A UPLC-MS/MS METHOD DEVELOPMENT AND VALIDATION FOR THE ESTIMATION OF SOFOSBUVIR FROM HUMAN PLASMA

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ABSTRACT

Objective: The present work aimed to develop a simple, rapid, specific and precise ultra-performance liquid chromatography-tandem mass spectrometric (LC-MS/MS) validated method for quantification of sofosbuvir and internal standard (ISTD) Sofosbuvir-d3 in human plasma.

Methods: Samples prepared by employing liquid-liquid extraction (LLE) using 2.5 ml of ethyl acetate. Chromatographic separation was achieved on Gemini 5μ C18, 50 x 4.6 mm column using a mixture of 0.1% (v/v) formic acid in water to methanol at a ratio of 30:70 v/v as the mobile phase. The flow rate was 0.50 ml/min. The LC eluent was split, and approximately 0.1 ml/min was introduced into Tandem mass spectrometer using turbo Ion Spray interface at 325 °C. Quantitation was performed by transitions of 428.35/279.26 (m/z) for sofosbuvir and 431.38/282.37 (m/z) for sofosbuvir-d3.

Results: The concentrations of ten working standards showed linearity between 4.063 to 8000.010 ng/ml (r² ≥ 0.9985). Chromatographic separation was achieved within 2 min. The average extraction recoveries of three quality control concentrations were 75.3% for sofosbuvir and within the acceptance limits. The coefficient of variation was ≤15% for intra-and inter-batch assays. The %CV of ruggedness ranges 0.35% and 3.09%. The % stability of short term and long term stock solution stability studies was found to be 97.25% and 98.81% respectively.

Conclusion: The results obtained for specificity, linearity, accuracy, precision, ruggedness and stability studies were within the acceptance limits. Thus the validated economical method was applied for pharmacokinetic studies of sofosbuvir.

Keywords: Sofosbuvir, LC-MS/MS, Human plasma, Stability studies

INTRODUCTION

Sofosbuvir, a phosphoramide prodrug is chemically described as (S)-Isopropyl 2-((S)-(2R, 3R, 4R, 5R)-5-((2-dioxo-3,4-dihydro-pyrimidin-1(2H)-yl)-4-fluoro-3-hydroxy-4 methyl tetrahydropuran-2-yl)-methoxy)-(phenoxypyrrophosphorylamino) propanoate [1-2]. Literature survey reveals two HPLC methods for determination of sofosbuvir from its bulk and pharmaceutical dosage forms [3-4]. Three UPLC-MS/MS method were reported for quantification of sofosbuvir from its metabolites and along with other drugs from human plasma [5-7]. Described here is a simple, sensitive, and selective UPLC-MS/MS method for sofosbuvir in the human plasma concentration range of 4.063 to 8000.010 ng/ml. As there is no literature on stability and validation details of sofosbuvir estimation from human plasma, this study performed assay validations, according to the FDA guidelines [8]. While this method with validation details were economical and applied for pharmacokinetic studies of sofosbuvir.

MATERIALS AND METHODS [5]

Apparatus and software

The UPLC (Waters, Model Acquity) was coupled with Mass spectrometer (Waters Quattro Premier XE) having Turbo Ion Spray (Waters Quattro Premier XE). The chromatographic integration was performed by MassLynx V4.1 software.

Chemicals and reagents

Sofosbuvir and Sofosbuvir-d3 (IS) were procured from Mylan Laboratories Ltd, Hyderabad, Formic acid, Methanol and ethyl acetate was procured from Merck Specialities Pvt. Ltd, Mumbai, India. Water used was collected from water purification systems (Milli Q, MilliPore, USA) installed in the laboratory. Pooled drug-free expired frozen human plasma (K2-EDTA as anticoagulant) was obtained from Blood Bank, Hyderabad, was used during validation and study sample analysis. The plasma was stored into−70±5 °C.

Standards and working solutions

Calibration standard solutions

Stock solutions of sofosbuvir and Sofosbuvir-d3 internal standard (IS) were prepared in methanol. Further dilutions were carried out in 50% methanol. Calibration standards often concentration levels were prepared freshly by spiking drug-free plasma with a sofosbuvir stock solution to give the concentrations of 4.063, 8.125, 62.5, 125.0, 250, 500, 1000, 2000, 4000 and 8000ng/ml.

Quality control standards

Lowest quality control standards, Median quality control standards and highest quality control standards were prepared by spiking drug-free plasma with sofosbuvir to give a solution containing 11.488, 52.180 and 7252.503 ng/ml respectively. They were stored at −20 °C till the time analysed.

Chromatographic conditions

Chromatographic separation was performed on Gemini 5μ C18, 50 x 4.6 mm, analytical column and the mobile phase was a mixture of 0.1% (v/v) formic acid in water to methanol at a ratio of 30:70 v/v. Injection volume was 10μL. The flow rate was 0.50 ml/min. Total analysis time of single injection was 2.0 min. Column oven temperature and autosampler temperature was set to 30 °C and 10 °C, respectively.

Mass spectrometric conditions

The LC eluent was split, and approximately 0.100 ml/min was introduced via electrospray ionisation using a Turbo Ion Spray interface set at 325 °C to generate positive ions [M+H]+. The Mass spectrometric parameters were optimised as shown in table no 1.
Sample preparation method

To 250 µl of plasma, 50 µl of ISTD (1 µg/ml) and 50 µl of 0.1% formic acid was added and vortexed. The drug was extracted with 2.5 ml of ethyl acetate, followed by centrifugation at 2000 rpm/min on a cooling centrifuge for 15 min at 4 °C. The supernatant of 2 ml was withdrawn and evaporated at 50 °C 15 psi of nitrogen until dryness at LV evaporator. The residue was reconstituted with 500 µl of mobile phase, and respective samples were injected into the column.

Validation [9-13]

Specificity

A solution containing 4.063 ng/ml was injected onto the column under optimised chromatographic conditions to show the separation of sofosbuvir from impurities and plasma. The specificity of the method was checked for the interference from plasma.

Linearity

Spiked concentrations were plotted against peak area ratios of sofosbuvir to the internal standard and the best fit line was calculated. Wide range calibration was determined by solutions containing 4.063 to 8000.010 ng/ml.

Recovery studies

The % mean recoveries were determined by measuring the responses of the extracted plasma Quality control samples at HQC, MQC and LQC against un-extracted Quality control samples at HQC, MQC and LQC.

Precision and accuracy

The between-run (Inter-day) accuracy and precision evaluation were assessed by the repeated analysis of human Ks EDTA plasma samples containing different concentrations of sofosbuvir on separate occasions. A single run consisted of a calibration curve plus six replicates of the lower limit of quantitation, low, medium and high-quality control samples.

Within-run (Intraday) accuracy and precision evaluations were performed by analysing replicate concentrations of sofosbuvir in human Ks EDTA plasma. The run consisted of a calibration curve plus a total of 24 spiked samples, six replicates of each of the LLOQ, lower, medium and higher quality control samples.

Matrix effect

The matrix effect for the intended method was assessed by using chromatographically screened human plasma. Concentrations equivalent to LLOQ of Sofosbuvir were prepared with seven different plasma batches/ lots. Samples were analysed along with one set of freshly spiked QC Standards prepared in the screened biological matrix.

Ruggedness

The ruggedness of the method was assessed by analysing a precision and accuracy batch using a different column, by the different analyst in another instrument.

Stability studies

Short-term stock solution stability of sofosbuvir

Solutions of sofosbuvir were prepared in methanol (Stability Samples) and were kept at room temperature for 6 h 30 min. A freshly prepared solution of sofosbuvir (Comparison Samples) and stability samples were diluted at approximately the same analyte concentration and analysed in a single run; analyte responses were used to determine % stability over time.

Long-term stock solution stability of internal standard

Solutions of internal standard (Sofosbuvir-d3) were prepared in methanol (Stability Samples) and were kept at refrigerator (2-8 °C) for 10 D 02 H. A freshly prepared solution of internal standard (Comparison Samples) and stability samples were diluted at approximately the same analyte concentration and analysed in a single run; analyte responses were used to determine % stability over time.

Long-term stock solution stability of sofosbuvir

Solutions of Sofosbuvir were prepared in methanol (Stability Samples) and were kept at refrigerator (2-8 °C) for 10 D 02 H. A freshly prepared solution of sofosbuvir (Comparison Samples) and stability samples were diluted at approximately the same analyte concentration and analysed in a single run.

Freeze-thaw stability

Samples were prepared at low and high-quality control levels, aliquoted and frozen at -70 °C. Some of the aliquots of quality control samples were subjected to five freeze-thaw cycles (stability samples). A calibration curve and quality control samples were analysed and processed with 6 replicates of stability samples and analysed in a single run.

RESULTS AND DISCUSSION

The chromatography observed during the course of validation was acceptable and representative chromatograms of standard blank, HQC, MQC, LQC and LLOQ are shown in fig. 1-3.
Fig. 1: Chromatograms of standard blank and HQC matrix

Fig. 2: Chromatograms of MQC and LQC

Fig. 3: Chromatograms of LLOQ
The method developed was validated for specificity, accuracy and precision, linearity, ruggedness and stability as per FDA guidance [9-11]. The results of validating parameters are given below.

**Specificity**

Nine different lots of plasma were analysed to ensure that no endogenous interferences were present at the retention time of sofosbuvir and Sofosbuvir-d3. Nine LLOQ (4.063 ng/ml) level samples along with plasma blank from the respective plasma lots were prepared and analysed. (table 2). In all plasma blanks, the response at the retention time of sofosbuvir was less than 20% of LLOQ response and at the retention time of IS, the response was less than 5% of mean IS response in LLOQ. The typical chromatogram of plasma blank and the chromatogram of LLOQ was shown in (fig. 1).

| S. No. | Drug response | ISTD response |
|-------|---------------|---------------|
|       | STD BL Area | LLOQ Area | STD BL RT | LLOQ RT | % Interference | STD BL Area | LLOQ Area | STD BL RT | LLOQ RT | % Interference |
| 01    | 0            | 298         | 0.800     | NIL     | 0            | 61776       | 0.800     | NIL     | 0     | 66613 |
| 02    | 0            | 290         | 0.800     | NIL     | 0            | 70621       | 0.800     | NIL     | 0     | 67694 |
| 03    | 0            | 334         | 0.800     | NIL     | 0            | 64807       | 0.800     | NIL     | 0     | 65249 |
| 04    | 0            | 267         | 0.807     | NIL     | 0            | 67694       | 0.800     | NIL     | 0     | 68774 |
| 05    | 0            | 271         | 0.800     | NIL     | 0            | 62927       | 0.800     | NIL     | 0     | 62927 |
| 06    | 0            | 303         | 0.800     | NIL     | 0            | 37012       | 0.800     | NIL     | 0     | 37012 |
| 07    | 0            | 281         | 0.800     | NIL     | 0            | 66441       | 0.800     | NIL     | 0     | 66441 |
| 08    | 0            | 255         | 0.800     | NIL     | 0            | 62927       | 0.800     | NIL     | 0     | 62927 |
| 09    | 0            | 147         | 0.800     | NIL     | 0            | 37012       | 0.800     | NIL     | 0     | 37012 |
| 10    | 0            | 283         | 0.800     | NIL     | 0            | 66441       | 0.800     | NIL     | 0     | 66441 |

**Linearity**

The calibration curve (peak area ratio Vs Concentration) was linear over working range of 4.063 to 8000.010ng/ml with ten point calibration used for quantification by linear regression, shown in (fig. 2). The regression equation for the analysis was:

\[ Y=0.0011227x-0.000164437 \]

\[ r^2 = 0.9985 \]

**Recovery**

The % mean recovery for sofosbuvir in LQC, MQC and HQC was 75.47%, 74.37% and 76.26% respectively (table 3).

| S. No. | HQC Aqueous area ratio | MQC Aqueous area ratio | LQC Aqueous area ratio | HQC Extracted area ratio | MQC Extracted area ratio | LQC Extracted area ratio |
|--------|------------------------|------------------------|------------------------|-------------------------|-------------------------|-------------------------|
| 01     | 13.466                 | 8.226                  | 0.981                  | 0.598                   | 0.021                   | 0.013                   |
| 02     | 13.541                 | 8.082                  | 1.010                  | 0.590                   | 0.022                   | 0.013                   |
| 03     | 13.318                 | 7.995                  | 0.995                  | 0.571                   | 0.021                   | 0.012                   |
| 04     | 13.133                 | 8.248                  | 1.001                  | 0.599                   | 0.021                   | 0.013                   |
| 05     | 12.997                 | 7.994                  | 0.985                  | 0.600                   | 0.021                   | 0.013                   |
| Mean   | 13.291                 | 8.109                  | 0.994                  | 0.597                   | 0.021                   | 0.012                   |
| SD     | 0.2265                 | 0.12243                | 0.0178                 | 0.0128                  | 0.00045                 | 0.00045                 |
| % CV   | 1.70                   | 1.51                   | 1.18                   | 2.06                    | 2.11                    | 3.49                    |
| % Mean Recovery | 76.26                | 74.37                  | 75.47                  | 75.36                   |                         |                         |
Intraday (within run) and Inter-day (between run) precision and accuracy

The within-run coefficients of variation ranged between 1.06% and 5.06% for sofosbuvir. The within-run percentages of nominal concentrations ranged between 97.21% and 105.93% for sofosbuvir. Results are presented in table 4.

The between-run coefficients of variation ranged between 2.04% and 5.48% for sofosbuvir. The between-run percentages of nominal concentrations ranged between 98.34% and 100.58% for sofosbuvir. Results are presented in table 4.

Matrix effect

The % accuracy of LLOQ samples prepared with the different biological matrix lots were found within the range of 89.49% to 97.49% which were found within the range of 80.00-120.00% for the seven different plasma lots. % CV for LLOQ samples was observed as 2.87% which are within 20.00% of the acceptance criteria. Results are presented in table 5.

| QC ID | HQC | MQC | LQC | LLOQ QC |
|-------|-----|-----|-----|---------|
| Concentration (ng/ml) | 725.2503 | 522.180 | 11.488 | 4.136 |
| Within Batch Precision and Accuracy | Calculated Concentration (ng/ml) | 691.0342 | 511.080 | 11.360 | 4.290 |
| | 700.9484 | 518.984 | 10.484 | 3.998 |
| | 718.9506 | 514.176 | 11.501 | 4.116 |
| | 715.6740 | 511.840 | 11.892 | 4.132 |
| | 698.4985 | 504.031 | 11.887 | 4.477 |
| Mean | 705.0211 | 512.0222 | 11.4788 | 4.2026 |
| SD | 118.5622 | 5.42876 | 0.58102 | 0.18526 |
| % CV | 1.68 | 1.06 | 5.06 | 4.41 |
| % Mean Accuracy | 97.21 | 98.05 | 99.92 | 101.61 |
| PandA I | | | | |
| Concentration (ng/ml) | 723.4610 | 533.680 | 12.086 | 4.263 |
| Mean | 719.2185 | 531.929 | 12.605 | 4.266 |
| SD | 727.2508 | 523.890 | 12.1690 | 4.2034 |
| % CV | 1.08 | 1.11 | 2.94 | 1.99 |
| % Mean Accuracy | 100.47 | 101.39 | 105.93 | 101.63 |
| PandA II | | | | |
| Concentration (ng/ml) | 735.1433 | 522.452 | 11.705 | 4.172 |
| Mean | 730.8960 | 529.4556 | 12.1690 | 4.2034 |
| SD | 78.93435 | 5.88296 | 0.35753 | 0.08377 |
| % CV | 1.08 | 1.11 | 2.94 | 1.99 |
| % Mean Accuracy | 97.35 | 98.65 | 95.88 | 103.39 |
| PandA III | | | | |
| Concentration (ng/ml) | 716.1887 | 520.892 | 11.414 | 4.123 |
| Mean | 703.6505 | 514.024 | 11.006 | 4.395 |
| SD | 696.0208 | 497.103 | 10.554 | 4.354 |
| % CV | 1.51 | 2.06 | 2.79 | 2.86 |
| % Mean Accuracy | 97.35 | 98.65 | 95.88 | 103.39 |
| Between Batch Precision and Accuracy | | | | |
| Mean | 705.9597 | 515.1164 | 11.0150 | 4.2764 |
| SD | 106.7109 | 10.58730 | 0.30752 | 0.12214 |
| % CV | 1.51 | 2.06 | 2.79 | 2.86 |
| % Mean Accuracy | 97.35 | 98.65 | 95.88 | 103.39 |

Ruggedness

The coefficients of variation ranged between 0.35% and 3.09% for sofosbuvir. The percentages of nominal concentrations ranged between 93.2% and 99.29% for sofosbuvir. Results are presented in table 6.

Stability studies

Short-term stock solution stability of sofosbuvir and internal standard

Sofosbuvir and internal standard were found to be stable in methanol for 6 h 30 min at room temperature with a % stability of 97.25% and 97.0% respectively. Results are presented in table 7.

Long-term stock solution stability of sofosbuvir and internal standard

Sofosbuvir and internal standard were found to be stable in methanol with 10 D 02 H at refrigerator (2-8 °C) with a % stability of 98.81% and 107.96% respectively. Results are presented in table 8.

 Freeze-thaw stability

Sofosbuvir is found to be stable in human K3 EDTA plasma after five freeze-thaw cycles at-70 °C with coefficients of variation of 3.27% (LQC) and 3.86% (HQC) for sofosbuvir, and the percentages of nominal concentrations for sofosbuvir were found to be 103.17% (LQC) and 101.23% (HQC). Results are presented in table 9.
Table 6: Results of ruggedness with different column

| QC ID | HQC | MQC | LQC | LLOQ QC |
|-------|-----|-----|-----|---------|
| Conc.(ng/ml) | 7252.503 | 522.180 | 11.488 | 4.136 |
| Panda ID | Calculated concentration (ng/ml) | | | |
| Column | Acquisition batch ID: 031008PandADC01 | | | |
| Mean | 7128.3884 | 521.3342 | 11.4070 | 3.8546 |
| SD | 145.55342 | 4.13570 | 0.06040 | 0.11925 |
| % CV | 2.04 | 0.79 | 0.53 | 3.09 |
| % Mean Accuracy | 98.29 | 99.84 | 99.29 | 93.20 |

Table 7: Short-term stock solution stability of drug and ISTD

| S. NO. | Drug | ISTD | Nominal Conc (ng/ml) | Nominal Conc (µg/ml) |
|--------|------|------|----------------------|----------------------|
| Area ratio | Comparison samples | Stability samples | Area ratio | Comparison samples | Stability samples |
| 01 | 9.134 | 9.076 | 0.116 | 0.115 |
| 02 | 9.181 | 8.829 | 0.117 | 0.114 |
| 03 | 9.147 | 9.090 | 0.115 | 0.117 |
| 04 | 9.082 | 8.973 | 0.117 | 0.113 |
| 05 | 9.231 | 8.946 | 0.114 | 0.111 |
| 06 | 9.197 | 8.969 | 0.117 | 0.112 |
| Mean | 9.1620 | 8.9850 | 0.1160 | 0.1137 |
| SD | 0.05245 | 0.09532 | 0.00126 | 0.00216 |
| % CV | 0.57 | 1.06 | 1.09 | 1.90 |
| % Mean Stability | 97.25 | 97.00 |

Table 8: Long-term stock solution stability of drug and internal standard

| S. NO. | Drug | ISTD | Nominal Conc (ng/ml) | Nominal Conc (µg/ml) |
|--------|------|------|----------------------|----------------------|
| Area ratio | Comparison samples | Stability samples | Area ratio | Comparison samples | Stability samples |
| 01 | 9.219 | 9.049 | 0.108 | 0.111 |
| 02 | 9.116 | 9.111 | 0.107 | 0.110 |
| 03 | 9.228 | 9.026 | 0.108 | 0.115 |
| 04 | 8.918 | 9.141 | 0.112 | 0.119 |
| 05 | 9.208 | 9.073 | 0.111 | 0.119 |
| 06 | 9.138 | 9.022 | 0.113 | 0.114 |
| Mean | 9.1378 | 9.0703 | 0.1098 | 0.1147 |
| SD | 0.11700 | 0.04777 | 0.00248 | 0.00383 |
| % CV | 1.28 | 0.53 | 2.26 | 3.34 |
| % Mean Stability | 98.81 | 107.96 |

Table 9: Freeze-thaw stability at-70 °C

| S. No. | HQC | LQC | Nominal Conc (ng/ml) | Nominal Conc (ng/ml) | % accuracy | % accuracy |
|--------|-----|-----|----------------------|----------------------|------------|------------|
| 7252.503 | 11.488 | | | | | |
| Calculated Conc (ng/ml) | | | | | | |
| 1 | 7255.363 | 100.04 | 11.571 | 100.72 |
| 2 | 6985.35 | 96.32 | 11.547 | 100.51 |
| 3 | 7017.724 | 96.76 | 12.168 | 105.92 |
| Calculated Conc (ng/ml) | | | | | | |

CONCLUSION

Chromatographic separation was performed on Gemini 5µ C18, 50 x 4.6 mm, analytical column and the mobile phase was a mixture of 0.1% (v/v) formic acid in water to methanol at a ratio of 30:70 v/v. The drug was extracted from the sample with 2.5 ml of ethyl acetate. The specificity of the method was checked for the interference from plasma. Wide range calibration was determined by solutions containing 4.063 to 8000.010ng/ml. The % mean recovery for sofosbuvir in LQC, MQC and HQC was 75.47%, 74.37% and 76.26% respectively. The within-run coefficients of variation ranged between 1.06% and 5.06% for sofosbuvir. The between-run coefficients of variation ranged between 2.04% and 5.48% for sofosbuvir the % accuracy of LLOQ samples prepared with the different biological matrix lots were found within the range of 89.49 to 97.49%. Stability test were performed to assess the long term and short term stability of sofosbuvir sample solutions,
internal standard solutions. The developed method was validated for the quantitative determination of sofosbuvir from plasma was simple, rapid, specific, sensitive, accurate and precise. Hence, the method is quite suitable to detect the drug from plasma samples of human volunteers.

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CONFLICT OF INTERESTS

Declared none

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