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Specific stability indicating spectrofluorimetric method for determination of ledipasvir in the presence of its confirmed degradation products; application in human plasma

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ABSTRACT

A rapid and specific spectrofluorimetric method with higher sensitivity was developed for determination of ledipasvir (LDS) in tablets and human plasma. The proposed method relies on hydrogen bonding formations between the hydroxyl groups of polyoxyethylene 50 stearate and LDS, causing significant enhancement of its native fluorescence. The fluorescence intensity was measured at 430 nm after excitation at 340 nm. The fluorescence–concentration plot was rectilinear over the range 1–400 ng mL^{-1} with detection and quantification limits of 0.25 and 1.10 ng mL^{-1}, respectively. The high sensitivity of the proposed method permits its application for ledipasvir determinations in real human plasma even in the presence of co-administered drugs sofosbuvir and ribavirin. Moreover, the proposed method was further extended to stability studies of ledipasvir after exposure to different forced degradation conditions according to ICH guidelines, along with the structural elucidation of its degradation products utilizing IR and Mass spectra. A proposal for the degradation pathways was presented.

1. Introduction

Hepatitis C virus (HCV) is a RNA virus. It is estimated that about 170 million people are currently infected with hepatitis C worldwide [1]. There are approximately 700,000 people passing away every year from chronic liver disease [1]. A serious liver disease like cirrhosis and hepatocellular carcinoma, and consequence a liver transplantation is globally registered as a result of HCV infection [2].

The World Health Organization (WHO) says Egypt has the highest prevalence in the world, while 150,000 Egyptians are infected each year. Among those infected with HCV, 4000 people die every year [3].

The WHO has recently introduced ledipasvir (LDS) (Fig. 1) into WHO's core list of medicines [3]. The WHO recommended three regimens for the treatment of HCV; sofosbuvir (SFB), with daclatasvir, ledipasvir or ribavirin [4]. LDS is a direct-acting antiviral agent and has an ability to inhibit HCV-NS5A protein which is required for viral RNA replication and assembly of HCV virions [5].

Few methods were reported for determination of LDS in tablets and/or biological fluids depending mainly on chromatography [6–15] and UV spectrophotometry [16–19]. To date, one spectrofluorimetric method [20] was published concerning the determination of LDS in tablet dosage form. To the author's knowledge, there is no report concerning the spectrofluorimetric determination of LDS in human plasma until now. Therefore the proposed method is the first one for determination of the studied drug in human plasma. The reported spectrofluorimetric method [20] was based on native fluorescence with linearity range 100–800 ng mL^{-1} and quantitation limit 50 ng mL^{-1} with lower sensitivity than the proposed method (66 times). This linearity range is not sufficient enough to estimate the studied drug in real human plasma as the drug c_{max} [21] is 323 ng mL^{-1}. The presented method was successfully applied for stability indicating study of LDS under different stress conditions. Structural elucidation of its degradation products using IR and MS spectra also studied.

2. Experimental

2.1. Apparatus

The fluorescence measurements were recorded using a Fluorescence spectrometer FS-2 (Scinco, Korea), connected to Dell PC, grating excitation and emission monochromators. PMT voltage of 400 V. Slit width for both monochromators was set at 10 nm, and photomultiplier voltage was set to auto. Quartz cell 1 cm was used in all measurements. Jenwey PH meter model 350 (E.U) and laboratory centrifuge of 16,000 rpm

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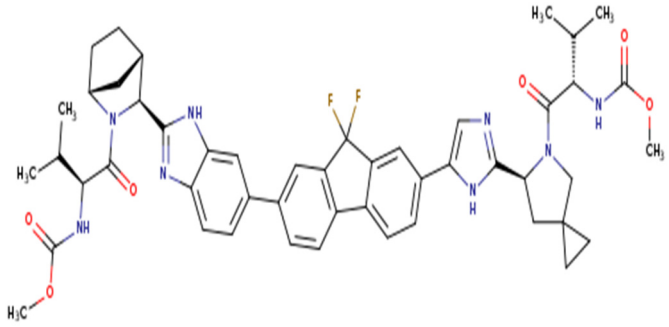

Fig. 1. Chemical structure of LDS.

speed (Bremsen ECCO, Germany). A Shimadzu UV-1601 PC UV–visible spectrophotometer (Tokyo, Japan) with 1 cm quartz cell.

CAMAG UV-lamp, dual wavelength (254/366), 2 × 8 W (Muttenz Switzerland) was used in the photo-stability study.

2.2. Material and Reagents

Analytical grade chemicals and double distilled water were used in this study:

Ledipasvir pure standard was purchased from Sell-eckchem Munich, Germany. With purity of 99.99% according to the comparison method [10]. Sofosbuvir pure standard was purchased from Virdev Intermediates Pvt. Ltd., India. Ribavirin pure standard was purchased from Modern Times Helpline Pharma; New Delhi; India.

Harvoni® tablets were manufactured by Gilead Sciences, limited IDA business & technology, Ireland, manufactured for Gilead Sciences international, Cambridge, UK (labeled to contain 90/400 mg ledipasvir and sofosbuvir respectively).

Methanol, ethanol, isopropanol, acetonitrile were purchased from El-Nasr company, Cairo, Egypt.

Acetate buffer solutions (0.2 M) with a pH range of 3.6–5.6 and borate buffer (0.2 M) with a pH range of 6.5–9.5 were prepared (BDH, UK).

Tween 80, PEG 6000, PEG 1200, sodium lauryl sulfate (SDS), polyoxyethylene 50 searate (PES), β-cyclodextrin (β-CD), carboxymethyl cellulose (CMC), hydrogen peroxide (30% v/v), sodium hydroxide, and hydrochloric acid 33% were manufactured by El-Nasr Chem. company, Cairo, Egypt.

The authors got the permission for using plasma sample of human volunteers from Assiut University Hospital according to institutional guidelines. In all cases, informed written consent was obtained from all participants.

2.3. Standard Solutions

A stock solution of 0.1 mg mL^{-1} of LDS was prepared. In to 100.0 mL volumetric flask; 10.0 mg of LDS was dissolved in 80 mL methanol and then completed to the mark with the same solvent. Then, further dilution with methanol was made to obtain working solutions in the concentration range of 10–4000 ng mL^{-1}. All standards solutions were protected from light and stored at −20 °C. The maximum storage period was 30 day at −20 °C and 5 h at room temperature (25 °C).

2.4. Procedures

2.4.1. General Procedure

Into a series of 10.0 mL volumetric flasks, aliquot volumes of standard solutions were transferred, then 1.0 mL of PES (2% w/v) was added. The contents of each flask were diluted to the mark with distilled water and mixed well to obtain final concentrations in the range of 1–400 ng mL^{-1}. The fluorescence intensities were measured at 430 nm after excitation at 340 nm against reagent blank treated similarly.

2.4.2. Procedure for Tablets

Ten pulverized Harvoni® tablets were finely powdered and weighed. A weighed quantity of the powdered tablets equivalent to 10.0 mg of LDS was transferred into 100.0 mL volumetric flask, dissolved in 80 mL methanol and sonicated for 10 min. The flask content was completed to the mark with distilled water and filtered. Aliquot volume in the working concentration range of the studied drug was analyzed using the procedure described under Section 2.4.1.

2.4.3. Procedure for Spiked Human Plasma

Into 10.0 mL stoppered calibrated tube, 1.0 mL of the drug-free plasma was added, then spiked with 1.0 mL of LDS standard (0.4 μg mL^{-1}), vortex for 1 min. Two milliliters of acetonitrile was added to precipitate proteins. The resultant mixture was diluted to 10.0 mL with methanol to achieve final concentration range of 40–4000 ng mL^{-1}. Then, centrifugation at 4000 rpm for about 10 min (procedure under Section 2.4.1) was followed. A blank experiment was carried out on plasma free drug in the same manner. The relative fluorescence intensity was plotted against the final concentration of the drug.

2.4.4. Procedure for Real Human Plasma

Ledipasvir is well absorbed after oral administration and median peak concentrations were observed 4 to 4.5 h post-dose. After multiple oral 90 mg once daily doses, with C_{max} [21] of 323 ng mL^{-1}, plasma samples were taken from four hepatitis C patients during treatment with SFB and LDS (Harvoni® tablet) regimen. One Harvoni® tablet contains 90/400 mg LDS/SFB was taken orally once daily for 12 weeks. Into heparinized tube, 5.0 mL of human blood sample was taken after an average of 4.5 h following multiple oral administrations and then centrifuged at 4000 rpm for 10 min. Then, 1.0 mL of plasma obtained was quantitatively transferred to an aluminum foil wrapped centrifuge tube. For protein precipitation 2 mL of acetonitrile was added, vortex for 1 min. Then the mixture was centrifuged at 4000 rpm for 10 min. The clear supernatant was carefully transferred to a 10.0 mL volumetric flask and further processing as under Section 2.4.1.

2.4.5. Stress Degradation Studies

2.4.5.1. Acidic and Alkaline Degradation Study. Aliquots of LDS equivalent to 50 μg mL^{-1} were transferred into series of a test tube, 5.0 mL of 0.5 M HCl or 0.5 M NaOH was added, the contents of tubes were boiled at 100 °C for different time ranges 10, 20, 30, 40, 50, and 60 min, the solutions were cooled and neutralized to pH 7 with either 0.5 M NaOH or 0.5 M HCl. The solutions were transferred to 25.0 mL volumetric flask and completed to the mark with distilled water. Then, into 10.0 mL volumetric flask 1.5 mL was added and the volume was completed to the mark with distilled water (the final concentration 300 ng mL^{-1}), then the general procedure was followed.

To investigate the degradation pathway of the studied drug under acidic and alkaline condition, powder equivalent to 50 mg of ledipasvir was dissolved in the least amount of ethanol, transferred into 100-mL round flask and 50 mL of 0.5 M NaOH and/or 0.5 M HCl was added to the flask. The contents of the flask were heated under reflux at 80 °C for 6 h. The solution was cooled and adjusted to pH 7–8 with 0.5 M HCl or 0.5 M NaOH and evaporated to dryness under vacuum. The dry residue was extracted twice with ethanol (2 × 20 mL), filtered into a 50-mL volumetric flask and completed to the volume with ethanol to obtain an acid or alkali-hydrolyzed solution equivalent to 1 mg mL^{-1} of the LDS drug. This solution was tested by TLC, IR, and MS.

2.4.5.2. Oxidative Degradation Study. Aliquots of LDS equivalent to 50 μg mL^{-1} were transferred into test tube series, 5.0 mL of 10%