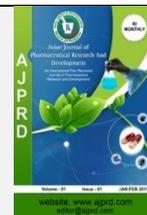


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Research Article

## Quantification of plasma levels of antiviral drug sofosbuvir and its metabolite GS331007 in patients of chronic hepatitis C with chronic kidney disease using UPLC-MS/MS method

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### ABSTRACT

Sofosbuvir based regimens are the only treatment available in India and some other Asian countries for curing chronic hepatitis C viral (HCV) infection. The main excretion route of sofosbuvir is renal so treatment of HCV infection is challenging in patients on hemodialysis. A simple and sensitive UPLC-MS/MS method was developed and validated according to USFDA guidelines on Linear Ion trap Quadrupole tandem mass spectrometer (QTRAP4500) which was applied to estimate the drug concentration of sofosbuvir and its metabolite GS331007 in patients of chronic kidney disease with HCV infection. Clopidogrel was used as internal standard (IS) for this study. All analytes and IS were separated on UPLC C18-HSS column (2.1mmX50mm, 1.7µm) with retention time of 2.07, 0.29 and 1.58 min, respectively, by using mobile phases of 5mM ammonium acetate in 0.2% formic acid in water (A) and 5mM ammonium acetate in 0.2% formic acid in methanol (B) on gradient elution mode at a flow rate of 0.6 mL/min. Calibration curve was plotted over the range of 0-100 g/mL for both of the analytes and equation was calculated by applying linear regression method. Detections of daughter ions (sofosbuvir-530 to 243m/z, GS331007-261 to 112.1m/z and clopidogrel- 322 to 154.9m/z) were done in multiple reactions monitoring (MRM) mode and weights were analyzed by using Linear ion trap quadrupole mass spectrometer with turbo spray ion source. The developed method has been successfully used for quantification of drug concentration of sofosbuvir and GS331007 to see the safety of sofosbuvir in patients of chronic HCV infection with renal failures.

**Keywords:** sofosbuvir, GS331007, renal dysfunction, hemodialysis, UPLC-MS/MS

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### INTRODUCTION

Hepatitis C virus (HCV) was discovered 25 years ago<sup>1</sup> and affects up to 185 million individuals worldwide.<sup>2-4</sup> This infection has been a source of high levels of morbidity and mortality due to hepatic complications (cirrhosis, hepatocellular carcinoma) and also has been responsible for extra hepatic

manifestations.<sup>5,6</sup> Infection of HCV is highly prevalent in chronic kidney disease (CKD) patients specifically in patients on hemodialysis.<sup>7-9</sup> It has been associated with an increase in morbidity and mortality in patients with renal failure and renal transplant recipients as well.<sup>10-13</sup> Interferon therapy in combination with ribavirin had been the ancient treatment for HCV infection but had

higher dropout rates in specifically patients of renal dysfunction.<sup>14, 15</sup> The pressing need for safe and effective antiviral therapies has led to advances in treatment of HCV infection with advent of direct antiviral agents (DAAs).<sup>16</sup> Sofosbuvir is a cytidine analogue, pan-genotypic, nucleoside polymerase inhibitor. It is a nucleotide-phosphoramidate prodrug having high solubility and low permeability. It is extensively metabolized in hepatocytes in the pharmacologically active nucleotide analog uridine-triphosphate. Later dephosphorylation results in formation of predominant inactive metabolite GS331007. Its main route of elimination is renal.<sup>17-19</sup>

However, there are previous reports on development of method on UPLC-MS/MS for sofosbuvir in rat plasma and in health human plasma.<sup>20-25</sup> To the best of our knowledge, there is no published evidence on development/ validation of UPLC-MS/MS method for quantification of sofosbuvir and its metabolite and its application in quantification of drugs in patients of c HCV infection with renal dysfunctions from the Indian subcontinent. Sofosbuvir based regimens are the only available treatment for hepatitis C in India. Nowadays, these are widely used. Owing to renal excretion of sofosbuvir, it is very crucial to know the concentration of sofosbuvir in patients of renal failure. Therefore, the present study was designed to develop a simple and sensitive UPLC-MS/MS method for quantification of sofosbuvir and its predominant metabolite GS331007 in plasma of patients of chronic HCV infection with chronic kidney disease taking sofosbuvir as their treatment in different combinations.

### Methodology

This prospective observational study was carried out at a public teaching tertiary care hospital. The study was conducted according to ICMR ethical guidelines for biomedical research on human participants. The study was approved by institutional ethics committee. The confidentiality of patients was respected and no information that discloses the patients' name and identity was released or published in any form, whatsoever. Written informed consent was obtained from the subjects before enrolling in the study.

### Materials

Sofosbuvir and its metabolite GS331007 were received as gift sample for educational purpose from M/s Cadila Health Care Ltd., Ahmedabad and clopidogrel was obtained likewise from M/s Ind Swift Laboratories. All other chemicals were of analytical-reagent grade. Methanol, ethyl acetate, and acetonitrile used were LCMS grade. Ammonium acetate was purchased from Sigma Aldrich. Water obtained from ELGA water purification unit (Elga Ltd., Bucks) was utilized to prepare all the solutions.

### Instruments

The major instrument used in the study included ultra performance liquid chromatograph (UPLC) with tandem mass spectrometer (MS/MS) under linear ion trap quadrupole detection (ABSciex QTRAP 4500) at the biochemistry department of advanced pediatrics centre at PGIMER, Chandigarh. Ion source was turbo spray and scan type was multiple reactions monitoring (MRM).

Source temperature was of 400°C and curtain, GS1, GS2 and collision gas were set at 25, 30, 14 and 10 respectively. The ion spray capillary voltage was 4000V and CAD was used at 8mL/min.

### Patients' sample collection and storage

Blood samples were collected from patients at Hepatology department after taking informed consent and samples were taken into heparinized tubes at one time point i.e. after 2 hours of the administration of drugs. In case of patients on hemodialysis, sample was taken before hemodialysis after 2 hours of administration of drug and after completion of hemodialysis. Plasma was separated from blood by centrifugation at 7000 rpm for 8 minutes at 4°C stored in an ultra deep freezer at -80°C till the analysis time at.<sup>26-28</sup>

### UPLC-MS/MS conditions

A gradient UPLC method was developed for the analysis of sofosbuvir and GS331007 along with internal standard from plasma samples of CHC patients with CKD. The drug was extracted from the biological matrix in methanol by using protein precipitation, and liquid-liquid extraction method. Analytes were separated using UPLC HSS C18 (2.1mm×50mm, 1.7µm) column (Thermo Fisher Scientific) with 5mM ammonium acetate in 0.2% formic acid in water (A) and 5mM ammonium acetate in 0.2% formic acid in methanol (B) in gradient elution mode at a flow rate of 0.6 mL/min. Injection volume was kept at 5µL and run time was of 5 minutes. Gradient elution program included 0-1.0 min (90%A-10%B), 1.0-2.0 min (50-50%), 2.0-4.0 min (5-95%), and 4.0-5.0 min (90-10%). Linear ion trap quadrupole mass spectrometer with turbo spray ion source was used to analyze the concentration of the analytes. Detections were done in multiple reactions monitoring (MRM) mode and dwell time for each transition was 100ms. MRM transitions were m/z 530 to 243 for sofosbuvir, m/z 261 to 112 for GS331007, and 321 to 154.9 for clopidogrel (IS). The mass spectrometer was operated at ion spray capillary voltage of 4000V, source temperature 400°C, and CAD gas flow was 8mL/min. The analyst software version 1.6.2 was used to analyze the data.

### Method validation

According to the US Food and Drug Administration (FDA) guidelines for the bioanalytical method development and validation, the linearity of the developed method was evaluated and a calibration curve was plotted.<sup>29</sup>

### Preparation of standard stock and working solutions for calibration curve

Stock solutions of sofosbuvir, GS331007 and internal standard (IS, clopidogrel) were prepared with 1mg/mL concentration in the methanol. Further dilution of the same was done in 100% methanol (CH<sub>3</sub>OH) to get secondary stock solutions having concentration of 100µg/mL. Working stock solutions were prepared fresh at each time of use. All samples were stored at -20°C.

### Preparation of calibration curve (CC) and quality control (QC) samples

An aliquot of 20µL from each working standard was spiked in 50µL of blank human plasma to yield calibration

curve samples of concentration ranging from 5 to 100 µg/mL (0, 5, 10, 50, 75, and 100 µg/mL of sofosbuvir and GS331007). Further, blank sample (plasma sample processed without internal standard) and zero sample (plasma processed only with internal standard) were prepared. All samples were stored at 4°C during experimentation.

### Sample extraction from plasma

QC and calibration standard plasma samples were extracted by using liquid liquid extraction technique. To each tube containing 50 µL plasma (blank plasma, sample plasma, and plasma spiked with sofosbuvir and GS331007 for preparation of calibration and QC samples), 100 µL aliquot of extracting solvent ethyl acetate, containing 25 µg/mL clopidogrel (IS) solution was added. The mixture was vortex-mixed for 10 seconds followed by 2 minutes at room temperature. Then 250 µL aliquot of precipitator methanol was added followed by 1 min vortex and kept for 2 min at room temperature. This mixture was centrifuged at 14000rpm for 8min.

The supernatant 200 µL was transferred into another centrifuge, out of which 05 µL was injected into the TMS system for analysis.<sup>27</sup>

### Selectivity

The selectivity of the developed method was evaluated by using six different human plasma samples from different sources to assess the interferences from plasma components between peaks of analytes and internal standard.

### Linearity

For the evaluation of linearity, calibration curves were constructed (0-100g/mL) by plotting the peak area ratio of sofosbuvir and GS331007 to IS versus concentration. Calibration curves containing six points were assayed in triplicate. Concentration of analytes were calculated in accordance with calibration curves.

### Carry-over

Carry over was assessed by injecting blank samples after run of calibration standards at upper limit of quantification (ULOQ) of the linearity range. The carry over should be less than 20% of the peak response of the lower limit of quantification (LLOQ) of linearity range.<sup>29</sup>

### Extraction recovery and matrix effect

The extraction recovery of sofosbuvir, GS331007, and clopidogrel from human plasma was assessed by comparing the peak area of extracted samples and unextracted standards at equivalent concentrations. Matrix

effects were evaluated by comparing peak areas of samples spiked with standard solutions of drugs with peak areas of reference standard solution at equivalent concentrations.

### Estimation of plasma concentration of sofosbuvir and GS331007

Analytes' concentrations at a particular time were calculated using linearity plot. Hemodialysis extraction ratio was calculated with paired pre-HD and post-HD plasma concentrations as (preHD-postHD) / preHD.<sup>30</sup>

## RESULTS AND DISCUSSION

### Optimization of the chromatographic conditions

Several trials were carried out for selection of mobile phase and internal standard. IS was selected on the basis of chromatographic separation parameters after multiple trials with various drugs. The pKa and structure of the compound was found comparable to that of the analyte. Clopidogrel (5.14) was selected as internal standard (IS) as it has basic pKa same as sofosbuvir (9.38). Hence, it showed somewhat similar retention behaviour on the UPLC column. Clopidogrel had separate retention time than analytes and eluted well with selected mobile phase. In addition, a significant advantage of this IS was elution time which was longer than metabolite.

Different mobile phases were tried. Firstly, 0.1% formic acid with acetonitrile was used but resolution of extraction of metabolite was low. Different concentrations of formic acid for mobile phase with different flow rates were tested. Many trials were conducted at isocratic mode and different gradient mode with different mobile phases at different flow rates. It was very difficult to fix the mobile phases and their concentration for optimum pH to resolve out all analytes included sofosbuvir, its metabolite, and internal standard (clopidogrel). Parameters of mass spectrometer were optimized in respect to positive or negative ion mode. Finally, ESI positive mode was used to achieve the method. Different amount of collision energies were used to fix the daughter ions.

The best resolution for all the analytes was achieved on UPLC HSS C18 (2.1mm×50mm, 1.7µm) with gradient method. Mobile phase composition was 10mM ammonium acetate in 0.2% formic acid in water (A) and 5mM ammonium acetate in 0.2% formic acid in methanol (B) in flow rate of 0.6 mL/min. As shown in Fig 1(a), 1(b), 1(C), the sofosbuvir, GS331007 and IS (clopidogrel) were separated on the developed method, respectively. The developed method showed sufficient resolution in three peaks. (Figure 1)

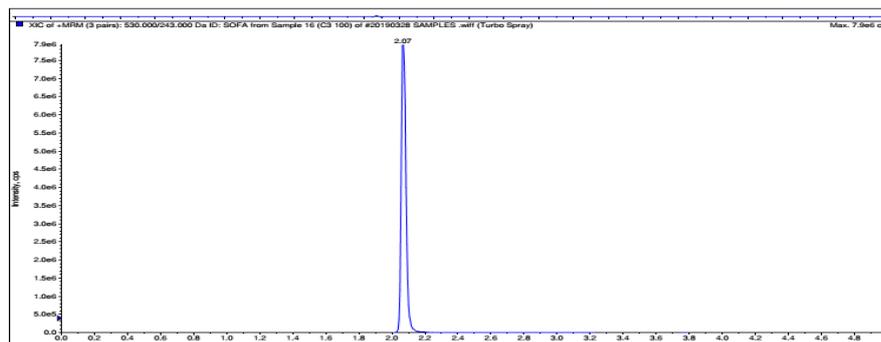


Figure 1:(a)

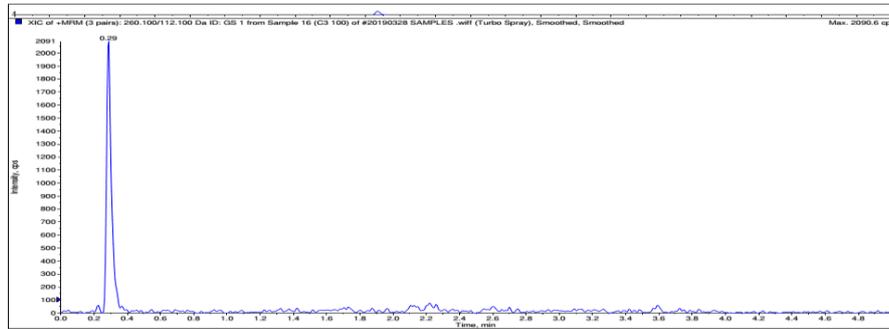


Figure 1:(b)

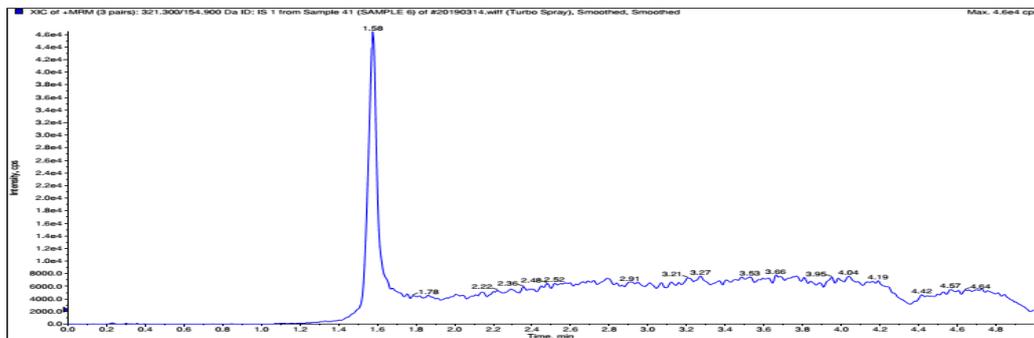


Figure1:(c)

Figure 1: Chromatograms of drug standard solutions

Figure 1(a) Chromatogram of sofosbuvir , Figure 1(b) Chromatogram of GS331007, Figure 1(c) Chromatogram of clodipogrel on developed method

Various ways of plasma extraction were tested under the category of liquid-liquid extraction. Acetonitrile and methanol were used as precipitating solvents. Different drying mechanisms including Turbovap Nitrogen sample concentration system and Eppendorf sample concentrator were tested. Finally, ethyl acetate as extracting solvent

and methanol as precipitation solvent were used in liquid-liquid extraction procedure. After spiking blank human plasma with equal concentration of drug standard solutions, chromatograms on developed method follows as in Figure 2.

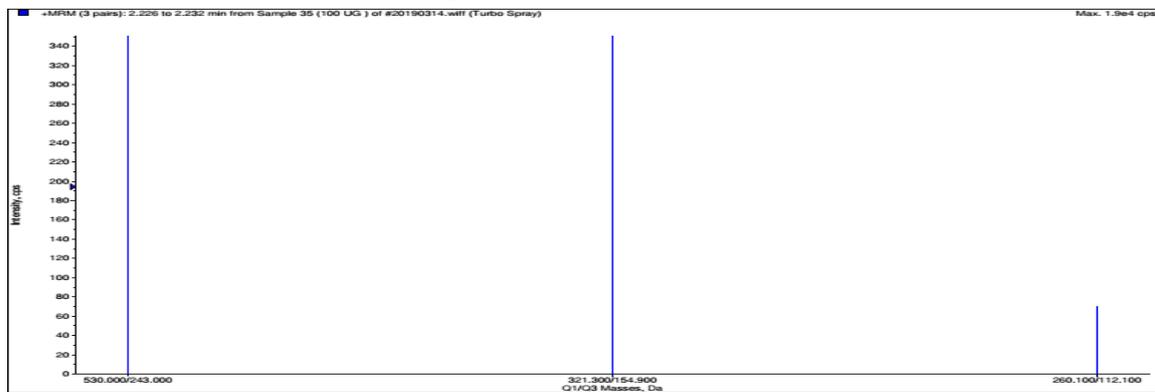


Figure 2 (a) Mass spectra of sample spiked with LLOQ on developed method

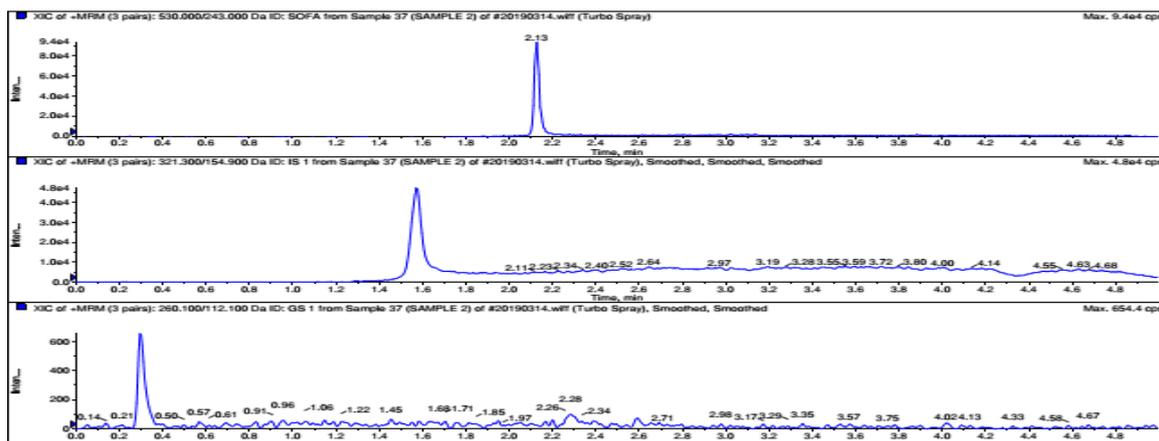


Figure 2 (b) Chromatogram of sample spiked with LLOQ on developed method

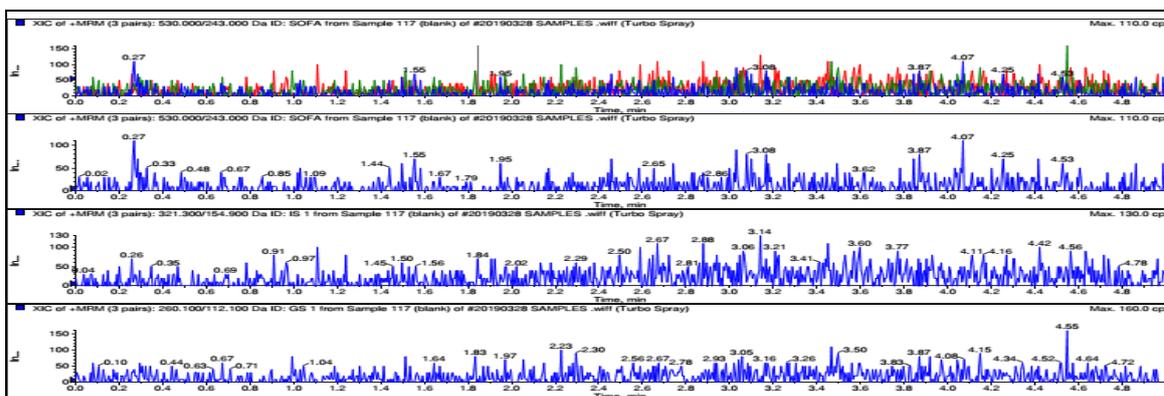


Figure 2 (c): Chromatogram of blank plasma sample on developed method

Figure 2: Mass Chromatograms of analytes and blank plasma

The overlaid chromatograph with mass spectra for non-zero sample (plasma spiked with LLOQ concentrations of both analytes along with IS) and blank human plasma shown in Fig 2(a), Fig 2(b) and Fig 2(c), respectively.

**Method Validation**

**Selectivity**

There was no significant interference from human plasma on retention time and peak response of analytes. The retention time for sofosbuvir, GS331007 and IS was 2.07, 0.29 and 1.58 min, respectively.

**Linearity of calibration curve and LLOQ**

The linearity of the developed method was evaluated by preparing standard curves in the concentration range of 0-100µg/mL of sofosbuvir and GS331007. Good linearity was established across the studied range in plasma. The calibration curve for both drugs (as depicted in Fig. 3a and 3b) were constructed between concentrations of drug

(µg/mL) vs area ratio of analyte to IS as shown in Table 1. Data was analyzed by linear regression to get the regression equation and coefficient of correlation. Value of LLOQ was 5µg/mL and ULOQ was 100µg/mL.

**Carry over**

Blank plasma samples were injected after calibration standards at ULOQ (100µg/mL) for all analytes. The carryover was less than 20% of LLOQ in the blank samples.

**Extraction recovery and matrix effect**

Matrix effect was seen by using blank samples from six different volunteers. The average matrix effects of all analytes were from 97.2% to 102.4% at different corresponding concentration ranges. The average extraction recoveries of all the three analytes including IS was ranged from 78.4% to 93.7%.

Table 1: Calibration Curve data for analytes

Concentration (µg/mL)	Average analyte area (Sofosbuvir)	Average analyte area (GS331007)	Average-area (Internal standard)	Area Ratio (analyte/IS)	Area Ratio (analyte/IS)
0	0	0	0	0	0
5	1656230.4	298.867	1762.729	721.35453	0.169597277
10	2566148.1	635.123	1955.365	1312.36265	0.324808184
50	8440941.6	1340.294	1531.369	5513.3514	1.005878511
75	14878015	2930.713	1799.979	8265.56389	1.627777778
100	19511552	4348.178	1991.332	10615.6431	2.183827223

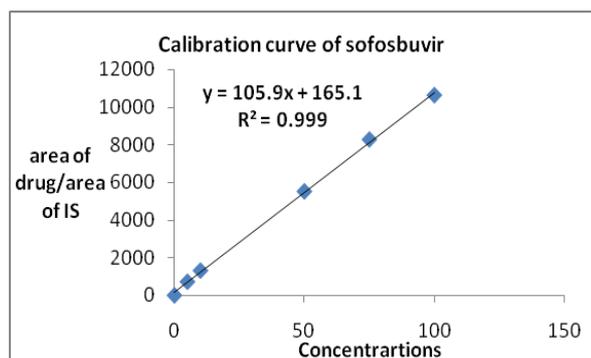


Figure 3(a) Calibration curve for sofosbuvir

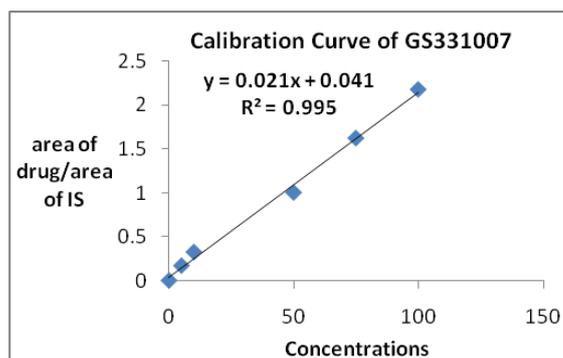


Figure 3 (b) Calibration curve for GS331007

Figure 3: Calibration curves for determination of sofosbuvir and GS331007

**Estimation of Plasma Concentration of sofosbuvir and GS331007 from plasma of patients of hepatitis C with chronic kidney diseases (Pharmacokinetic study)**

This is first Indian study to successfully establish bioanalytical method on UPLC-MS/MS for sofosbuvir and its metabolite in human plasma. The validated UPLC-MS/MS bioanalytical method was used for estimation of the concentration of analytes in human plasma, 30 patients of CHC with CKD who were administering sofosbuvir. The mean plasma concentrations as per their stage of renal dysfunction were illustrated in Table 2 and Table 3.

This study found the concentration of sofosbuvir only before the hemodialysis and concentration was not detected after the dialysis. This present study was compared with one report which showed comparable results.<sup>30</sup> The concentration of sofosbuvir was not detected in patients with normal renal function and mild renal impairment.(Table 2, 3) It was detected in limited number of patients with severe renal impairment and ESRD i.e. only before hemodialysis. Hemodialysis extraction ratio for metabolite was found to be 50%, which was comparable to results of one other report.<sup>17, 30</sup>

**Table 2:** UPLC-MS/MS data of CHC patients on dialysis

Samples #	Sofosbuvir (µg/mL)(m/z530Da)		GS331007 (µg/mL) (m/z 260Da)	
	Before HD	Post HD	Before HD	Post HD
1	<LLOQ	<LLOQ	12.56	12.45
2	<LLOQ	<LLOQ	8.55	7.8
3	<LLOQ	<LLOQ	34.56	27.05
4	<LLOQ	<LLOQ	20.92	13.84
5	<LLOQ	<LLOQ	13.86	11.87
6	<LLOQ	<LLOQ	5.4	2.62
7	<LLOQ	<LLOQ	20.03	14.52
8	1.77	<LLOQ	9.37	5.19
9	<LLOQ	<LLOQ	13.85	6.03
10	<LLOQ	<LLOQ	3.79	2.85
11	<LLOQ	<LLOQ	4.19	2.02
12	0.99	<LLOQ	19.08	10.64
13	<LLOQ	<LLOQ	14.27	8.95
14	<LLOQ	<LLOQ	8.13	3.68
15	0.45	<LLOQ	2.86	0.81
16	0.86	<LLOQ	6.21	1.08
17	<LLOQ	<LLOQ	11.53	6.37
18	<LLOQ	<LLOQ	40.94	17.62
19	<LLOQ	<LLOQ	6.5	4.75
20	<LLOQ	<LLOQ	19.43	18.97
21	<LLOQ	<LLOQ	3.45	1.16
22	0.72	<LLOQ	8.68	7.78
23	0.65	<LLOQ	10.43	10.4
24	<LLOQ	<LLOQ	2.18	1.13

**Table 3:** UPLC-MS/MS data of CHC patients without hemodialysis

CKD Patients with CHC not on HD	Sofosbuvir concentration (µg/mL)	GS331007 Concentration (µg/mL)
GFR>30(400mg)	<LLOQ	15.93
GFR>30(400mg)	<LLOQ	11.88
GFR>30(400mg)	<LLOQ	8.09
GFR>30(200mg)	<LLOQ	4.33
GFR<30 (400mg)	0.73	22.06
GFR<30 (200mg)	<LLOQ	11.76

HD-hemodialysis, GFR unit- mL/min./1.73m<sup>2</sup>, 200mg-half dose of sofosbuvir, 400mg-full dose of sofosbuvir

**Limitations of the study:** This report did not include results on accuracy and precision of the developed method. The authors expect that this gap shall be addressed in future studies.

**CONCLUSIONS**

As per USFDA guidelines, a simple and sensitive method on UPLC-MS/MS for pharmacokinetic study of antiviral

drug sofosbuvir and its metabolite GS331007 in human plasma has been successfully developed and validated. This method was successfully used for estimation of drug concentration in patients in the present study so this method can be used for bioequivalence studies and therapeutic drug monitoring studies.

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