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Simultaneous Determination of Ledipasvir/Sofosbuvir by LC/MS/MS in Human Plasma and its Pharmacokinetics Application

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Abstract

Background: The rapid growth of COVID-19 infections may result in second wave of infection and an overwhelmed health care providing systems. Ledipasvir and sofosbuvir can be a good choice for management of COVID-19 patients. Development of simple, sensitive, and rapid assay for simultaneous determination of ledipasvir / sofosbuvir to investigate their pharmacokinetic parameters in human plasma, and aid in therapeutic drug moitoring in COVID-19 patients seems to be essential. Besides, its application in bioequivalence study of ledipasvir 90mg / sofosbuvir 400mg film coated tablets generic and reference products to ensure bioanalytical method reliability. ***Methods:*** After extraction of ledipasvir and sofosbuvir from human plasma, it was chromatographed with mobile phase consisting of ammonium formate pH 2.8 : acetonitrile (10 : 90 V/V) at flow rate 0.55ml/min, ESI positive mode, and m/z 889.8→130.1, 530.3→243.1, 739.4→565.3 for ledipasvir, sofosbuvir and daclatasvir (internal Standard) respectively. The bioequivalence study was conducted in a partial replicated crossover design invovlving 36 volunteers. The criteria used to assess bioequivalence of the two products were AUC $_{0\text{-}t}$, AUC $_{0\text{-}inf}$, C_{max}, and T_{max} for sofosbuvir, and AUC $_{0\text{-}72}$, C_{max}, and T_{max} for ledipasvir. ***Results:*** The described method of analysis showed that the average recovery of Ledipasvir and Sofosbuvir from human plasma was 95.180%, and 94.721%. The limit of quantitation was 0.1ng/ml for both drugs, and the correlation coefficient (r2) was equal to 0.999 for ledpasvir and sofosbuvir. Statistical analysis (ANOVA) of the measured parameters showed that there was no significant difference between the two products. ***Conclusion:*** The LC/MS/MS method presented is direct, simple, reproducible, sensitive, and linear for determination of ledipasvir / sofosbuvir in plasma, and is adequate for its clinical pharmacokinetic studies, and use in therapeutic drug monitoring. Besides the generic product was found to be biologically equivalent to the reference product regarding their kinetic behavior.

Keywords: COVID-19, Ledipasvir, Sofosbuvir, SARS-CoV-2, bioanalytical, therapeutic drug monitoring, LC/MS/MS.

1. BACKGROUND

Postulations suggested that there is no COVID-19 disease recurrence after the first wave of the disease. But this sugesstions proved to be fault. People ignored the necessary protective measures and involved in some misbehavior, like mixing socially with others or holiday outings. The result lead use dramatical increase in COVID-19 infctions and increased demand on health care facilities and endanger lives of many people worldwide [1].

It was found that about 177,866,160 confirmed cases of COVID-19 worldwide with a death rate of 2.17% according to the situation report of World Health Organization on June 22, 2021[(2)]. Different continentals showed different prevalence percentages for example; Africa showed a 3,791,054 confirmed cases with a mortality rate of 2.42%, Europe showed a 55,325,145 confirmed cases with a mortality rate of 2.12%, the Americas showed a 70,663,034 confirmed cases with a mortality rate of 2.63% till 22_{th} of June, 2021 [2].

Ledipasvir is an antiviral agent that targets the hepatitis C virus's (NS5A) protein involved in viral replication by lowering the virus amount in infected hosts. Sofosbuvir is an antiviral drug (nucleotide analogue) used in combination with other therapies to treat chronic HCV. Since the efficacy of interferon-containing regimens may be affected by adverse side effects, there is interest among researchers in the study of other medications to be used simultaneously with sofosbuvir [3].

Both drugs are available in combination, and is marketed in the form of tablets as a Ledipasvir 90 mg / Sofosbuvir 400 mg, under brand name HARVONI® 90 mg / 400 mg Tablets [4]. Ledipasvir and Sofosbuvir are indicated for the treatment of chronic HCV genotype 1 in adults [5].

The advantage of ledipasvir/sofosbuvir lies in its fixed-dose once-daily regimen, which does not require ribavirin co-administration [6]. Compared to the previous standard of care requiring treatment for 48 weeks, duration of chronic HCV-1 infection treatment with LDV/SOF is just 12 or 24 weeks, as recommended by EASL, American Association for the Study of Liver Diseases, and Infectious Diseases Society of America [7, 8]. Among treatment-naïve (TN) patients with HCV RNA <6 million IU/mL without cirrhosis, treatment duration can even be reduced to 8 weeks [9-11].

The combination of LDV/SOF has few drug interactions compared to the first generation of HCV protease inhibitors; this benefit contributes to a wide range of prescription possibilities among HCV-1 or -4-infected patients with comorbidities. [(12)] Furthermore, co-administration with carbamazepine, phenytoin, amiodarone, rifampicin, rosuvastin, tipranavir, simeprevir, and St John's wort, should be avoided, and co-administration with dabigatran etexilate, digoxin, and TDF should be closely monitored [13-15].

The approved dose for the treatment of chronic HCV genotype 1 infection in adults is 1 tablet (ledipasvir 90 mg/sofosbuvir 400 mg) orally once per day with or without food. The duration of treatment depends on the patient's baseline condition; treatment-naïve patients both with and without cirrhosis require 12 weeks, treatment-experienced patients (ie, individuals who have failed treatment with either peginterferon-α and ribavirin or an HCV protease inhibitor, peginterferon-α, and ribavirin) without cirrhosis require 12 weeks, and treatment-experienced patients (ie, individuals who have failed treatment with either peginterferon-α and ribavirin or an HCV protease inhibitor, peginterferon-α, and ribavirin) with cirrhosis require 24 weeks [16].

After single dose administration of ledipasvir 90 mg/sofosbuvir 400 mg Tablets, mean Ledipasvir C_{max}, AUC_{o-t}, AUC_{o-inf}, $T_{1/2}$ was 246.25 ng/ml, 7258.61 ng.hr/ml, 8648.33 ng.hr/ml, 45.73 hr respectively. Median T_{max} was equal to 5 hours. The mean Sofosbuvir C_{max}, AUC_{o-t}, AUC_{o-inf}, $T_{1/2}$ was 1279.71 ng/ml, 2487.13 ng.hr/ml, 2504.08 ng.hr/ml, 0.49 hr respectively. Median T_{max} was equal to 0.54 hours [17].

Different analytical methods are developed for assay of ledipasvir / sofosbuvir in biological fluids; methods include spectrophotometric, and MS detection of the single analytes, and for simultaneous quantification of ledipasvir / sofosbuvir [18-20].

Many analytical methods used for determination of sofosbuvir in biological samples using HPLC-UV method and sample extraction procedure with liquid-liquid extraction technique, shows a lower quantitation limits LLOQ of 20ng/ml [21]. Another analytical method used for determination of ledipasvir and sofosbuvir in biological samples using HPLC-DAD method, and sample extraction procedure with protein precipitation shows a lower quantitation limits LLOQ of 1000ng/ml [22].

To obtain more sample clean up, an analytical method used for determination of sofosbuvir and daclatasvir in biological samples using LC/MS/MS method, and sample extraction procedure with liquid-liquid extraction shows a lower quantitation limits LLOQ of 0.3ng/ml for sofosbuvir, and 3ng/ml for daclatasvir [23].

Another sensitive LC/MS/MS assay developed for determination of sofosbuvir in human plasma in which drug were extracted by liquid-liquid extraction from human plasma. Chromatographic separations were achieved on a C_{18} column. The method was fully validated. The multiple reaction monitoring was based on m/z transition of 530.21 → 243.21 for Sofosbuvir, and m/z 415.13 → 163.19 for eplerenone (Internal standard). The total run time was 1 min, and the LLOQ was 0.25 ng/mL. The method showed linearity within the range of 0.25 to 3500 ng/mL [24].

This study was performed to investigate the bioequivalence of ledipasvir / sofosbuvir between a generic product; ledipasvir 90mg / sofosbuvir 400mg film coated tablets and reference product. The study protocol called for 36 healthy volunteers in a partial replicate study design. The subjects received one film coated tablets of generic product in one study period, and two film coated tablets of reference product in the other two study periods, in a randomized fashion with a washout period of two weeks. Thirty-Six healthy male subjects completed the crossover [25-27].

Bioanalysis of plasma samples would be carried out through the development of an LC/MS/MS method, which was developed and validated in following international guidelines [(28)]. Pharmacokinetic parameters, determined using non-compartmental analysis module in WinNonline software. The analysis of variance (ANOVA) statistics were calculated using SAS software. The 90% confidence limits for the ratio (or difference) between the generic and reference product pharmacokinetic parameters of AUC_{0-t} and AUC_{0-inf} and C_{max} were calculated and found to be within the 80 to 125% confidence limits [27].

2. MATERIALS AND METHODS

2.1. Chemicals and reagents

Purified Water for LC/MS/MS grade, Human plasma (Vacsera Blood Bank), Methanol (SIGMA

Aldrich, Germany), Acetonitrile (Scharlab, Spain), Formic acid (Scharlab, Spain), Diethyl ether (Scharlab, Spain), Dichloromethane (Scharlab, Spain).

2.2. Equipments:

Variable pippettes (200ul, and 1000ul), plastic pipettes tips - Yellow (range 5 to 200 µL) & Blue (range 200 to 1000 µL), glass test tubes 120 x 12 mm, Vortex mixer (Boeco, Germany), Vacuum pump (Boeco, Germany), PH-meters (Boeco, Germany), Water purifier (Purelab option- R7ELGA, U. K.), Sonicator (Crest, U.S.A.), Analytical balance (Sartorius, U.S.A.), Concentrator Plus/Vacufuge® Plus (Eppendorf, Germany), LC-MS/MS Agilent 6410B Triple Quad, USA.

2.3. Bioanalytical method

2.3.1. Chromatographic conditions:

In house developed chromatographic conditions was used. Mobile phase composition is Ammonium formate pH 2.8 : Acetonitrile (10: 90 V/V). The flow rate was set at 0.55ml/min. Injection volume was set at 12 ul. MS/MS 6410B detector was operated at ESI positive mode, m/z was 889.8 → 130.1, 530.3 → 243.1, and 739.4 → 565.3, for ledipasvir, sofosbuvir, and daclatasvir (internal Standard) respectively.

Fragmentor energy was set at 150, 135, and 140. Collision energy was set at 70, 6, and 38 for ledipasvir, sofosbuvir, and daclatasvir (internal Standard) respectively.

2.3.2. Preparation of solutions

2.3.2.1. Master Standard solution of Sofosbuvir:

Accurately weighed 10mg of sofosbuvir standard were transferred to a volumetric flask (100 ml), about 80 ml methanol was added, and sonication was done for 10 minutes. Volume was completed with methanol in order to solution contains 100ug/ml sofosbuvir "Solution A".

- From “Solution A” 20 ml were transferred to a volumetric flask (100 ml) and volume completed with methanol to obtain a solution of 20ug/ml “Solution B”.
- From “Solution A” 0.05 ml were transferred to a volumetric flask (100 ml) and volume completed with methanol to obtain a solution of 0.05ug/ml “Solution C”.

2.3.2.2. Working Solutions of Sofosbuvir:

Master Solution used	Milliliters taken	Final concentration obtained (ng/ml)	Final volume (ml)
"Solution C"	0.2ml	1	10
"Solution C"	2ml	10	10
"Solution C"	5ml	25	10
"Solution B"	0.025ml	50	10
"Solution B"	0.125ml	250	10
"Solution B"	0.25ml	500	10
"Solution B"	0.5ml	1000	10
"Solution B"	1ml	2000	10
"Solution B"	4ml	8000	10
"Solution B"	5ml	10000	10
"Solution B"	10ml	20000	10

All dilutions are done with Methanol.

2.3.2.3. Master Standard solution of Ledipasvir:

- Accurately weighed 10.65mg of ledipasvir acetone (equivalent to 10mg ledipasvir) standard were transferred to a volumetric flask (100ml), about 80 ml methanol was added, and sonication was done for 10 minutes. Volume was completed with methanol in order to obtain a solution contains 100ug/ml ledipasvir *"Solution A"*.
- From “Solution A” 10 ml were transferred to a volumetric flask (100ml) and volume completed with methanol to obtain a solution containing 10ug/ml ledipasvir “Solution B”.
- From “Solution A” 0.01 ml were transferred to a volumetric flask (100ml) and volume completed with methanol to obtain a solution containing 0.01ug/ml ledipasvir “Solution C”.

2.3.2.4. Working Solutions of Ledipasvir:

Master Solution used	Milliliters taken	Final concentration obtained (ng/ml)	Final volume (ml)
"Solution C"	1ml	1	10
"Solution C"	10ml	10	10
"Solution B"	0.025ml	25	10
"Solution B"	0.05ml	50	10
"Solution B"	0.25ml	250	10
"Solution B"	0.5ml	500	10
"Solution B"	1ml	1000	10
"Solution B"	2ml	2000	10
"Solution B"	4ml	4000	10
"Solution B"	6ml	6000	10

All dilutions are done with Methanol.

2.3.2.5. Daclatasvir Hydrochloride Standard Solution:

Accurately weighed 10mg of daclatasvir hydrochloride standard were transferred to a volumetric flask (100ml), and about 80 ml of methanol were added. Sonication was done for 10 minutes. Volume was completed with methanol to obtain asolution contains 100ug/ml daclatasvir solution (A). From solution (A) 3 ml were transferred to a volumetric flask (100ml) and volume were completed with methanol to obtain 3ug/ml daclatasvir hydrochloride solution (B).

2.3.3. Preparation of Ledipasvir / Sofosbuvir Standard concentrations in human plasma:

The standard samples in plasma were prepared by transferring a 50 ul aliquot of the working standard solutions at concentrations ranging from 1 to 6000 ng/ml, and 1 to 20000ng/ml for of ledipasvir / sofosbuvir respectively, to a centrifuge tubes containing 0.5 ml of blank plasma.

2.3.4. Sample Preparation

Volunteers human plasma samples, standard samples (500 ul) were transferred into appropriate centrifuge test tubes 50 ul of the internal standard (Daclatasvir working solution 3000ng/ml), were added. Then samples were vortex-mixed for approximately 60 seconds. 3 ml [(Diethyl ether / Dichloromethane) 70/30 V/V] were added and vortex-mix was done for approximately 1 to 2 minutes. Samples centrifugation was performed at 3500 rpm for 5 minutes; then a clear layer of organic supernatant was transferred to a test tube and evaporated till dryness. The remaining dry residue was reconstituted with 200ul mobile phase and transfered to insert vial for injection on LC/MS/MS.

2.3.5. Quantitation

Unkown plasma samples drug concentrations calculated using the equation y=ax+b, where; **Y**: response ratio, **X**: unkown plasma drug concentration, **a:** calibration curve slope, **b:** Y-Intercept

2.4. Bioequivalence study

2.4.1. Study Ethics

This study was conducted according to ICH and GCP guidelines adopted by the EMEA, and Declaration of Helsinki after ethics committee approval on the study protocol of ledipasvir/ sofosbuvir 90 / 400 mg tablet (Study Code: MAR-COPE-BES-1216/0238). Essential documents and records were all archived according to drug research center (DRC) internal procedures for authorized direct access.

Written informed consents were signed by the participant and clinical investigator, and all study aspects where discussed with participants before starting of screening. There were no any obligations on volunteers to continue the study if they didn't want to.

Clinical Investigator, study director (principal investigator), licensed physicians responsible for physical examination and following-up of the subjects for appearance of any side or adverse effects, measurement of vital signs throughout the study including blood pressure, pulse rate, body temperature, respiratory rate before and all over the study and registered nurses were responsible for blood sampling.

2.4.2. Inclusion Criteria

Volunteers age should be within 18 to 55 years, and calculated body mass index should lie within normal acceptable limits, no history of contribution in any pharmacokinetics study, and normal physiological examination, laboratory data within normal limits. Subjects should not be alcoholic or drug abusers, and shouldn't have any known history for both. It is preferred to select non-smoker subjects, and if subjects are smokers, so they should not smoke more than 8 cigarettes per day.

2.4.3. Exclusion Criteria

A known drug hypersensitivity, GIT problems, auto-immune diseases, kidney diseases or kidney dysfunction, CVS diseases, diabetics, hepatic disease, hematological abnormalities, respiratory diseases, alcohol intake or drug abuse history, positive HIV-I, (smoking and if including they should be identified), abnormal laboratory values, subject administered any medication less than two weeks of the study starting date, subjects who have donated blood or who participated in clinical studies that requires more than 500 ml of blood to be withdrawn within month and half preceding study starting date.

2.4.4. Subjects

Thirty-six healthy adult, male volunteers participated in this study after being subjected to complete medical and laboratory assessment, and insuring that they are in compliance with the required inclusion/exclusion criteria. No concurrent medication was allowed during the study duration. Subjects started the recommended high-fat / high-calorie meal 30 min before administration of the drug product, and finished eating this meal in 30 min or less. The drug product administered 30 min after start of the meal with 240 mL of water. Food were not permitted for a minimum of 4 hrs post-dose. At 7:30 a.m. they received the recommended high-fat / high-calorie meal followed by the drug doseing, and at 12:00 p.m. another meal. The written informed consent for the intended study were reviewed, discussed and then signed by the participant and clinical investigator before the beginning of screening procedure without any obligation on the volunteers to continue if they didn't want to.

2.4.5. Study design

This study was an open-label, randomized, single-dose study with three-period partial replicated crossover design comparing the bioavailability of

ledipasvir and sofosbuvir between two products, in 36 healthy adults, male volunteers under fed conitions with two weeks washout period between dosing. The number and disposition of the blood collections and the wash out period were designed with respect to pharmacokinetic parameters of ledipasvir and sofosbuvir.

2.4.6. Smaple collection:

The number of blood collections for drug analysis was 22 samples in each study period. The volume of blood taken for the determination of ledipasvir and sofosbuvir in plasma was 5ml per sample. The following blood samples for the analysis of ledipasvir and sofosbuvir in plasma were collected at the following intervals: 0 (directly prior to dosing), 10min, 20 min, 30min, 45 min, 1, 1.5, 2, 2.5, 3, 3.5, 4, 4.5, 5, 6, 7, 8, 10, 12, 24, 48 and 72 hr after the administration. The total amount of blood withdrawn during the whole study did not exceed 330 ml.

Samples were gathered into test tubes spiked with EDTA disodium as an anticoagulant and centrifuged for 10 minutes at approximately 4000 r.p.m. Plasma samples are kept at -80°C in a freezer at the research site until analysis time.

2.4.7. Analysis of Plasma Samples*:*

The withdrawan volunteers' samples plasma were analyzed by using LC-MS/MS technique for the quantitation of ledipasvir and sofosbuvir in human plasma.

2.4.8. Pharmacokinetic Calculations:

The following pharmacokinetic parameters (variables) of sofosbuvir were assessed; C_{max}, t_{max}, $t_{1/2e}$, K_e, AUC_{0-t}, MRT, $AUC_{0-\infty}$, and AUC_{0-72} for ledipasvir.

2.4.9. Tolerability and safety:

The participant medical histories, laboratory reports, physical examination, and all incidents of probable adverse effects to the study drug were reported.

2.4.10. Statistical Analysis of Data

Analysis of variance (ANOVA) were performed by using SAS software. Bioequivalence could be demonstrated for sofosbuvir within the prescribed 90% confidence interval of 80.00% to 125.00% for AUC_{0-t}, AUC_{0-inf} and C_{max}, and for ledipasvir $AUC_{0-72,}$ and C_{max} with respect to the parametric method on Ln-transformed data.

3. RESULTS

3.1. Analytical method validation:

3.1.1 Chromatograms of Ledipasvir / Sofosbuvir:

Ledipasvir and sofosbuvir (Figures 9 and 10) were well separated and their retention time was 1.6, and 1.3 min. chromatography showed symmetrical and sharp peaks with a good baseline resolution and minimum tailing.

3.1.2 Linearity, Accuracy and Precision:

Peak area ratios of varying amounts of ledipasvir and sofosbuvir in human plasma ranging from 0.1 to 600 ng/ml, and 0.1 to 2000ng/ml was highly linear (r^2 was equal to 0.9999) for both drugs. The results of three replicate analysis at three different days over one week period were obtained, where the average CV% was 0.752%, and 0.747% for ledipasvir and sofosbuvir respectively. Accuracy and precision was assessed at three different concentrations in the range of predicted drug concentrations on within, and between-day basis. Intra-day accuracy results showed an average recovery percentage of 99.989, and 100.324% for ledipasvir and sofosbuvir respectively. Inter-day accuracy results showed an average recovery percentage of 99.674, and 98.917%, with an average CV% of 0.752, and 0.747% for ledipasvir and sofosbuvir respectively. Stability study results in plasma showed that the average stability percentage of ledipasvir and sofosbuvir was greater than 95% providing that both drugs are stable in the studied conditions.

3.2. Bioequivalence Study:

3.2.1. Clinical observation:

The drug was well tolerated by all participating subjects, blood sampling was obtained during the two periods completely at the proper time, and no adverse events was observed during the study time course.

3.2.2. Pharmacokinetic data and assessment of bioequivance:

For ledipasvir (Tables 1, 2, 3) the mean (C_{max}) was 253.814±58.557ng/ml, 251.634±56.983ng/ml, and 253.749±62.569ng/ml, (t_{max}) 4.806±0.467hr, 4.667±0.609hr, and 4.611±0.465hr, ($t_{1/2e}$) 47.529±9.930hr, 48.821±10.577hr, and 48.254±11.346hr, (AUC_{0-72}) 6498.690±1152.277ng.hr/ml, 6479.679±1182.046ng.hr/ml, and 6580.804±1229.018ng.hr/ml for generic and the two reference products adminstrations respectively.

For sofosbuvir (Tables 4, 5, 6), the mean (C_{max}) was 1317.038±150.341ng/ml, 1355.967±180.741ng/ml, and 1316.699±169.770ng/ml. (t_{max}) 1.264±0.280hr, 1.174±0.304hr, and 1.229±0.335hr. ($t_{1/2e}$) 0.877±0.296, 0.881±0.254hr, and 0.845±0.897hr. (AUC_{0-t}) 2672.513±527.577ng.hr/ml, 2585.363±557.100ng.hr/ml, and 2745.991±469.307ng.hr/ml. (MRT) 1.995±0.411hr, 1.797±0.386hr, and 2.006±0.422hr. ($AUC_{0-\infty}$) 2673.137±527.672ng.hr/ml, 2586.444±557.644ng.hr/ml, and 2747.260±470.182ng.hr/ml for generic and the two reference products adminstrations respectively.

3.2.3. Tolerability and safety:

Side effects and adverse events didn't appear on any participant during the whole study periods.

3.2.4. Statistical analysis

The results of 2-way ANOVA on C_{max}, AUC_{0-t}, AUC_{0-inf} for ledipasvir and sofosbuvir (Tables 7 and 8) showed that there was no significant difference between generic and reference product. For ledipasvir the point estimate (%) results for C_{max}, and AUC_{0-72} were 100.14, and 99.96% respectively. The 90% confidence limits of parametric means of C_{max}, and AUC_{0-72}, were 99.75 to 100.54%, 99.70 to 100.21% respectively. For sofosbuvir the point estimate (%) results for C_{max}, AUC_{0-t}, AUC_{0-inf} were 99.85, 100.07, and 100.06% respectively. The 90% confidence limits of parametric means of C_{max}, AUC_{0-t}, AUC_{0-inf} were 99.48 to 100.23%, 99.44 to 100.69%, 99.44 to 100.69% respectively.

Table 1: Pharmacokinetic parameters of Ledipasvir following administration of single oral dose of Ledipasvir/Sofosbuvir 90/400 mg Film Coated Tablet (Generic product) to 36 volunteers

Subject	T_{max} (hr)	C_{max} (ng/ml)	AUC_{0-72} (ng.hr/ml)	K_e (hr^{-1})	$T_{1/2}$ (hr)
Mean	4.806±0.467	253.814±58.557	6498.690±1152.277	0.015±0.003	47.529±9.930
CV%	9.722	23.071	17.731	20.810	20.893
Range (Median)	4.000-6.000 (4.750)	170.670-397.496 (235.690)	3375.282-8696.970 (6536.603)	0.009-0.023 (0.015)	30.694-75.466 (46.249)

Table 2: Pharmacokinetic parameters of Ledipasvir following administration of single oral dose of Ledipasvir/Sofosbuvir 90/400 mg Tablet (Reference product) administered for the first time (in period I and II) to 36 volunteers

Subject	T_{max} (hr)	C_{max} (ng/ml)	AUC_{0-72} (ng.hr/ml)	K_e (hr^{-1})	$T_{1/2}$ (hr)
Mean	4.667±0.609	251.634±56.983	6479.679±1182.046	0.015±0.003	48.821±10.577
CV%	13.060	22.645	18.242	20.420	21.665
Range (Median)	3.500-6.000 (4.750)	160.548-386.858 (243.681)	4275.662-9424.254 (6320.275)	0.008-0.021 (0.015)	32.557-84.285 (47.329)

Table 3: Pharmacokinetic parameters of Ledipasvir following administration of single oral dose of Ledipasvir/Sofosbuvir 90/400 mg Tablet (Reference product) administered for the second time (in period II and III) to 36 volunteers

Subject	T_{max} (hr)	C_{max} (ng/ml)	AUC_{0-72} (ng.hr/ml)	K_e (hr^{-1})	$T_{1/2}$ (hr)
Mean	4.611±0.465	253.749±62.569	6580.804±1229.018	0.015±0.004	48.254±11.346
CV%	10.076	24.658	18.676	23.710	23.512
Range (Median)	3.500-6.000 (4.500)	160.680-403.899 (231.774)	3871.888-8582.216 (6447.153)	0.010-0.024 (0.015)	28.528-72.059 (47.103)

Table 4: Pharmacokinetic parameters of Sofosbuvir following administration of single oral dose of Ledipasvir/Sofosbuvir 90/400 mg Film Coated Tablet (Generic product) to 36 volunteers

Subject	T_{max} (hr)	C_{max} (ng/ml)	AUC_{0-t} (ng.hr/ml)	AUC_{0-inf} (ng.hr/ml)	K_e (hr^{-1})	$T_{1/2}$ (hr)	MRT_{inf} (hr)
Mean	1.264±0.280	1317.038±150.341	2672.513±527.577	2673.137±527.672	0.874±0.265	0.877±0.296	1.995±0.411
CV%	22.150	11.415	19.741	19.740	30.344	33.719	20.626
Range (Median)	1.000-2.000 (1.250)	1064.450-1642.271 (1300.051)	1484.592-3609.683 (2695.365)	1484.752-3610.862 (2696.519)	0.445-1.499 (0.897)	0.462-1.558 (0.773)	1.297-2.848 (1.875)

Table 5: Pharmacokinetic parameters of Sofosbuvir following administration of single oral dose of Ledipasvir/Sofosbuvir 90/400 mg Tablet (Reference product) administered for the first time (in period I and II) to 36 volunteers

Subject	T_{max} (hr)	C_{max} (ng/ml)	AUC_{0-t} (ng.hr/ml)	AUC_{0-inf} (ng.hr/ml)	K_e (hr^{-1})	$T_{1/2}$ (hr)	MRT_{inf} (hr)
Mean	1.174±0.304	1355.967±180.741	2585.363±557.100	2586.444±557.644	0.841±0.204	0.881±0.254	1.797±0.386
CV%	25.874	13.329	21.548	21.560	24.244	28.835	21.484
Range (Median)	0.750-2.000 (1.000)	1040.981-1655.426 (1396.029)	1659.215-3790.073 (2507.495)	1660.073-3790.900 (2508.228)	0.394-1.317 (0.853)	0.526-1.758 (0.813)	1.205-2.717 (1.695)

Table 6: Pharmacokinetic parameters of Sofosbuvir following administration of single oral dose of Ledipasvir/Sofosbuvir 90/400 mg Tablet (Reference product) administered for the Second time (in period II and III) to 36 volunteers

Subject	T_{max} (hr)	C_{max} (ng/ml)	AUC_{0-t} (ng.hr/ml)	AUC_{0-inf} (ng.hr/ml)	K_e (hr^{-1})	$T_{1/2}$ (hr)	MRT_{inf} (hr)
Mean	1.229±0.335	1316.699±169.770	2745.991±469.307	2747.260±470.182	0.897±0.249	0.845±0.290	2.006±0.422
CV%	27.233	12.894	17.091	17.115	27.774	34.334	21.029
Range (Median)	0.750-2.000 (1.000)	1029.441-1728.383 (1305.600)	1823.628-3622.659 (2723.187)	1824.269-3626.123 (2723.388)	0.402-1.529 (0.940)	0.453-1.724 (0.738)	1.290-3.001 (1.960)

Table (7): 90% C.I for Ledipasvir generic and reference Products

Pharmacokinetic Parameter	90% Confidence intervals of parametric means		
	Point estimate (%)	Lower limit (%)	Upper limit (%)
C_{max}	100.14	99.75	100.54
AUC_{0-72}	99.96	99.70	100.21

Table 8: 90% C.I for Sofosbuvir generic and reference Products

Pharmacokinetic Parameter	90% Confidence intervals of parametric means		
	Point estimate (%)	Lower limit (%)	Upper limit (%)
C_{max}	99.85	99.48	100.23
AUC_{0-t}	100.07	99.44	100.69
AUC_{0-inf}	100.06	99.44	100.69

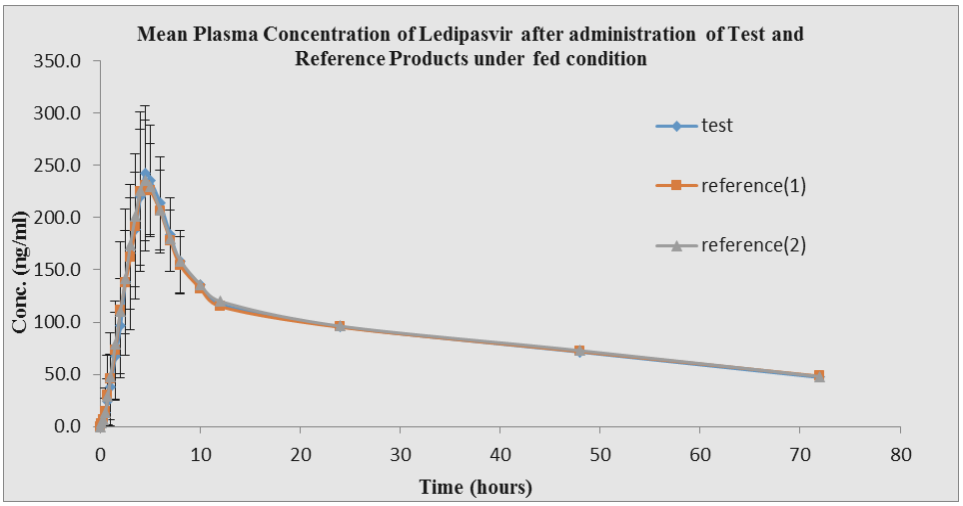

Figure 1: Mean Plasma concentration of Ledipasvir following single dose administration of Ledipasvir/Sofosbuvir 90/400 mg Film Coated Tablets generic and reference products

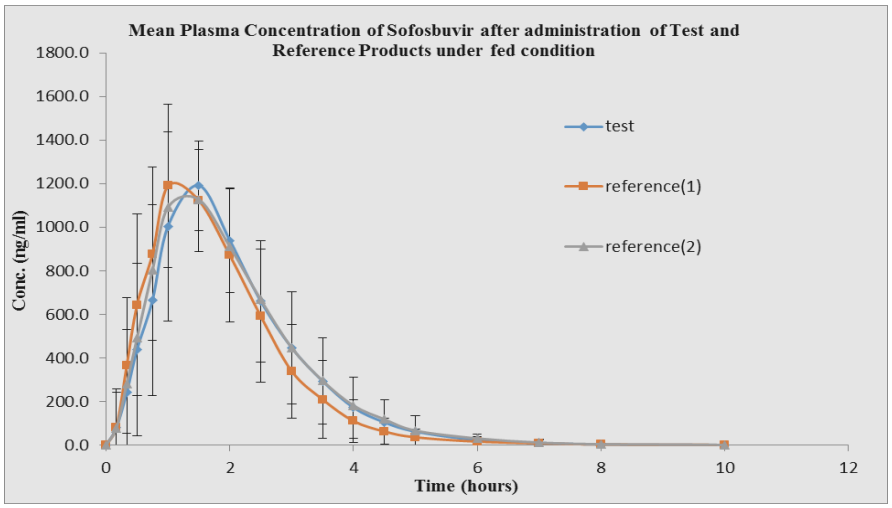

Figure 2: Mean Plasma concentration of Sofosbuvir following single dose administration of Ledipasvir/Sofosbuvir 90/400 mg Film Coated Tablets generic and reference products

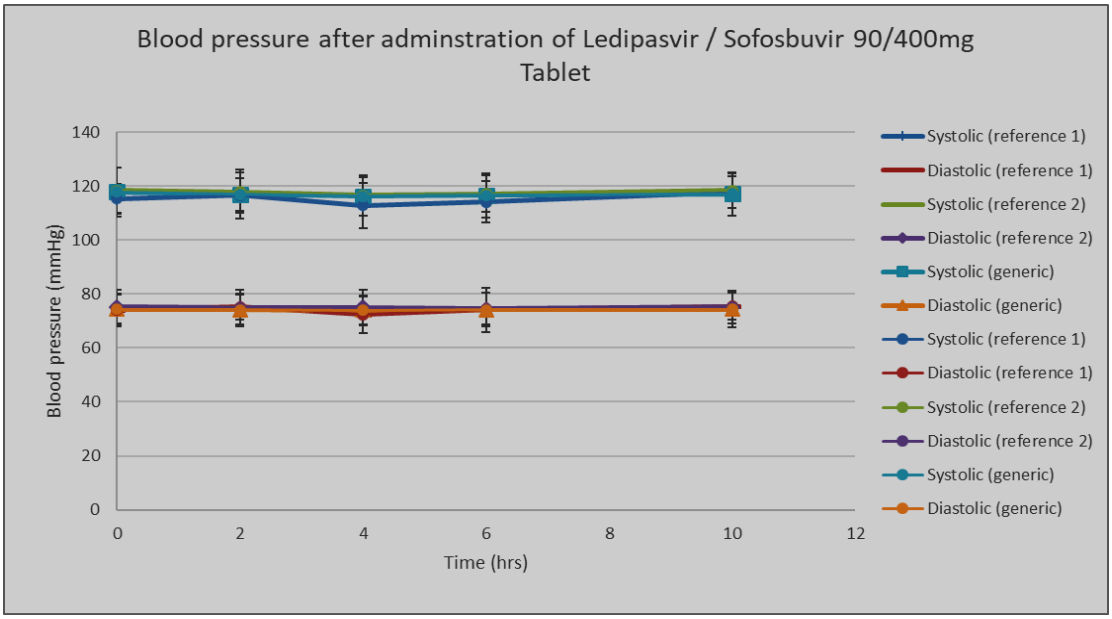

Figure 3: Blood pressure after administration of Ledipasvir/Sofosbuvir 90/400 mg Film Coated Tablets generic and reference products

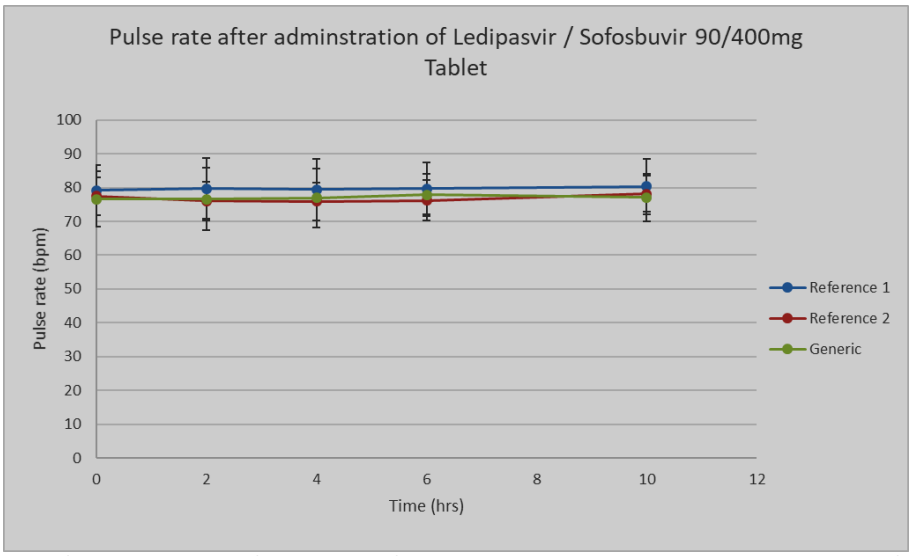

Figure 4: Pulse rate after administration of Ledipasvir/Sofosbuvir 90/400 mg Film Coated Tablets generic and reference products

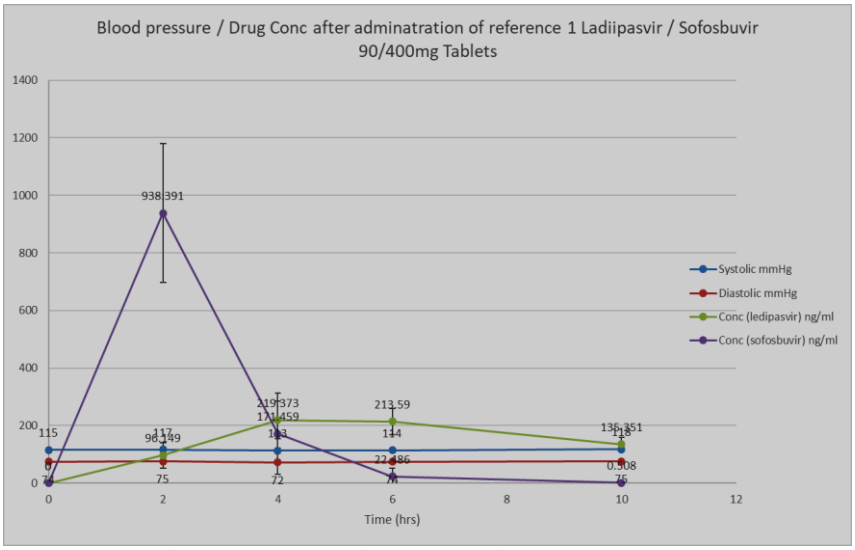

Figure 5: Blood pressure / Drug Conc after administration of Ledipasvir/Sofosbuvir 90/400 mg Film Coated Tablets reference 1 product

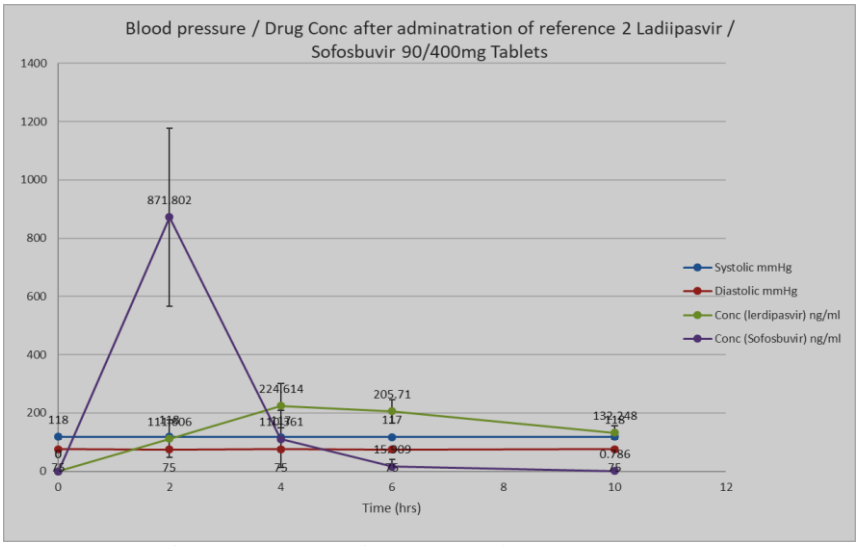

Figure 6: Blood pressure / Drug Conc after administration of Ledipasvir/Sofosbuvir 90/400 mg Film Coated Tablets reference 2 product

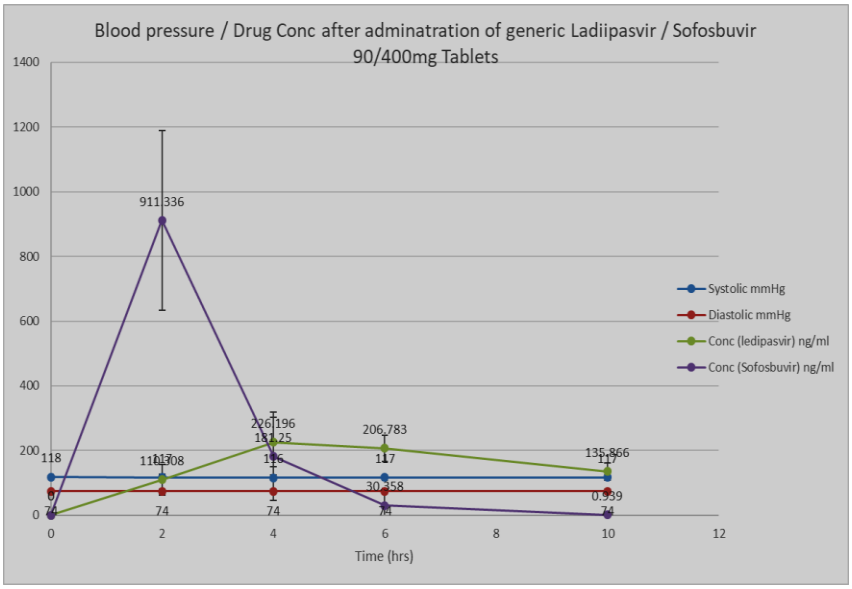

Figure 7: Blood pressure / Drug Conc after administration of Ledipasvir/Sofosbuvir 90/400 mg Film Coated Tablets generic product

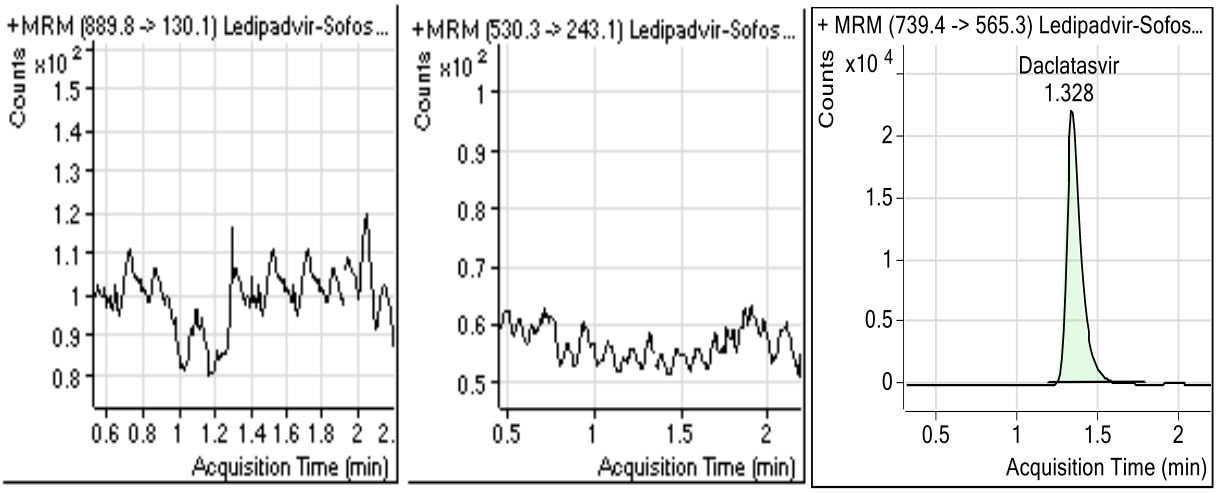

Figure 8: Sample chromatogram representing an MRM data of blank human plasma sample spiked with internal standard Daclatasvir

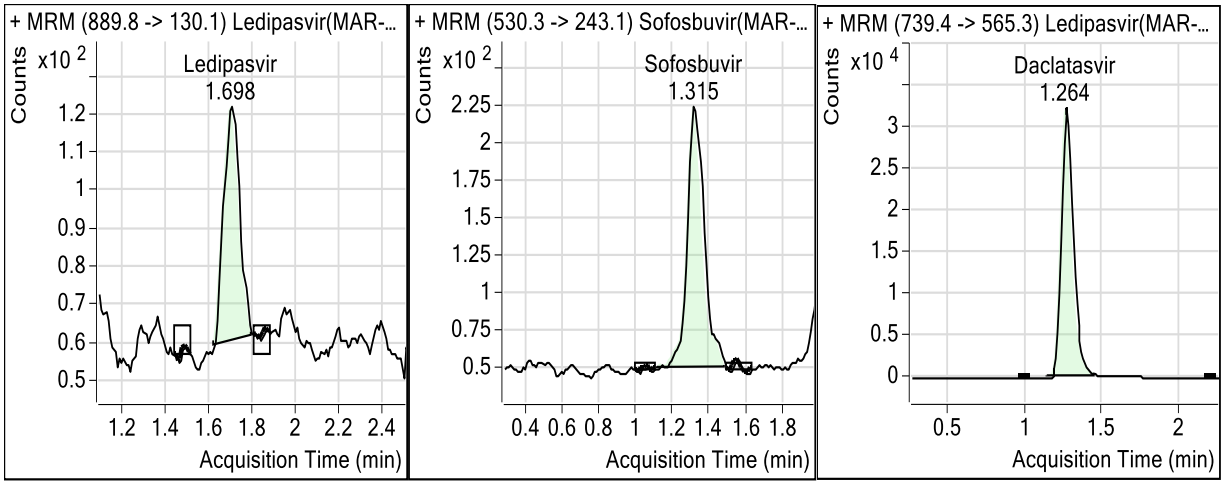

Figure 9: Sample chromatogram representing an MRM data of blank human plasma sample spiked with 0.1ng/ml Ledipasvir, 0.1ng/ml Sofosbuvir and internal standard Daclatasvir

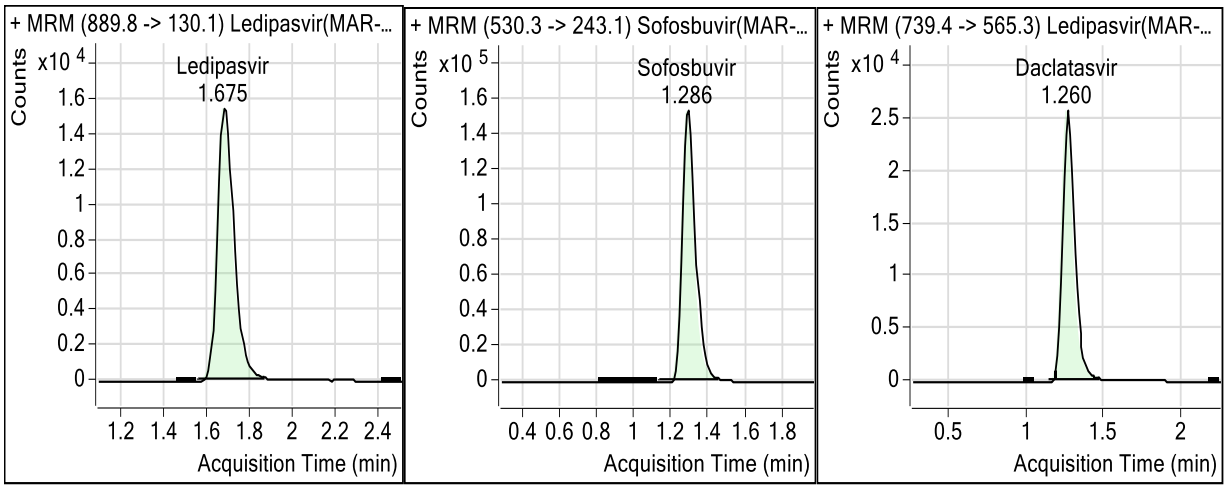

Figure 10: Sample chromatogram representing an MRM data of blank human plasma sample spiked with 200ng/ml Ledipasvir, 1000ng/ml Sofosbuvir and internal standard Daclatasvir

4. DISCUSSION

The LC/MS/MS method used in this study was simple, of excellent sensitivity, specificity, precision and accuracy. The calibration curve was linear over the concentration range of 0.1 to 600 ng/ml, and 0.1 to 2000ng/ml for ledipasvir and sofosbuvir respectively, and r^2 was equal to 0.9999 for both drugs, which is in accordance with the latest FDA Guidelines [28], and so it could be used for pharmacokinetic and bioavailability studies of ledipasvir and sofosbuvir.

Literature showed a sensitive and simultanioues determination of ledipasvir / sofosbuvir in biological samples, using LC/MS/MS and liquid-liquid extraction. The method used a Reversed Phase C_{18} column with an isocratic mobile phase mixture of 10 mM Amm acetate, adjusted to pH 4.0 by acetic acid : acetonitrile : 0.1% methanolic formic acid (12 : 25 : 63 v/v/v). Mass parameters were set on multiple reaction monitoring mode (MRM) and Positive ESI, using the respective mass to charge ratios, m/z 889.2 → 889.2 for ledipasvir, m/z 530.3 → 243.1 for sofosbuvir, and m/z 243.1 → 565.3 for daclatasvir (Internal standard), with a quantitation limit of 0.5 ng/ml, and 5 ng/ml and a linearity of 0.5 to 2500 ng/ml for sofosbuvir and 5 to 2100 ng/ml for ledipasvir [29].

The in-house developed chromatographic conditions, is more sensitive, specific than published litreature methods [21-24, 29] after modifying extaction procedure and chromatographic conditions. The technique used for sample cleanup is in accordance with published literature [29] which applied liquid - liquid extraction technique for sample preparation.

As shown previously, the clinical importance of Ledipasvir / Sofosbuvir as an antiviral agent indicated for the treatment of chronic HCV genotype 1 in adults [5]. Besides there is no requirement for coadministration of ribavirin [6], and shortening the duration of treatment to 12 or 24 weeks [7, 8]. It is worthy to mention the importance of the developed bioanalytical assay in order to ensure accurate and precise therapeutic drug monitoring, and testing the validity of generic drug products for commertical use, obtaining better clinical outcomes.

The results of ledipasvir / sofosbuvir pharmacokinetic parameters obtained was nearly in accordance with reported literature which stated that for ledipasvir T_{max} is 5 hours, C_{max} 246.25 ng/ml, $T_{1/2}$ 45.7 hours, and for sofosbuvir T_{max} were found to be 0.5 hours, C_{max} 1279.71 ng/ml, $T_{1/2}$ 0.49 hours [17].

In bioequivalence study 90% confidence limite of 80 to 125% for AUC_{0-t}, AUC_{0-inf} and C_{max} with respect to the parametric method on Ln-transformed data should be fulfilled. The 90% confidence intervals of parametric means of C_{max}, AUC_{0-t}, AUC_{0-inf} were lying within FDA acceptance limits (80 to 125%) [28].

Clinical studies showed that sofosbuvir is capable of suppressing families of positive-stranded RNA viruses; flaviviridae, togaviridae, and coronaviruses. Therbay we can postulate that SARS-CoV-2 RdRp is very likely to be effectively inhibited by Sofosbuvir [30]. Morover, it was reported that (ledipasvir and sofosbuvir) could be candidates that can inhibit 3C-like protease of the virus, and treat COVID-19 with minimal side effects [31].

Sofosbuvir at a 400 mg daily dose is generally well tolerated in a 24-week treatment plan. Besides, sofosbuvir active metabolite shows a high degree of intracellular stability which leads to a proposal that SARS-CoV-2 infection could also likely to be cured by sofosbuvir. Also, it was reported that sofosbuvir does not affect the main cytochromal metabolizing enzymes as cytochrome P450 system [32].

The 3C-like cleavage sites on the coronaviral polyproteins are highly conserved. Some specific inhibitors previously developed for the SARS-CoV enzymes can be used for SARS-CoV-2. Ledipasvir/Sofosbuvir could be very effective choice due to their dual inhibitory actions on two viral enzymes [33].

Furthermore, other direct antiviral drugs, such as lopinavir/ritonavir, shown a variety of pharmacological interactions with other therapeutic medications used to treat co-morbid conditions such as diabetes. Ledipasvir/sofosbuvir combination have the advantage over other direct antiviral agents in terms of minimal drug interaction and does not affect CYP450 metabolic enzymes. Besides, ledipasvir/sofosbuvir demonstrated efficacy, safety, and tolerability in several cases, and therefore may be preferable to the other direct antiviral medications described in the treatment of COVID-19 [34].

5. CONCLUSION

It can be concluded that the bioanalytical method developed for the quantification of ledipasvir and sofosbuvir in human plasma is valid, sensitive, specific, precise and accurate, and could be used for the quantification of drug pharmacokinetic parameters. Besides, results of the bioequivalence study of ledipasvir / sofosbuvir 90/400 mg F.C.T generic product compared to reference product are bioequivalent. Moreover, ledipasvir / sofosbuvir is suggested to be superior over other direct antivral agents in management of COVID-19 due to its minemal side effects and less drug interactions, hence the importance of a valid bioanalytical method emerges for accurate therapeutic drug monitoring.

DECLARATIONS

Consent for publication: Not applicable

Availability of data and materials

The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

Declaration of Competing Interest: The authors have no conflicts of interest to declare.

Author Contributions

Nagwa Ali Sabri designed and supervised the research. Mohamed Ahmed Raslan carried out the experiments and performed data analysis. Sara Ahmed Raslan and Eslam Mansour Shehata provided resources and wrote the manuscript. Nagwa Ali Sabri revised the manuscript. All authors have read and approved the final manuscript.

ABBREVIATIONS

COVID-19: Coronavirus Disease 2019.
SARS-CoV-2: Severe Acute Respiratory Syndrome Coronavirus 2.
LC/MS/MS: Liquid Chromatography Tandem Mass Spetrometry.
ANOVA: Analysis of Variance.
Cmax: Maximum (or peak) Plasma Drug Concentration.
Tmax: Time Taken to Reach Cmax.
AUC: Area Under the Curve.
T1/2el: Elimination Half-life
MRT: Mean Residence Time
LDV: Ledipasvir
SOF: Sofosbuvir
ICH: International Council for Harmonisation
GCP: Good Clinical Practice
EMEA: European Medicines Agency.
GIT: Gastrointestinal Tract.
CVS: Cardiovascular System.
HIV: Human Immunodeficiency Virus.
HCV: Hepatitis C Virus.
FDA: U.S. Food and Drug Administration.
CYP450: Cytochrome P450.
F.C.T: Film Coated Tablet.

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