as an internal standard. Sofosbuvir and ledipasvir (ISTD) were separated from plasma using solid phase extraction technique and The chromatographic separation was accomplished by using a Zorbax eclipse XDB C18 Column (250×4.6 mm i.d, 5 µm particle size) with 1% orthophosphoric acid (pH6.4) and Acetonitrile in the ratio of 30:70% v/v, as mobile phase in an isocratic elution mode pumped at a flow rate 0.7 mL/min and the column oven temperature was maintained at 25°C. The wavelength selected for quantitation was 254 nm. Retention times of sofosbuvir and internal standard were found to be 3.8min and 7.4min, respectively. The standard curves were found to be linear in the range of 30.566-2000.381 ng/ml for sofosbuvir in human plasma. This method performed an intra-day and inter-day precision within the range of 2.44–11.14 and 5.01–8.70%, respectively. Additional intra-day and inter-day accuracy was within the range of 87.99–105.93% and 94.97–99.18% respectively. Total percentage mean recovery of sofosbuvir from spiked plasma was found to be 86.54%. All the validated parameters were found to be within the Acceptance limit.

Keywords: Human plasma, RP-HPLC, Sofosbuvir, Solid phase extraction.

*Corresponding Author Email: shaktipalpatil@yahoo.com

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INTRODUCTION

Sofosbuvir (SOF) is a hepatitis C virus (HCV) non-structural protein (NS) 5B polymerase inhibitor. It was first approved for commercial marketing in the United States (US) on 06 December 2013 and in the European Union (EU) on 17 January 2014. Sofosbuvir is indicated for the treatment of genotypes 1 to 4 (US), and genotypes 1 to 6 (EU) HCV infection¹. SOF is a prodrug and is extensively metabolized in the liver to form the pharmacologically active nucleoside analog triphosphate GS-461203. The active triphosphate GS-461203 was undetectable in plasma during both nonclinical and clinical studies. The two major metabolites are GS-331007 and GS-566500². Systematic (IUPAC) name: Isopropyl (2S)-2-[[[(2R,3R,4R,5R)-5-(2,4-dioxypyrimidin-1-yl)-4-fluoro-3-hydroxy-4-methyl-tetrahydrofuran-yl] methoxy-phenoxy-phosphoryl]amino] propanoate (Figure 1)³. It is freely soluble in Methanol and chemical formula is C₂₂H₂₉FN₃O₉P and the molecular weight is 529.458 g/mol.

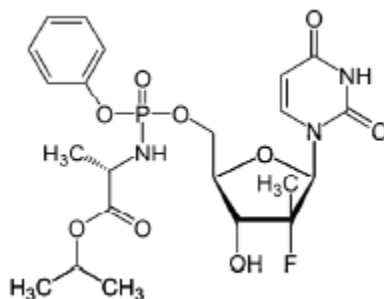


Figure 1: Chemical structure of Sofosbuvir

Literature review reveals that few analytical methods have been reported for the determination of sofosbuvir, which includes high performance liquid chromatography coupled with ultra violet (UV) detection and liquid chromatography-mass spectrometry (LC-MS) and ultra-high performance liquid chromatography tandem mass spectrometric detection (UPLC-MS/MS)^[4-13]. The present study was aimed to develop a novel, simple, economic and validated RP-HPLC method for the estimation of Sofosbuvir in human plasma according to USFDA guidelines¹⁴.

MATERIALS AND METHOD

Sofosbuvir and Ledipasvir bulk drug was kindly provided as gift sample by Sequent Labs Pvt. Ltd., Mangalore, India. Analytical grade of Ammonium acetate purchased from Rankem Ltd., India and HPLC grade of Methanol and Acetonitrile purchased from Merck Specialties Private Limited, India. Analytical grade Ortho phosphoric acid purchased from Rankem Ltd., India, Matrix: Human plasma (K₂EDTA - Anticoagulant) obtained from a registered blood bank.

Instrument

Quantitative HPLC was performed on Shimadzu HPLC system with SPD-M10 A VP system PDA and LC-MS solution software. An XDB column of 250mm 4.6mm: i.d and 5 μ particle size was used. Micro Weighing Balance (MX5) - Mettler Toledo, an Ultrasonic bath sonicator, Digital pH meter - Mettler Toledo, Deep Freezer (-20 \pm 5 $^{\circ}$ C) - Thermo Fisher Scientific, Refrigerator (2-8 $^{\circ}$ C) - Thermo Fisher Scientific and Vortex - Spinix, Swirlex, Heidolph, Heidolph Reax top and Solid phase extraction(SPE) unit- Orochem Technologies Ezypress 48 were used in the study.

Chromatographic parameters

Equipment: Shimadzu HPLC system with SPD-M10 A VP system PDA detector

Column: Zorbax eclipse XDB C18 Column (250 \times 4.6 mm, 5 μ m)

Mobile Phase: Acetonitrile and 1% orthophosphoric acid (pH6.4) (70:30% v/v)

Flow rate: 0.7mL/min

Wavelength: 254 nm

Injection volume: 20 μ l

Run time: 10 Minutes

Standard solution preparation

Accurately weighed and transferred 5mg of Sofosbuvir into 5ml dry volumetric flask, The solution is made upto the mark using Methanol at a concentration of 1.0 mg/mL and stored at 2-8 $^{\circ}$ C.

Working standard preparation

The working solutions for the calibration standards containing sofosbuvir were prepared by dilution of the stock solution with the diluent 60% methanol solution (v/v). Calibration standards in human plasma were prepared by diluting the corresponding working solutions with blank plasma samples at the following concentrations: 30.300, 60.600, 120.905, 250.048, 500.095, 1000.190, 1500.286 and 2000.381 ng/mL for sofosbuvir. The working solutions for the quality control (QC) samples were prepared at the following concentrations: 30.566, 80.376, 908.442 and 1700.417 ng/mL for sofosbuvir. IS working standard solution at a concentration of 500 ng/mL was prepared by diluting the stock solution (1.0 mg/mL) with the diluent 60% methanol solution (v/v). All stock solutions, working solutions, calibration standards and QCs were immediately stored at 2-8 $^{\circ}$ C.

Sample preparation:

Exactly 0.400mL of sample was aliquoted and transferred into a polypropylene tube and 50.00 μ L of ISTD solution was added, except for standard blank, to which 50.0 μ L of 60% methanol solution (v/v) was added and vortexed. To this 0.200mL of 20mM ammonium acetate (w/v) was added and vortexed. Sample clean-up was conducted using the following SPE procedure, the cartridges (Oasis

HLB) were first conditioned with methanol 1mL then equilibrated by water 1mL, in a positive pressure manifold. Plasma samples were loaded onto SPE cartridges. Then, the cartridges were washed with water 1mL twice and followed by 10% methanol solution (v/v) 1mL to wash the interferences and the analyte was successively eluted with 0.300mL of methanol with elution Rial vial contained 50.00 μ L of 1% orthophosphoric acid (pH6.4) and vortexed. Finally 20 μ L was injected into the HPLC system.

METHOD VALIDATION

Method validation was done as per the criteria of industrial guidance for bioanalytical method validation.

Selectivity and system suitability

Selectivity of the method was established by analyzing human plasma samples six from normal source, two from lipemic source and two from haemolysed source, to investigate the potential interferences at the LC peak region for sofosbuvir and IS. System suitability experiment was performed by injecting six consecutive injections using a standard aqueous mixture equivalent to Middle concentration of the calibration curve of sofosbuvir and IS during the start of the method validation and at the start of the each day. The percentage CV of system suitability have within the limits.

Carryover effect

Carryover effect was performed in order to eliminate the carryover from previous injection to next injection. Extracted blank sample, extracted LLOQ, and extracted ULOQ samples were prepared from same biological matrix lot. These samples were injected in the order of mobile phase, extracted blank plasma, extracted LLOQ, extracted ULOQ and extracted blank plasma samples during the start of the method validation and before using different equipment. The response of interfering peaks at the retention time of analyte have $\leq 20\%$ of response of extracted LLOQ.

Calibration curve

Calibration curves were acquired by plotting the peak area ratios of the transition pair of sofosbuvir to that of IS against the nominal concentration of calibration standards. The concentrations used for SOF calibration curves were 30.300, 60.600, 120.905, 250.048, 500.095, 1000.190, 1500.286 and 2000.381 ng/mL, while 30.566, 80.376, 908.442 and 1700.417 ng/mL were used for LLOQ, LQC, MQC and HQC, respectively. Blank sample (without IS) and zero samples (with IS) were run with each calibration curve. The acceptance criterion for each back-calculated standard concentration was $\pm 15\%$ deviation from the nominal value except at LLOQ, which was set at $\pm 20\%$.

Precision and accuracy

Intra-assay precision and accuracy were estimated by analyzing six replicates containing sofosbuvir at the lower level of quantification (LLOQ) in addition to three different QC levels in human plasma. The inter-assay precision and accuracy were determined at the four levels as described above on different days. The parameter was checked on different batches and within the batches. The criteria for acceptability of the data included accuracy $\pm 15\%$ standard deviation (SD) from the nominal values and a precision $\leq 15\%$ coefficient of variation % (CV) and at LLOQ, which was set at $\pm 20\%$.

Recovery

The extraction efficiency of sofosbuvir and IS extraction from human plasma was determined by comparing the responses of the analytes extracted from six replicate QC samples at LQC, MQC and HQC level with the response of analytes from post-extracted plasma standard sample at equivalent concentrations.

Dilution integrity

Dilution integrity was investigated to ensure that samples could be diluted with blank matrix without affecting the final concentration. Sofosbuvir spiked human plasma samples were prepared at concentrations of 8502.084 ng/mL, respectively. These solutions were further diluted with pooled human plasma five folds in six replicates and analyzed. The six replicates have precision $\leq 15\%$ and accuracy of $100 \pm 15\%$.

Ruggedness

Ruggedness was assessed by a precision and accuracy batch. The batch was monitored using different column and different analyst.

Stability experiments

The stability of sofosbuvir and IS in the injection solvent was estimated by injecting replicate preparations of processed samples up to 30 h (in auto-sampler) after the initial injection. The peak areas of the analytes and IS obtained at initial cycle were used as the reference to determine the relative stability of the analytes at subsequent points. Stability of analytes in the plasma after 8 h 11 min at room temperature (bench top) was determined at two QC concentrations (LQC and HQC) in six replicates. Freezer stability of the analytes in plasma was assessed by analyzing the QC samples (LQC, HQC and DIQC) stored at $-20 \pm 5^\circ\text{C}$ for at least 26 days. The stability of analytes in plasma following repeated six freeze-thaw cycles (stored at $-20 \pm 5^\circ\text{C}$) was assessed using QC samples (LQC and HQC) spiked with analytes. Samples were processed as described above. Samples were considered to be stable if assay values were within the acceptable limits of accuracy (i.e. $\pm 15\%$ SD) and precision (i.e. $\leq 15\%$ CV).

RESULTS AND DISCUSSION

Selectivity and system suitability

The selectivity of the method was demonstrated on ten blank plasma samples obtained from different lots: the chromatograms were found to be free from endogenous significant interferences. These batches were further selected for preparation of standard CC and QC samples. The % CV of system suitability was observed in the range of 0.20 to 0.84% for retention time of sofosbuvir and IS and 0.61 to 4.09% for area ratio, which is not more than 5.00% as per the acceptance criteria.

Carryover effect

There was no carryover observed for the interference peak obtained at the retention time (RT) of analyte is not more than or equal to 20% of extracted LLOQ and interference at the RT of ISTD is less than or equal to 5% of internal standard response of extracted LLOQ in re injected blank sample

Linearity

The optimized method was found to be linear in the concentration range of 30.300 to 2000 ng/mL. A straight line was witnessed when the plot was made through the data points by 1/x²weighing method and was observed to be in the range of best fit for sofosbuvir. The results found for correlation coefficient (r^2) was greater than 0.99 as per the limits were. Thus, the method was found to be linear in the mentioned range (Table 1) and Figure (1, 2 & 3).

Table: 1 Precision and Accuracy data of back-calculated concentration of calibration samples for Sofosbuvir from four representative calibration curves

Nominal concentration (ng/mL)	Concentration found (ng/mL) (mean \pm SD)	Precision (% RSD)	Accuracy (%)
30.300	29.711	1.56	98.06
60.600	56.439	2.45	93.13
120.905	109.657	4.04	90.70
250.048	235.075	2.80	94.01
500.095	477.527	5.21	95.49
1000.190	1003.244	3.54	100.31
1500.286	1462.844	3.57	97.50
2000.381	1932.351	1.38	96.60

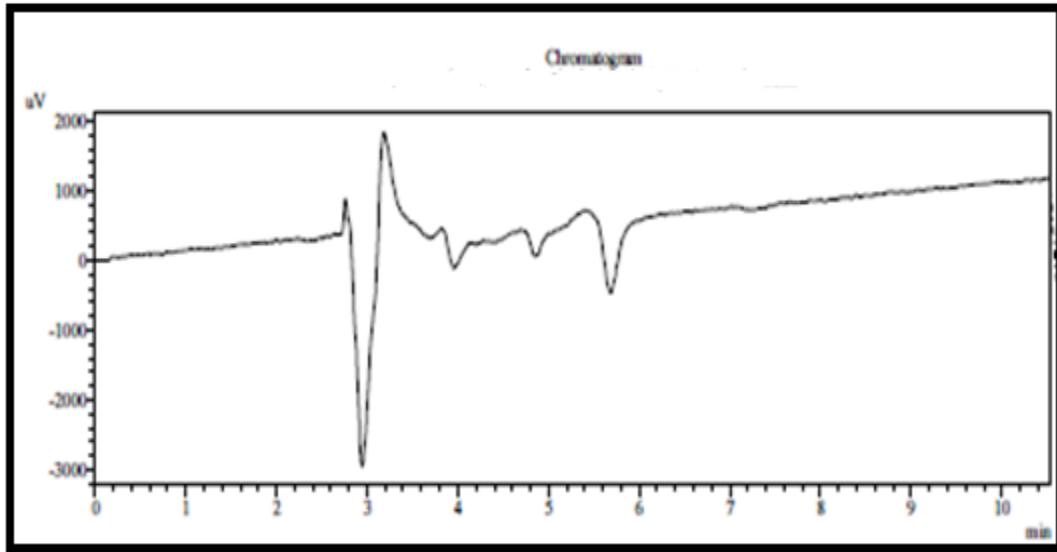


Figure 2: Extracted Blank of Sofosbuvir

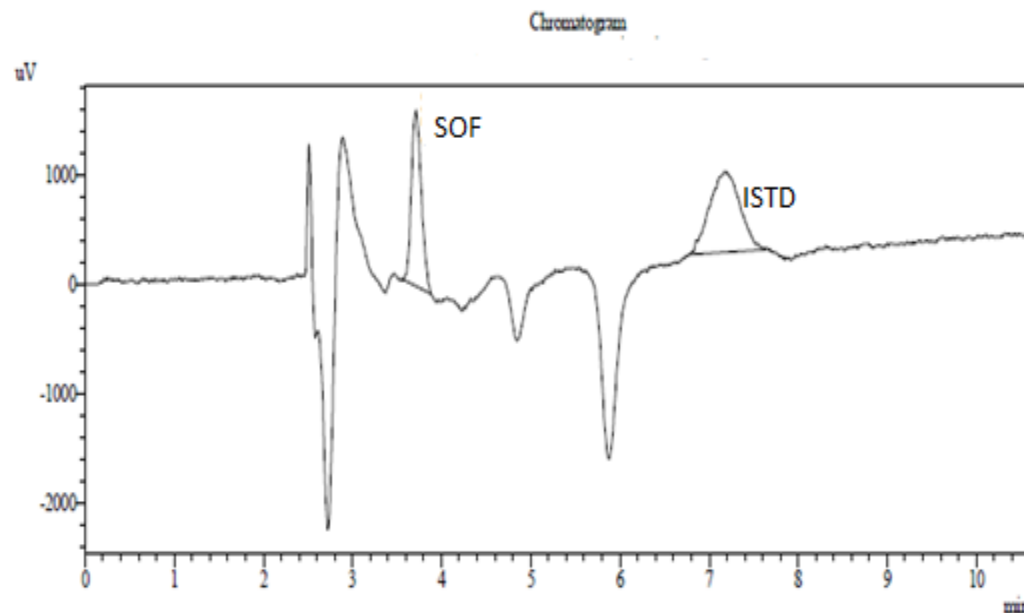


Figure 3: Extracted LLOQ of Sofosbuvir

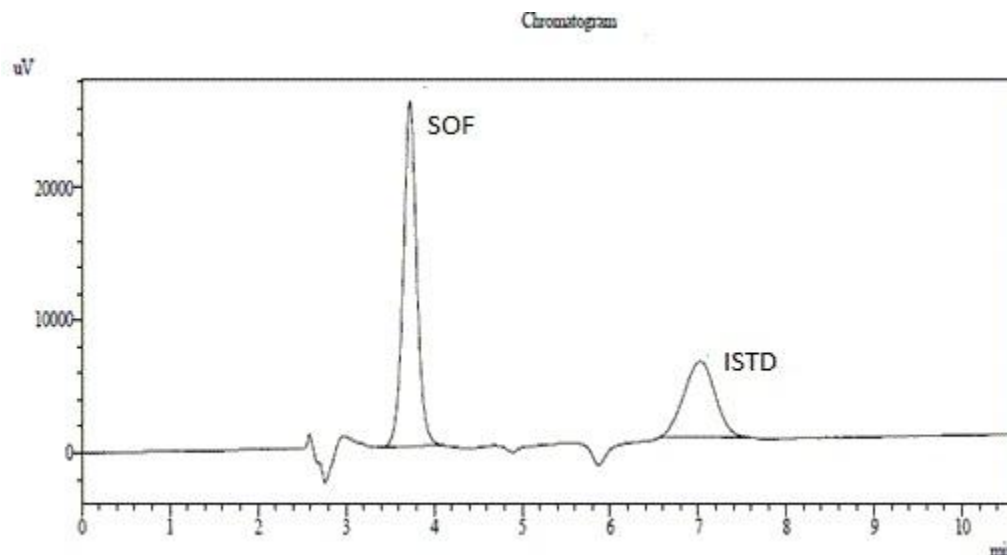


Figure 4: Extracted ULOQ of Sofosbuvir

Precision and accuracy

Precision and accuracy under inter-batch and intra-batch studies were determined and were expressed as percentage CV and percentage accuracy respectively. The percentage CV found for sofosbuvir for inter-batch QC samples were 8.70, 5.82, 5.59, and 5.01 for LLOQ, LQC, MQC and HQC respectively. Similarly, the results obtained for intra-batch QC samples ranged as 4.13-11.14, 3.45-6.49, 2.89-5.78 and 2.44-8.32 for LLOQ, LQC, MQC and HQC respectively. Accuracy in terms of percentages for sofosbuvir found under inter-batch studies was 97.21%, 94.97%, 98.38% and 97.51% for LLOQ QC, LQC, MQC and HQC respectively. Similarly, the results obtained for intra-batch QC samples ranged as 87.99-101.87%, 89.93-101.36%, 90.44-103.59% and 94.00-103.93% for LLOQ, LQC, MQC and HQC respectively (Table 2 and 3).

Table-2 Inter-day precision and accuracy (n =24)

QC ID	Nominal concentration (ng/mL)	Concentration found (ng/mL) (mean \pm SD)	Precision (% RSD)	Accuracy (%)
LLOQ	30.566	29.713	8.70	97.21
LQC	80.376	76.334	5.82	94.97
MQC	908.442	893.712	5.59	98.38
HQC	1700.417	1658.155	5.01	97.51
DIQC	8502.084	8432.230	7.20	99.18

Table-3 Intra-day precision and accuracy-(n =24)

QC ID	Nominal concentration (ng/mL)	Precision (% RSD)				Accuracy (%)			
		PA-01	PA-02	PA-03	PA-04	PA-01	PA-02	PA-03	PA-04
LLOQ	30.566	8.04	11.14	4.13	5.93	87.99	101.87	99.10	99.87
LQC	80.376	3.45	3.92	6.49	4.20	89.93	101.36	96.92	91.67
MQC	908.442	3.49	2.89	3.81	5.78	98.73	90.44	100.76	103.59
HQC	1700.417	2.44	8.32	3.13	2.44	96.06	94.00	103.93	96.06
DIQC	8502.084	5.63	6.53	2.53	5.71	96.17	105.93	92.36	102.26

Recovery

The mean % recovery for LQC, MQC, HQC samples of Sofosbuvir were 84.63%, 85.55% and 89.43% respectively. The mean overall recovery of SOF was 86.54% with a precision of 2.95% and the internal standard, mean peak area of MQC level extracted samples was compared to the mean peak area of MQC level post extracted IS and the mean % recovery is 88.99%.

Dilution integrity

Samples were analysed along with freshly spiked CC Standards and DIQC samples prepared in the screened biological matrix. The percentage accuracy was found inter-batch and intra-batch samples were 99.18% and 92.36-105.59%. Precision percentage CV was observed inter-batch and intra-batch samples were 7.20% and 2.53-6.53%. as 2.87%. The results found at both the levels are within the acceptance limit.

Ruggedness

During all two cases the precision and mean accuracy for CC Standards of SOF were ranged from 2.44 to 5.93 and 91.67 to 103.59% (Table 3).

Stability studies

Stability studies were experimented and studies performed are wet extract, short time stability, long term stability, auto sampler, freeze and thaw cycles, and bench top stability. The analyte passes all stability tests viz., Auto-injector (30 h), bench-top (8 h) and freezer stability at $-20^{\circ}\text{C} \pm 5^{\circ}\text{C}$ for at least for 26 days and was found unaltered to a significant extent with good percentage precision and accuracy. The freeze thaw stability results indicated that sofosbuvir was stable for six cycles. Results showed that plasma samples under investigation could be frozen and thawed without losing the reliability of the analyte under circumstances as per the optimized method (Table 4).

Table 4 Stability Samples Results for Sofosbuvir

Stability Experiment	QC ID	Nominal concentration (ng/mL)	Concentration found (ng/mL) (mean \pm SD)	Precision (% RSD)	Accuracy (%)
Bench top	LQC	80.376	77.955	3.12	96.99
Stability	HQC	1700.417	1637.730	8.34	96.31
Auto sampler	LQC	80.376	79.026	4.93	98.32
Stability	HQC	1700.417	1699.955	8.07	99.97
Wet extract	LQC	80.376	72.689	3.57	90.44
Stability	HQC	1700.417	1772.107	6.96	104.22
Freeze thaw	LQC	80.376	75.221	3.43	93.59
Stability	HQC	1700.417	1722.162	9.11	101.28
Long term	LQC	80.376	81.614	3.76	101.54
plasma	HQC	1700.417	1640.399	8.07	96.47
stability	DIQC	8502.084	8502.823	5.88	100.01

CONCLUSION

A sensitive and simple HPLC method for measurement of sofosbuvir in Human plasma has been developed and validated according to FDA Guidance for industry on bioanalytical method validation. The precision and accuracy of the method are well within the limits required for bioanalytical assays. The extraction procedure is simple, with suitable recovery. The use of the proposed method as a routine assay for pharmacokinetic, bioequivalence and drug interaction studies for the estimation of Sofosbuvir from Huma plasma.

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