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Simultaneous quantification of Daclatasvir and Sofosbuvir in human plasma and pharmacokinetic study by LCMS/MS

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In the treatment of hepatitis C, direct-acting antivirals (DAA) are highly efficient and well tolerated with a series of DAA combinations available for treatment. A sensitive high-performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS) method has been developed and validated for the simultaneous quantification of Sofosbuvir (SOF) and Daclatasvir (DAC) in human plasma. Sofosbuvir D6 (SOF D6) and Daclatasvir $^{13}C_2{}^2H_6$ (DAC $^{13}C_2{}^2H_{6)}$ are used as internal standard (IS). Quantification for both the analytes has been attained with MS-MS detection in positive ion mode using an Acquity UPLC system (Waters) equipped with Waters Xevo TQ MS system with a Gemini NX 5µ C18 (50 × 2.0mm) (Phenomenex) column, and a gradient mobile phase consisting 5 mM Ammonium Formate buffer: Acetonitrile at a flow rate of 0.300 mL/min is used as mobile phase to separate the analytes and detection is performed by electrospray ionization technique using the mass spectrometer. Full validation is performed for bio-analytical methods with respect to linearity, precision, accuracy, selectivity, carry-over, stability and dilution integrity. Linearity is obtained over a concentration range of 10.002 -3000.488 and 10.004 -3001.218 ng mL^{-1} for SOF and DAC respectively by applying weighted least-squares linear regression method (1/*x*2). The developed method was applied successfully in bioequivalence and/or clinical studies in 48 male subjects for the simultaneous quantification of SOF and DAC.

Keywords: Bioequivalence, Daclatasvir, Good clinical practice, High performance liquid chromatography, Liquid Chromatography, Lower limit of quantification, Mass Spectrometry, Sofosbuvir

The significant efforts in field of HCV treatment has led to the discovery of new direct-acting antivirals, with better safety profile and improved antiviral potency[1]. In an approximation 170 million people worldwide are effected by chronic infection with hepatitis C virus (HCV) leading to cirrhosis and hepatocellular carcinoma. In the United States HCV-related deaths are increased as compared to human immunodeficiency virus (HIV) infection[2]. For chronic HCV infection approximately 40% of patients with genotype 1 infection and 75% of patients infected with genotype 2 or 3 had sustained virologic response after treatment with Peginterferon alfa–ribavirin[3,4]. The HCV NS5A replication complex inhibitor DAC is highly potent against six major HCV genotypes (1a, 1b, 2a, 3a, 4a, 5a)[5,6], with a pharmacokinetic profile obtained by once-daily dosing. The other HCV NS5B polymerase inhibitor SOF administered once daily orally has a good safety profile[7]. Both DAC and SOF have potent antiviral activity and broad genotypic coverage and are administered orally once daily. In treatment-naïve patients with HCV genotype 1, 2, or 3 the combination of DAC 60 mg once daily + SOF 400 mg once daily (with or without lead-in) ± weight-based ribavirin for 24 weeks was associated with high rates of sustained virologic response including patients showing no response to telaprevir or boceprevir therapy[8]. In both untreated and treated genotype 3-infected patients without cirrhosis combination of DAC with SOF for 12 weeks achieved high sustained virological response (SVR) rates[9]. Rational design of pharmacokinetic studies in humans is an essential component to determine the optimal dose and provide initial evaluation of efficacy on a therapeutic target. In this context measuring plasma drug concentration using bioanalytical techniques is an appropriate tool to understand drug-drug interactions and perceive the pharmacokinetic and/or pharmacodynamic properties of small molecules. Previously bioanalytical assays using LC-MS/MS have been developed for other DAA's orfor DAC and SOF separately or in combination[10-18], here we

developed and fully validated a bioanalytical method for the quantification of both DAC and SOF. The well validated method was further used to evaluate the bioequivalence of My Hep DVIR^{TM}(DAC/SOF) 60 mg /400 mg comprimes pellicules (tablets) of Mylan laboratories limited, India with daklinza ™ (DAC) tablets 60 mg (Bristol-Myers Squibb Company, Princeton, NJ 08543, USA) and Sovaldi® (SOF) 400 mg film-coated tablets (Gilead sciences international ltd. Cambridge CB 216GT United Kingdom), under fasting conditions in 48 normal healthy adult human subjects. The developed model is simple, sensitive, selective, efficient, and validated and is reliable for the determination of DAC & SOF and ensures precise & accurate for the determination of DAC & SOF. Figure 1 depicts the chemical structure of Sofosbuvir and Daclatasvir.

Experimental Section

The reference samples of DAC (99.2%) and SOF (99.4%) were obtained from Mylan laboratories ltd. and DAC-$^{13}C_2D_6$ (93.25%) and SOF-D_6 (96.20%) were obtained from Diacel chiral technologies. The Milli Q water purification system procured from Millipore (Bangalore, India) was used to prepare water for the LC-MS/MS analysis. Formic acid (suprapure grade) was purchased from Merck. Acetonitrile (HPLC grade) was purchased from Fisher or J.T. Baker (Phillipsburg, USA) while HPLC grade methanol was purchased from Merck, Fisher or J.T. Baker (Phillipsburg, USA). Ammonium Formate of grade GR was purchased from Sigma Aldrich. Human plasma sample for the control was obtained from Deccan's Pathological Lab's (Hyderabad, India).

LC–MS system and conditions

An Acquity UPLC system (Waters) equipped with Waters Xevo TQ MS system consisting of a Gemini NX 5µ C18 (50 × 2.0mm) (Phenomenex), a pump (Acquity binary sample Manager, Waters), and an auto sampler (Acquity sample manager) were used for the study. The injection volume (2 µL) for the processed samples were injected into the column, kept at ambient temperature (40 ± 5°C). The separation of the analytes wasperformed by using agradient mobile phase (5 mM Ammonium Formate buffer: acetonitrile) delivered at a flow rate of 0.300 mL/min. Electrospray ionization mode was selected for ionization of the analytes in the mass spectrometer. The positive ion mode was selected for quantification of both analytes and the internal standards with MS-MS detection using a Waters Xevo TQS system. The desolvation temperature was set to 500°C, desolvation gas flow rate at 1000(L/Hr) and capillary voltage at 3.50 KV. The ion spray voltage was set at 5500 V. The cone voltage for DAC and DAC $^{13}C_2.^2H_6$ was set to 30V while for SOF and $SOFD_6$ it was set to 25V. The collision energy for DAC and SOF were 50 and 12 KV respectively. The multiple-reaction monitoring mode (MRM) was used for the detection of the ions by monitoring the parent ion*m/z* 739.28 precursor ion to the *m/z* 339.20 for DAC, parent ion *m/z* 747.43 precursor ion to the *m/z* 339.17 for DAC $^{13}C_2.^2H_6$, and parent ion *m/z* 530.27 precursor ion to the *m/z* 243.10 for SOF, parent ion*m/z* 536.23 precursor ion to the *m/z* 243.06 for SOF D6 respectively. Analysis of the data obtained was processed by Mass Lynx 4.1 software.

Stock solutions

Stock solutions of DAC (1000.000 µg/mL) and SOF(1000.000 µg/mL) were prepared by dissolving 5.000 mg of DAC and SOF in 5.00 mL of Milli Q water and methanol respectively. DAC $^{13}C_2D_6$ and SOF D_6 stock solutions were prepared by dissolving in methanol at 400.00 µg/mL for both the IS. The DAC intermediate solutions 1 (DAC1) of concentration 250.00 µg/mL was prepared by using 500 µL of DAC stock solution (1000.000 µg/mL) and made the volumeto 2 mL by using diluent [methanol and water (60:40%, v/v)]. The DAC intermediate solutions 2 (DAC_2) of concentration 25.00 µg/mLwas prepared by using 200 µL DAC Intermediate Solution (250.000 µg/mL) and made the volume to 2 mL by using diluent [methanol and water (60:40%, v/v)].

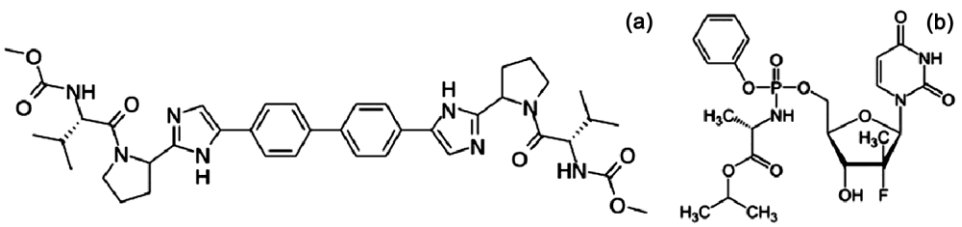

Fig. 1 — Chemical structure of (A) Daclatasvir (DAC) (B) Sofosbuvir (SOF).

The SOF intermediate solution 1 (SOF1) of concentration 100.00 µg/mL was prepared by using 500 µL of SOF stock solution (1000.000 µg/mL) and made the volume to 5 mL by using diluent [methanol and water (60:40%, v/v)]. Internal Standard Working Solution of DAC $^{13}C_2.^2H_6$ (5.000 µg/mL) and SOF D_6 (10.000 µg/mL) was prepared by dissolving respective stock (625 µL of DAC, 1250 µL of SOF) solutions in methanol and water (60:40) in a 50 mL volumetric flask. The preparation of working solutions with different concentrations was done by dilution of intermediate solutions [Diluent: methanol and water (60:40%, v/v)].

Sample pretreatment

Aliquot of human plasma sample (100 µL) was mixed with 25 µL of working internal standard (DAC $^{13}C_2.^2H_6$, 5.000 µg/mL+SOF D6 10.000µg/mL) and vortexed for few seconds. To this, 100 µL of 1.0% Formic Acid extraction additive was added and vortexed for few seconds and kept aside for solid phase extraction (SPE).

In the SPE procedure the cartridges (Strata-X, 30 mg, 1cc) were conditioned and equilibratedwith 1mL of methanol followed by 1mL of Milli-Q water. The spiked plasma samples of volume 225 µL (100 µL aliquot of human plasma +25 µL of Working internal standard +100µL of 1.0% Formic Acid extraction additive) were loaded into cartridge and washed twice with 1.0 mL of Milli-Q/HPLC grade water followed by elution with 0.5 mL methanol. To the elute 0.5 mL of reconstitution solution [(Acetonitrile: 5mM Ammonium formate, 50:50, v/v): Methanol, 50:50, v/v], was added, vortexed and loaded into the UPLC vial. The injection volume of 2.0 µL was set for the chromatographic system. The developed method was validated on different parameters such as linearity, selectivity, sensitivity, precision and accuracy, recovery, matrix effect, stability, and dilution integrity.

Calibration curves and limit of quantitation

This assay was validated with a standard curve range of 10.004 to 3001.218 ng/mL for DAC and 10.002 to 3000.488 ng/mL for SOF. The standard curve consisted of nine non-zero calibration standards, along with matrix blanks (with and without the addition of IS). The lowest concentration standard (10.004 ng/mL or DAC and 10.002 ng/mL for SOF) defined the lower limit of quantitation (LLOQ) for the assay, while the highest standard concentration (3001.218 ng/mL for DAC and 3000.488 ng/mL for SOF) defined the upper limit of the assay (ULOQ).Linearity is defined as the square of the correlation coefficient (r) obtained from weighted linear regression of peak area ratio (analyte/internal standard) versus concentration. The criterion for acceptable linearity is $r \geq 0.99$. All validation standard curves used for accuracy and precision determinations surpassed this limit with r values of greater than 0.99. In any batch 75% of the standards samples and 67% of the QC samples (50% at each level should meet the acceptance criteria) were required to have an accuracy percentage deviation within ± 15% whereas for the LLOQ samples the accuracy percentage deviation should be within ± 20%.

Selectivity, Matrix Effect, Recovery and Carry Over

The selectivity experiment was performed usingfourteen different blank plasma lots (8 normal, 2 (1.0 %) Haemolysed, 2 (2.0%) Haemolysed & 2 lipemic) and the responses of the blank plasma lots were compared to the respective LLOQ standard mean area of DAC, SOF and IS.

To evaluate the matrix factor six lots (4 Normal, 1 Haemolysed & 1 Lipemic) of interference free blank matrix from individual donor were taken, processed in triplicate from each lot and extracted according to the analytical method procedure. The post extracted LQC & HQC samples were obtained by spiking the analyte and IS into the extracted blank plasma and compared with12 aqueous (without matrix) samples (6 LQC & 6 HQC). The matrix factor was determined by calculating the ratio between the peak area of post extracted to the mean peak area of unextracted samples for each lot. Additionally, IS normalized matrix factor was also determined by calculating the ratio of matrix factor of analytes by matrix factor of IS at each lot of matrix.The recovery percentage of DAC & SOF was estimated by analyzing the mean peak area of extracted versus unextracted LQC, M1QC, M2QC and HQC samples for DAC & SOF along with the IS (DAC and SOF -5.000 µg/mL and 10.000 µg/mL respectively).

Accuracy and Precision

The intra-assay precision and accuracy of the method was assessed by analyzing 6 QC replicates each of the LLOQQC, LQC, M1QC, M2QC and HQC in 4 sets along with 4 sets of calibration curves samples for DAC and SOF.The inter-assay precision and accuracy of the method was also determined by

analyzing 6 QC replicates each of the LLOQQC, LQC, M1QC, M2QC and HQC in 4 sets along with 4 sets of calibration curves samples for DAC and SOF.

The acceptance criteria for the calibration curve correlation coefficient (r^2) were set to be >0.98 and the obtained concentration values in the inter-day and intra-day assay for both accuracy and precision for LQC, M1QC, M2QC and HQC samples should be within ±15% of the nominal concentration and <15% RSD, and within ±20% of the nominal concentration and <20% RSD for LLOQ samples.

Ruggedness

The ruggedness experiment was evaluated by processing one P&A batch by different analyst and samples of one P&A batch was reinjected using different column of same make and specification. The experiment was performed by using nine non-zero calibration standards, along with matrix blanks calibration curve standard and 6 replicates of quality control samples (High (HQC), Medium (M1QC and M2QC) and low (LQC)). The calculation of the concentrations were performed for the standard and quality control samples to determine the precision and accuracy of the experiment.

Dilution integrity

The dilution integrity experiment was performed using six replicates of 5-fold dilution and 10 fold dilution of the ULOQ samples and concentrations were determinedusing the freshly spiked calibration curve samples. The obtained concentrations were compared with nominal concentrations to findif samples with concentrations higher than the ULOQ could be suitably determined by dilution with blank matrix.

Stability and re-injection reproducibility

The stability experiments for the analyte in matrix (plasma stability) and stock solutions were performed under different stability conditions to prove the stability of the analyte during the study sample analysis condition. The plasma stability was conductedby using bulk spiked samples where the accuracy of six replicates of LQC and HQC were evaluated immediately after preparation. The autosampler stability, bench top stability, reinjection stability, wet extract stability, freeze thaw stability were performed by using six replicates of bulk spiked LQC and HQC samples. The stability of samples was accepted if assay values were within the limits of accuracy (85-115%) and precision within ≤15% RSD.

Pharmacokinetic study design

A randomized, balanced, two-treatment, four-period, two-sequence, single-dose, full replicate, crossover oral bioequivalence study was planned as per the ICH GCP guidelines. Bioequivalence study was conducted on 48 male subjects under fasting conditions. In order to minimize the possibility of a carry-over effect, a minimum washout period of at least 15 days was selected for the study. In this study, the pharmacokinetic profile of the test product (A) DAC/SOF tablets 60 mg/400 mg was characterized relative to that of the reference products DAC and SOF, 60 mg and 400 mg given as separate dose to assess bioequivalence. Being a bioequivalence study with a crossover design, each subject act as his own control. Therefore, no control group was required for the study. The ethics committee approved the protocol and the volunteers provided with informed written consent. During each study period, 29 blood samples were collected. Blood samples were collected within 1.50 minutes prior to dose administration (0.00 hour) and after dose administration at study hours 0.083, 0.166, 0.25, 0.33, 0.50, 0.66, 0.83, 1.00, 1.25, 1.50, 1.75, 2.00, 2.33, 2.66, 3.00, 3.50, 4.00, 4.50, 5.00, 5.50, 6.00, 8.00, 10.00, 12.00, 16.00, 24.00, 36.00, and 48.00 hours in K_3-EDTA vacutainer collection tubes (BD, Franklin, NJ, USA). The plasma samples were collected and stored at –70 ± 10°C until use. The internal standard (IS) was spiked in plasma samples and extraction of both the analyte and IS was performed following the extraction procedure. The subject sample analysis was performed along with standard samples (calibration curve standards) and different level of QC samples (LQC, M1QC, M2QC and HQC) taken in triplicate.

Results and Discussion

LC-MS specification

During method development for the simultaneous quantification of DAC and SOF; different chromatography and mass detection parameters were optimized to improve the analyte chromatograms and sensitivity of the method. The tandem mass spectroscopy due to its high sensitivity and selectivity is a unique analytical tool for pharmacokinetic studies. A better response for both the analyte and IS was obtained in the positive ionization mode and the MRM parameters were further optimized to increasethe analyte and IS response. The MS/MS system

The specified mass for the analyte in the first quadrupole (Q1) is the precursor ion that collides with the in-quadrupole collision cell (Q2) with the collision gas, and by collision-induced dissociation (CID) undergoes further fragmentation. The structural information of the resulting daughter ions from the fragmentation is detected by a third quadrupole mass analyzer (Q3).To obtain the best selectivity multiple reaction monitoring scanning mode was used. The mass spectrometry specification for DAC and SOF and the respective internal standards are mentioned in Table 1. For bestretention, peak intensity and less chromatographic run time the chromatographic conditions, were optimized by changing the composition of the mobile phase by several trials.Gemini NX (5µC18 50 × 2.0mm, Phenomenex) gave good peak shape and response even at lowest concentration level for both the analytes and IS. The gradient mobile phase with different composition of 5 mM Ammonium Formate buffer: Acetonitrile was delivered into the electrospray ionization chamber of the mass spectrometer at a flow rate of 0.300 mL/min. The retention time of DAC and SOF were 2.15 and 1.40 mins respectively and for the internal standard (DAC $^{13}C_2D_6$ and SOF D_6) it was 2.12 and 1.35 mins respectively allowing a run time of 5.00 min. For both the analytes and respective IS the elution time frame variation was selected to be ± 30 secs.

Sample preparation

The extraction procedure for the analyte was checked for both liquid–liquid extraction (LLE) and solid phase extraction (SPE) and the results showed better recovery and low matrix effect for extraction of the drug and IS was obtained in the SPE technique.

The auto sampler wash solution having a composition of 50% Acetonitrile/water was optimized to prevent carry over effect. The extraction buffer of 100 µL of 1% formic acid was added to the plasma samples to extract the analyte and the IS. The analyte in the human plasma was detected with good accuracy and prediction by the optimized extraction procedure.

Calibration curves and limit of quantitation

This assay was validated with a standard curve range of 10.004 to 3001.218 ng/mL for DAC and 10.002 to 3000.488 ng/mL for SOF. The standard curve consisted of nine non-zero calibration standards, along with matrix blanks (with and without the addition of internal standard). The lowest concentration standard (10.004 ng/mL or DAC and 10.002 ng/mL for SOF) defined the lower limit of quantitation (LLOQ) for the assay, while the standard with the highest concentration (3001.218 ng/mL for DAC and 3000.488 ng/mL for SOF) defined the upper limit of the assay (ULOQ).The weighing factor selected was $1/x^2$. The preparation of the calibration curve (CC) standard samples were done by spiking appropriate volume of the working solutions (20 µL of DAC and 20 µL of SOF), in 960 µL of control human plasma resulting in final concentrations of 10.004, 20.008, 50.020, 250.102, 600.244, 1200.487, 1800.731, 2501.015, 3001.218 ng/mL for DAC, and 10.002, 20.003, 50.008, 250.041, 600.098, 1200.195, 1800.293, 2500.407 and 3000.488 ng/mL for SOF. The QC samples were prepared at of 9.999 (LLOQ), 29.996 (LQC), 299.959 (M2QC), 1499.796 (M1QC), 2259.693 (HQC) and 11998.371 (DQC) ng/mL for DAC and 10.012 (LLOQ), 30.035 (LQC), 300.347 (MQC-2), 1501.735 (M1QC), 2262.614 (HQC) and 12013.882 (DQC) ng/mL for SOF in blank plasma. The prepared plasma samples were kept at −70 ± 10°C. The criterion for acceptable linearity is $r \geq 0.99$. All validation standard curves used for accuracy and precision determinations surpassed this limit with r values of greater than 0.99.

Table 1 — Mass spectrometry specification for DAC and SOF and the respective internal standards.

Time(min)	Flow rate (per mL/min.)	% Pump A (Acetonitrile)	% Pump B (5 mM Ammonium formate buffer)
0.00	0.300	35	65
0.30	0.300	35	65
1.25	0.300	43	57
1.50	0.300	50	50
1.90	0.300	50	50
2.00	0.300	90	10
3.50	0.300	90	10
3.75	0.300	35	65
5.00	0.300	35	65

Selectivity, matrix effect, recovery and carry over

The developed method was selective as no significant interference was observed in any plasma lots (normal, lipemic, hemolyzed) for DAC, SOF and their respective IS. The matrix effect was examined to determine the ion suppression/enhancement on the ionization of the analytes and to make sure that selectivity, precision, and sensitivity are not affected by the matrix. For matrix effect no significant interference was observed at the RT of both the analytes as the % CV of IS normalized matrix factor for DAC and SOF was below 4% for both HOC and LQC level that was within acceptance criteria. (Table 2).

The absolute recovery for DAC ranged from 79.76 to 86.18 % while for SOF it ranged from 87.54 to 90.70 %. For DAC-13C2D6 and SOF-d6 the recovery rates are 81.58 and 84.92% respectively. The mean recovery values were approximately ≥ 83% for DAC and ≥ 89% for SOF respectively. In case of both the analytes and their respective IS the difference in the recovery %CV across each QC level was within 15%. (Table 2). The developed method showed no significant injector carry over for the analyte and IS.

Accuracy and precision

The inter- and intraday precision (%CV) and accuracy (% nominal value) of the method was determined by considering three batches having six replicates of quality controls samples at five different concentrations (LLOQQC, LQC, M1QC, M2QC and HQC) levelsand the results are summarized in Table 3. The accuracy was expressed as mean % nominal valueand was evaluated by considering the observed percentage deviation of mean from the theoretical spiked values. In both DAC and SOF across all QC levels the mean % nominal values were in range of 85-115% and %CV was less than 15% and met the acceptance criteria. Precision expressed as relative standard deviation (RSD) for the intra-day and inter day experimentswere below the acceptable limit of 15% at LQC, M1QC, M2QC and HQC levels, whereas for LLOQQC it was below 20%. The acceptable precision, accuracy result indicated the developed method is reproducible over the entire linearity range.

Ruggedness

The ruggedness of the developed method was evaluated using different column and different analyst

Table 2 — Matrix effect and recovery for DAC, SOF, DAC-$^{13}C_2D_6$ and SOF-d_6.

Analyte	Level	%CV of IS-normalized matrix factor	Recovery (%)/CV (%)	Mean recovery
DAC	HQC	0.60	86.18/5.19	83.05
	M1QC	-	82.28/6.03	
	M2QC	-	79.76/5.53	
	LQC	1.30	83.97/11.61	
SOF	HQC	0.74	90.70/6.78	89.24
	M1QC	-	89.38/8.40	
	M2QC	-	87.54/7.85	
	LQC	0.34	89.35/12.97	
DAC-$^{13}C_2D_6$	-	-	81.58/7.13	-
SOF -d_6	-	-	84.92/9.84	-

Table 3 — Precision and accuracy results for DRV and RTV.

Nominal concentration in ng/mL	Intra-day (n=6)			Inter-day (n=24)		
	Mean	CV (%)	Accuracy (%)	Mean	CV (%)	Accuracy (%)
Precision and accuracy of DAC						
HQC(2259.693)	2305.502	1.79	102.02	2259.668	1.91	99.99
M1QC(1499.796)	1537.209	2.95	102.49	1547.629	1.76	103.19
M2QC(299.959)	302.339	2.94	100.79	304.723	1.88	101.59
LQC(29.996)	30.118	3.03	100.41	30.397	2.53	101.34
LLOQQC(9.999)	9.913	1.42	99.14	10.099	4.93	101.00
Precision and accuracy of SOF						
HQC(2262.614)	2272.828	0.95	100.45	2238.989	1.59	98.95
M1QC(1501.735)	1550.380	3.55	103.23	1597.486	2.50	106.37
M2QC(300.347)	320.313	2.44	106.65	325.724	1.67	108.45
LQC(30.035)	30.483	1.92	101.49	30.537	1.66	101.67
LLOQQC(10.012)	10.207	2.84	101.95	10.238	3.09	102.26

by using calibration curve standard and 6 replicates of quality control samples at five different concentration levels (LLOQQC, LQC, M1QC, M2QC and HQC). For accuracy, the quality controls samples showed the mean % nominal value within 85-115% for all analytes.

The precision for ruggedness experiments were below the acceptable limit of 15% at LQC, M1QC, M2QC and HQC levels, whereas for LLOQQC it was below 20%. The ruggedness experiment for different column was performed byreinjecting samples of one P&A using a different column of similar make and specification. The ruggedness experiment for DAC & SOF **is** presented in Table 4.

Dilution integrity

The dilution integrity of the assay was performed at 5 times dilution and ten times dilutionon a concentration approx. 4 times ULOQ samples in six replicate and were calculated against freshly spiked calibration curve and compared with nominal concentrations. The result was within the acceptance criteria and is represented in Table 5.

Reinjection reproducibility and stability

The QC samples from the P&A batch was used to evaluate the partial reinjection reproducibility experiment. The QC samples were kept in the auto sampler for approx. 12 h 35 min at 5°C, and reinjected. The precision and accuracy of the samples were determined after reinjection. The concentration of both DAC and SOF after reinjection experiment showed no significant variability in auto sampler at 5°C (approx. 12 h 35 min).

The samples of the P&A batch were reinjected after keeping in the auto sampler for approximately 29 h 7 min to evaluate the whole batch reinjection reproducibility experiment for the whole batch after the initial analysis and determinedfor precision and accuracy by calculating the concentrations of the analyte. Both the analytes (DAC & SOF) showed reproducible concentration after reinjection and were found to be stable in autosampler for approx. 29 h 7 mins.

The short term and long term stock and working solutions stability for DAC, SOF, DAC $^{13}C_2{}^2H_6$ & SOF-D6 was found to be stable for 07 h 30 min and 11 days in Milli Q Water, methanol & methanol: water (60:40) respectively in the refrigerator at 0-10ºC. In order to evaluate the bench top stability stored QC samples (6 HQC and 6 LQC) were retrieved and kept for 27 h 16 minat room temperature

Table 4 — Ruggedness experiment for DRV and RTV.

	LLOQ QC	LQC	M2QC	M1QC	HQC
			DAC (Different analyst)		
Mean	10.198	30.564	305.221	1557.403	2266.78
Accuracy	101.9902	101.8936	101.7542	103.841	100.3136
%CV	4.18	2.01	0.95	0.66	1.14
			DAC (Different column)		
Mean	10.547	30.752	302.221	1539.695	2222.946
Accuracy	105.4805	102.5203	100.7541	102.6603	98.37381
%CV	6.43	3.20	1.14	1.41	1.09
			SOF (Different analyst)		
Mean	10.171	30.389	326.476	1607.613	2223.178
Accuracy	101.72	101.31	108.84	107.19	98.38
%CV	3.22	0.88	0.71	0.44	0.88
			SOF (Different column)		
Mean	10.542	30.808	326.063	1609.289	2196.014
Accuracy	105.43	102.71	108.70	107.30	97.18
%CV	2.17	2.13	0.88	1.03	0.58

Table 5 — Dilution integrity results for DAC and SOF.

Analyte	QC level	Dilution factor: 5			Dilution factor: 10		
		Mean	CV	% Bias	Mean	CV	% Bias
DAC	DQC*	12167.357	2.39	1.41	11954.477	1.86	-0.37
SOF	DQC*	11473.804	2.99	-4.50	13612.606	2.03	13.31

*For DAC the DQC concentration is 11998.371 ng/mL. For SOF the DQC concentration is 12013.882 ng/mL.

and compared with freshly processed comparison 6 LQC and 6 HQC samples along with freshly prepared calibration standards.The mean % change in concentrations were calculated to determine the stability period that was approx. 27 h 16 min at room temperature for DAC & SOF. The results are presented in Table 6.

The dry ice stability (coolant) was used to determine the stability of DAC and SOF during transportation in the biological matrix. The dry ice stability was determined by keeping 6 sets of LQC and HQC for approximately 69 h 51 min in dry ice after which the stability QC samples (6 HQC and 6 LQC) were processed and analyzed with freshly processed QC (LQC and HQC samples) and freshly prepared calibration standards. The results presented in Table 6. The dry ice stability of DAC&SOF were found to be approx. 69 h 51 min that indicates the stability time during shipment of the clinical samples.

The post extracted refrigerator stability in matrix was performed to check the stability of analytes after adding reconstitution solution to the processed samples and storing the samples in refrigerator for desired time duration at 0-10°C. The QC samples consisting of 6 LQC and 6 HQC were retrieved from deep freezer, and after sample processingand reconstitution were stored for approx. 27 h in refrigerator at 0-10°C. The samples were retrieved after specified time and compared along with freshly processed samples (LQC and HQC) and freshly prepared calibration standard samples. The results presented in Table 6. These results showed both the analytes DAC & SOF were stable during 27 h of storage in the refrigerator at 0-10°C. The freeze and thaw stability samples were determined by preparing six aliquots of LQC and HQC levels and stored at set temperature -70°C and -20°C and for each concentration (LQC and HQC) 5 freeze and thaw cycles (stability samples) were performed. These freeze and thaw subjected samples were processed and compared with LQC and HQC samples (freshly processed comparison samples) and freshly prepared calibration standards. The mean % change in concentrations was calculated and after 5 cycles DAC & SOF were found to be stable. Results are presented in Table 6.

In-injector Stability in Auto Sampler at 5ºC

The autosampler stability was determined by using six replicates of LQC and HQC (3 sets at each level). The processing of the first set of QC samples after kept in autosampler at 5°C after 80 h 6 min was compared with the second set of samples and with freshly prepared calibration standard samples. The mean % change in concentrations was calculated to determine the stability period. For both DAC and SOF at 5°C the stability of LQC & HQC samples was found to be approx. 80 h 6 min. Results are presented in Table 6. In whole blood stability six replicates of whole blood at Low and High QC level (Stability samples) were spiked and kept at the working bench.

After a period of 02 h 05 min spiking, six aliquots of whole blood at low and high QC (comparison samples) were aliquoted separately. The plasma was separated from both the stability and comparison

Table 6 — Stability study results for DAC and SOF.

Storage period and storage condition	QC level	DAC			SOF		
		Mean	CV (%)	Accuracy (%)	Mean	CV (%)	Accuracy (%)
Whole blood stability (RT)	HQC	1.4827	1.70	99.43	1.5390	2.84	98.18
(02 h 05 min)	LQC	0.0201	2.31	97.57	0.0218	1.92	98.34
Bench top stability	HQC	2218.64	1.72	98.18	2147.713	2.06	94.92
(27 h 16 min)	LQC	31.346	4.19	104.50	31.957	2.45	106.40
Auto sampler stability at 10ºC	HQC	2359.579	2.97	104.42	2345.350	1.97	103.66
(80 h 6 min)	LQC	30.542	2.79	101.82	31.709	3.83	105.57
Five freeze thaw cycles	HQC	2361.461	2.71	104.50	2368.093	1.80	104.66
(-20 ± 5^0C)	LQC	30.896	4.37	103.00	32.064	4.07	106.76
Five freeze thaw cycles	HQC	2214.548	4.81	98.00	2268.334	3.58	100.25
(-70 ± 10^0C)	LQC	28.906	2.65	96.37	30.984	3.57	103.16
Dry extract stability (RT)	HQC	2217.044	1.85	98.11	2138.510	1.95	94.52
27 Hrs 5 min	LQC	31.131	1.84	103.78	31.700	1.95	105.54
Post extracted stability (RF)	HQC	2230.552	1.38	98.71	2146.388	2.06	94.86
27 h	LQC	30.887	2.63	102.97	31.507	2.98	104.90
Coolant Stability	HQC	2221.800	4.97	98.32	2296.974	3.62	101.52
69 h 51 min	LQC	29.311	5.51	97.72	31.221	3.41	103.95

samples by centrifugation of samples at 4°C with a speed of 3000 RPM for about 10 minutes. The samples were processed and analyzed as per the analyte specific method procedure. The results are presented in Table 6.

The dry extract stability of DAC and SOF was determined (after processing but before adding reconstitution solution) during storage in the refrigerator (0-10ºC). The six sets of low (LQC) and high (HQC) for DAC & SOF were kept for approx. 27 h 5 min in a refrigerator at 0-10ºC prior to dry extract analysis and compared with freshly prepared calibration standards and freshly prepared comparison (LQC & HQC) samples. The results are presented in Table 6. These results showed dry extract stability for DAC & SOF during storage in the refrigerator (0-10ºC) was approx. 27 h 5 min.

Pharmacokinetic parameters

In a total 48 subjects study the mean and SD of pharmacokinetic parameters estimated for test product (A) and reference product (B) were as follows: The maximum plasma concentration C_{max} of the Test product A 1544.780±420.9965 ng/mL while for the reference product it was 1581.390±435.8123 ng/mL. The mean AUC_{0-t} of DAC was 16967.223±5427.0998 ng.hr/mL for the DAC (Test Product A) while for the reference dose it was found to be 16916.172±4957.6863 ng.h/mL. The $AUC_{0-\infty}$ was found to be 18258.116±5888.8902 ng.hr/mL and 18332.446±5751.2101 ng. h/mL for the test and reference dose regimen respectively. The reference and test dosage regimens have t_{max} values of 1.500 and 1.250 h respectively and a minimal difference in the $t_{1/2}$ values (10.655 hr and 10.461 hr for the test and reference product respectively). The K_{el} and (AUC_{0-t}/ AUC_{0-inf}) *100 values for the test and reference product are listed in Table 7.

For SOF the C_{max} was 1816.364 ng/ml and 1582.890 ng/ml for the test and reference product respectively. The T_{max} was 0.660 h and 0.830 h for the test and reference dosage regimens and little difference was observed for the $t_{1/2}$ values for the test (0.545 h) and reference product (0.514). The mean AUC_{0-t} was 2033.987 ng. h/mL for the test product and 1841.75 ng. h/mL for the reference product respectively. The AUC_{0-inf} for the test and reference product was 2045.382 ng. h/mL and 1852.852 ng. h/mL respectively. The K_{el} and (AUC_{0-t}/AUC_{0-inf})*100 values for the test and reference product are listed in Table 8.

The geometric least squares mean, percentage of test product (A) and reference product (B), (A /B) , 90% confidence intervals, for the log-transformed pharmacokinetic parameters $C_{max,}$ AUC_{0-t} and AUC_{0-inf} for DAC and SOF were summarized in Table 9.

Table 7 — Pharmacokinetic parameters for DAC in test and reference product under fasting conditions.

Parameters (Units)	Un-transformed Data (Mean ± SD)	
	Test Product(A) n=77	Reference Product (B) n=77
*T_{max} (h)	1.500(0.830-4.500)	1.250(0.830-6.000)
C_{max} (ng/mL)	1544.780±420.9965	1581.390±435.8123
AUC_{0-t} (ng.h/mL)	16967.223±5427.0998	16916.172±4957.6863
AUC_{0-inf} (ng.h/mL)	18258.116±5888.8902	18332.446±5751.2101
K_{el} (1/h)	0.067±0.0126	0.070±0.0143
$t_{½}$ (h)	10.655±2.0178	10.461±3.1493
(AUC_{0-t}/AUC_{0-inf})*100	93.291±5.6723	93.163±6.9722

*Median, Minimum and Maximum values reported for $T_{max.}$

Table 8 — Pharmacokinetic parameters for SOF in test and reference product under fasting conditions.

Parameters (Units)	Un-transformed Data (Mean ± SD)	
	Test Product(A) n=77	Reference Product (B) n=77
*T_{max} (h)	0.660(0.330-3.500)	0.830(0.250-3.000)
C_{max} (ng/mL)	1816.364±848.2540	1582.890±613.1162
AUC_{0-t} (ng.h/mL)	2033.987±829.1908	1841.75±640.7657
AUC_{0-inf} (ng.h/mL)	2045.382±828.8982	1852.852±640.9853
K_{el} (1/h)	1.331±0.2587	1.395±0.2577
$t_{½}$ (h)	0.545±0.1422	0.514±0.0972
(AUC_{0-t}/AUC_{0-inf})*100	99.323±0.4454	99.308±0.4542

*Median, Minimum and Maximum values reported for $T_{max.}$

The ratio of geometric least squares mean for the C_{max} of test product (A) and reference product (B) treatments of log-transformed pharmacokinetic parameter C_{max} for DAC was 98.06%. The two one-sided 90% confidence interval for the ratio of the geometric least squares mean was found 91.86-104.68 %. The ratio of geometric least squares mean of test product (A) and reference product (B) treatments of log-transformed pharmacokinetic parameter AUC_{0-t} was 99.34%. For AUC_{0-t} the two one-sided 90% confidence interval for the ratio of the geometric least squares mean was found 93.99-105.00. For the log-transformed pharmacokinetic parameter AUC_{0-inf} the ratio of geometric least squares mean of test product (A) and reference product (B) treatments was 99.09 % and the two one-sided 90% confidence interval for the ratio of geometric least squares mean was found 93.68-104.81 %. In DAC for all the pharmacokinetic parameters the 90% confidence interval is within the acceptance limits as represented in Table 9.

For SOF the ratio of geometric least squares mean of test product (A) and reference product (B) treatments of log-transformed pharmacokinetic parameter C_{max} and AUC_{0-t} was 111.41%. and 106.14% respectively. The two one-sided 90% confidence interval for the ratio of the geometric least squares mean for the C_{max} and AUC_{0-t} was found 100.60 -123.38 % and 99.53-113.19% respectively. In case of log-transformed pharmacokinetic parameter AUC_{0-inf} the ratio of geometric least squares mean of test product (A) and reference product (B) treatments of was 106.13 %. The two one-sided 90% confidence interval for the ratio of geometric least squares mean for AUC_{0-inf} was found 99.59-113.11 %. For SOF also all the pharmacokinetic parameters are within the acceptance limits of as represented in Table 9. The linear and semi log plot of mean plasma concentration versus time curves of DAC and SOF after administration of test product (A) and reference product (B) under fasting conditions are represented in Fig. 2 that indicates the test product (A) compared to the Reference product (B), met the bioequivalence criteria under fasting conditions.

The study presents the development, validation, and clinical application of a novel bioanalytical method of DAC and SOF usingLC-MS/MS in the positive ionization mode with DAC-13C2D6 and SOF-d6 as the respective internal standards.Results from clinical studies as well as preliminary real-life data regarding the combination of SOF (a nucleotide polymerase inhibitor) and DAC, a first-in-class NS5A replication complex inhibitor, demonstrate that it is one of the most promising antiviral therapies, with once-daily oral dosing, a low pill burden, good tolerability, and limited drug–drug interactions, in addition to high antiviral potency, with 90% sustained virologic response rates. This combination has high pangenotypic antiviral potency regardless of the severity and patient characteristics.In a 24 week extended study outcome SOF plus DAC+ribavirin obtained 100% efficacy in genotype 3 hepatitis C cirrhosis, with very limited side effects[19]. Thus it appeared of interest to develop a highly sensitive, reliable and selective bioanalytical methods and its application for pharmacokinetic measurements and optimization of dosages in clinical study. This study exclusively reports the well validated method for the simultaneous quantification of DAC and SOF and the application of the method to study the pharmacokinetic parameters in 48 male subjects. During method development an extensive method optimization was performed to select the best extraction procedure for selective determination of DAC and SOF from human plasma. Depending on the extraction efficiency SPE was tested on (Strata-X, 30 mg, 1cc) cartridge for their simultaneous determination in human plasma.

Table 9 — Statistical results for DAC and SOF test and reference products under fasting condition.

Parameters (Units)	Ratio of geometric least squares means			Acceptable Lower BE limit (%)	Acceptable Upper BE limit (%)	90% Confidence limits (A vs. B)
	Test product (A)	Reference product (B)	(A/B)%			
			DAC			
C_{max} (ng/mL)	1476.4399	1505.6365	98.06	75.81	131.91	91.86-104.68
AUC_{0-t} (ng.h/mL)	15978.9751	16084.7272	99.34	80.00	125.00	93.99-105.00
AUC_{0-inf} (ng.h/mL)	17172.9780	17330.7229	99.09	80.00	125.00	93.68-104.81
			SOF			
C_{max} (ng/mL)	1625.4665	1458.9713	111.41	69.83	143.21	100.60 -123.38
AUC_{0-t} (ng.h/mL)	1847.5201	1740.6457	106.14	80.00	125.00	99.53-113.19
AUC_{0-inf} (ng.h/mL)	1860.2153	1752.7127	106.13	80.00	125.00	99.59-113.11

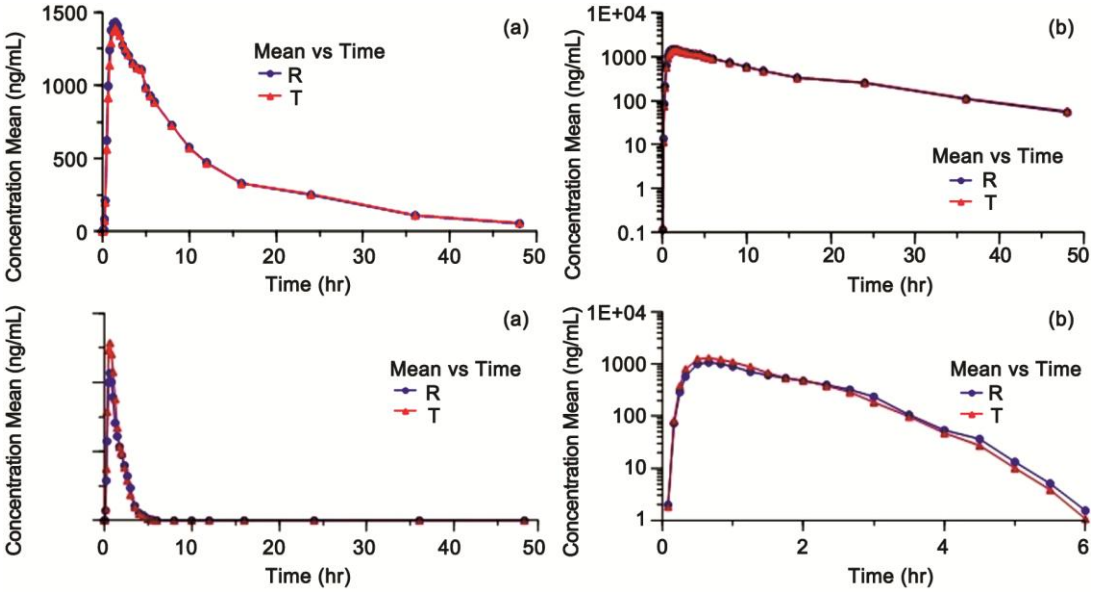

Fig. 2 — (a)Linear and (b) Semi log plot of mean plasma concentration versus time curves of DAC after administration of test product (T) and reference product (R) under fasting conditions. (c)Linear and (d) Semi log plot of mean plasma concentration versus time curves of SOF after administration of test product (T) and reference product (R) under fasting conditions.

An Acquity UPLC system (Waters) equipped with Waters Xevo TQ MS system consisting of a Gemini NX 5µ C18 (50 × 2.0mm) (Phenomenex), a pump (Acquity binary sample Manager, Waters), and an auto sampler (Acquity sample manager) were used for the study. Aliquots of the processed samples (2 µL) were injected into the column, which was kept at ambient temperature (40 ± 5 ºC). A gradient mobile phase consisting 5 mM Ammonium Formate buffer: Acetonitrile was used to separate the analytes and delivered at a flow rate of 0.300 mL/min after severaloptimizations to achieve, adequate response and complete separation. All mass parameters were suitably optimized to obtain a stable and adequate response for the analytes. The difference in retention time of SOF and DAC were 1.40± 0.30 and 2.15± 0.30 mins respectively and for the Internal standard (SOF D6 and DAC $^{13}C_2{}^2H_6$) (1.35± 0.30 and 2.12± 0.30 mins) respectively allowed a good separation for both the analytes. Further, use of deuterated internal standards helped to compensate any variability during extraction and UPLC-MS/MS analysis.

The validation was carried out as per US FDA guidelines[20]. The parameters determined were selectivity, sensitivity, matrix effect, linearity, precision, accuracy, recovery, stability and dilution integrity. The selectivity and sensitivity results established the method to be selective for both these analytes with adequate response. No Matrix effect was observed for six different lots of K3-EDTA plasma and the blank plasma samples were also analyzed to confirm the absence of direct interferences. The results of the three P&A batches confirm the reproducibility of the method with an excellent ruggedness for different analyst and column and suggested a suitable method for the quantification of DAC and SOF in human plasma. This optimized and validated LC–MS method was applied to quantify plasma DAC and SOF concentration for a bioequivalence study in 48 healthy subjects after oral administration of (DAC/SOF) 60 mg /400 mg of Mylan laboratories limited, India with daklinza ™ (DAC) tablets 60 mg (Bristol-Myers Squibb Company, Princeton, NJ 08543, USA) and sovaldi® (SOF) 400 mg film-coated tablets (Gilead Sciences International Ltd. Cambridge cb 216gt United Kingdom), under fasting conditions.

Conclusion

A simple, rapid and sensitive LC-MS/MS assay method is described in this paper for the quantification of DAC and SOF in human plasma and full validation of the method was performed following the FDA guidelines. To the best of our knowledge, this report is the first one to describe the simultaneous quantification of DAC and SOF in human matrix. The developed method showed good linearity with

reproducible and consistent recoveries of DAC and SOF from plasma. The method was applied for pharmacokinetic studies of DAC and SOF in human's plasma. The developed method with desired precision and accuracy could be helpful for the bioequivalence (BA/BE) studies/bioavailability and routine therapeutic drug monitoring.

Abbreviations

DAC: Daclatasvir; SOF: Sofosbuvir; FDA: Food and Drug Administration;Cmax: Maximum plasma concentration; ICH: International Conference on Harmonisation; HPLC: High Performance Liquid Chromatography; USA: United State on America; LCMS: Liquid Chromatography; Mass Spectrometry; K_3EDTA: Tripotassium Ethylene Diamine Tetra Acetate; BE: Bioequivalence;LLOQ: Lower Limit of Quantification; MS: Mass Spectrometry; GS: Gas; DP: Clustering Potential; EP: Entrance Potential; CE:Collision Energy; CXP: Cell Exit Potential; QC: QualityControl, CS: Calibration Standard; WIS: Working InternalStandard; HLB: Hydrophilic Lipophilic Balance;EMA: European Medicines Agency; LQC: Low QC;MQC: Med QC; HQC: High QC; DQC: Diluted QC;ULOQ: Upper Limit of Quantification; PB: PlasmaBlank; GCP: Good Clinical Practice, CV: Co-efficientof Variation; SD: Standard Deviation

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