Original Research Article

Validated Stability – Indicating Methods for Determination of Sofosbuvir by UPLC and HPTLC in Pure Form and Tablet Dosage forms.

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Aims: Two simple and sensitive stability- indicating methods were developed and validated for the quantitative determination of sofosbuvir in presence of its degradation products.

Study design: Ultra high performance liquid chromatography (UPLC), High performance thin layer chromatography (HPTLC) are developed for determination of sofosbuvir in presence of its degradation products, laboratory-prepared mixtures and in tablet dosage forms.
Place and Duration of Study: Analytical Chemistry Department, Faculty of Pharmacy (Girls), Al-Azhar University, between August 2018 and March 2019.
Methodology: Two simple and sensitive stability- indicating methods were developed and validated for the quantitative determination of Sofosbuvir in presence of its degradation products. The first method was an Ultra Performance Liquid Chromatography (UPLC) method, in which efficient separation was carried out on phenomenex kinetex 2.6 µm C18 100 A column using a mobile phase consisting of filtered and degassed mixture of 0.1% ortho-phosphoric acid in water and methanol with the ratio of (40:60% v/v) adjusted to pH 3.5, at a flow rate of 1 mL min^{-1} and UV detection at 260 nm at ambient temperature. The second method is a high performance- thin layer chromatographic one (HPTLC) in which chromatographic separation was performed on silica gel 60 F_{254} plates, with methanol – chloroform – ammonia (2.5: 6: 1.5 % v/v/v) as a developing system followed by densitometric determination at 261 nm. Sofosbuvir was subjected to stress conditions including alkaline, acidic and oxidative degradation.

Results: Beer's law was obeyed over the range of 1-20 µg mL^{-1} for UPLC and 2-12 µg / spot for HPTC with good accuracy and precision using the two procedures, respectively. Results obtained was statistically analysed and found to be in accordance with those given by the reported method.

Conclusion: The proposed methods were successfully applied for the determination of sofosbuvir in bulk powder, laboratory prepared mixtures and pharmaceutical dosage form with good accuracy and precision. The methods were validated according to ICH guidelines. The results obtained were compared with those of the reported method and were found to be in good agreement.

Keywords: Sofosubvir; UPLC; HPTLC; Degradates,Tablet dosage form.

1. INTRODUCTION

Sofosubvir, chemically described as (*S*)-isopropyl-2-(*S*)-(2*R*, 3R, 4R, 5R)-5-(2,4-dioxo-3,4dihydropyrimidin-1(2*H*)-yl)-4-fluoro-3hydroxy4methyltetrahydrofuran 2yl)methoxy)zphenoxy) phosphoryle amino) propanoate (1), Fig.(1). Sofosbuvir is a direct-acting antiviral agent against HCV for the treatment of adult patients with chronic hepatitis C virus (HCV) infection. It interferes with the HCV lifecycle, restraining viral replication. (2). Literature survey revealed that sofosbuvir has been determined by spectrophotpmetry and HPLC methods (3-6). It was also assayed in combination with other drugs by RP- HPLC (7, 8), LC – MS/MS method (9) and bio-analytical UPLC-ESI-MS/MS methods (10-12). Moreover, few stability HPLC indicating methods were reported for its analysis (13-15). The primary goal of this work was to develop a simple, rapid and sensitive methods for the determination of sofosbuvir in presence of its alkaline, acidic and oxidative degradates as well as in its tablet dosage forms.

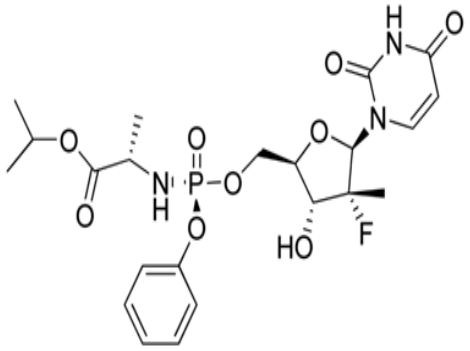


Fig. 1: Structure of Sofosbuvir

2. EXPERIMENTAL

2.1. Instrumentation

- Agelint 1290 Ultra HPLC with binary pump and UV detector (USA).
- Camag TLC scanner 3, with WINCATS computer software (Switzerland).
- Precoated TLC plates, silica gel 60 GF254 (20 × 20 cm), (Fluka chemie, Switzerland). Using Hamilton 50-µL micro syringe (Germany).
- UV lamp with short wavelength (254 nm), (Desega, Germany).
- pH meter combined plus electrode (Adwa model AD1030 pH mv), (UK).

2.2. Material and Reagents

2.2.1. Pure and Market samples

Sofosbuvir B.N. OP-G-LD/12/17/099 was kindly supplied by Marcyrl Company for Pharmaceutical Industry, El Obour City, Egypt, with purity of 98.2% as stated by the supplier. MPIVIROPACK® tablets; B. N. 1830486, each tablet labeled to contain 400 mg Sofosbuvir, product of Marcyrl Company for Pharmaceutical Industry, El Obour City, Egypt.

2.2.2. Degraded samples

Ten mg of pure sofosbuvir was accurately weighed and refluxed separately with 25 mL of 5N NaOH for 3 h, or with 25 mL 5N HCL for 7 h. For oxidative degradation 10 mg of pure drug was set aside with 25 mL of 3% H_2O_2 for one week at room temperature. The alkaline and acidic hydrolysed solutions were cooled and neutralized to about pH 7 with 5N HCL or 5N NaOH and evaporated to dryness under vacuum. The obtained residues were extracted three times each with 25 mL methanol then filtered separately into 100 mL volumetric flask and diluted to the volume with methanol to obtain a stock solution claimed to contain degradates derived from 0.1mg mL^{-1} intact drug which was used for UPLC and HPTLC methods. Also oxidative degradate was exposed to dryness at room temperature then the residue was extracted three times each with 25 mL methanol then filtered into 100 mL volumetric flask and diluted to the volume with methanol to obtain a stock solution claimed to contain degradates derived from 0.1mg mL^{-1} of intact drug which was used for the proposed methods.

2.3. Chemicals and reagents

All reagents used were of analytical grade, solvents were of spectroscopic grade and water used throughout the procedure was freshly distilled.

- Methanol; HPLC grade (Sigma-Aldrich, USA).
- Ortho phosphoric acid (Fisher, UK)
- Sodium hydroxide, hydrochloric acid, chloroform and ammonia (El-Nasr Co., Egypt).

2.4. Standard solutions

Stock solution of the drug (1 mg mL^{-1}) was prepared in methanol. This solution was stable for 1 week in refrigerator or at room temperature. It was diluted with methanol to obtain working standard solution of concentration 0.1mg mL^{-1}.

2.5. Procedures

2.5.1. Chromatographic conditions

i. UPLC method

At ambient temperature, isocratic separation was carried out on Kinetex 2.6µ C_{18} column 100A (4.6-mm × 100-mm) using a mobile phase composed of mixture of 0.1% ortho-phosphoric acid in water and methanol with the ratio of (40:60 % v/v) pH adjusted at 3.5 with ortho phosphoric acid. The mobile phase was pumped at a flow rate 1.0 mL min^{-1} with an injection volume of 10 µL and the detection at 260 nm.

ii. HPTLC method

Analysis was performed on precoated (20 × 20 cm) TLC aluminum silica gel 60 GF254 plates. Samples were applied to the plates using Hamilton micro syringe (50 µL). Plates were spotted 2 cm apart from each other and 1.5 cm apart from the bottom edge. The chromatographic tank was pre-saturated with the mobile phase consisting of methanol - chloroform - conc. ammonia (2.5: 6: 1.5 % v/v/v) for 20 minutes, and then developed by ascending chromatography The plates were air dried, and detected under UV- lamp 254 nm. Densitometry was performed at 261 nm in absorption mode. The silt dimension was 6.0 × 0.3 µm and the scanning speed was 20 mm/s with data resolution: 100 nm / step.

2.5.2. Linearity

i. UPLC method

Aliquots of working standard drug solution (0.1mg mL^{-1}) containing (0.01-0.2 mg) were transferred into a series of 10- mL volumetric flasks and diluted to the volume with methanol. 10 µL of each solution were injected into the UPLC system in triplicate and chromatographed under the above mentioned conditions. Calibration curve was obtained by plotting the peak area against concentration of the drug.

ii. HPTLC method

Aliquots of stock standard drug solution (1 mg mL^{-1}) equivalent to (2.0- 12 µg mL^{-1}) were introduced into a series of 10 - mL volumetric flasks diluted to volume with methanol. 10µL were spotted on a TLC plate following the above mentioned specific chromatographic conditions and scanned at 261nm. Calibration curve was constructed by plotting area under the peak versus the corresponding drug concentrations in µg / spot.

2.5.3. Assay of laboratory prepared mixtures

i. UPLC method

Different aliquots of intact sofosbuvir working standard solution (0.1mg mL^{-1}) containig (0.01-0.19 mg) were transferred into a series of 10 mL volumetric flasks containing alkaline or acidic or oxidative degradate derived from (0.19 -0.01mg) drug. Ten µL of each solution were injected into the UPLC column and the corresponding chromatograms were recorded at 260 nm; Fig. (2). The intact drug concentrations were calculated from the corresponding regression equation.

ii. HPTLC method

Different aliquots of stock drug solution (1 mg mL^{-1}) containing (1.0 –11 mg) were introduced into a series of 10 mL volumetric flasks containing (11 –1.0 mg) of the alkaline or acidic or oxidative degraded sofosbuvir and complated to the volume with methanol. Ten µL of each mixture was applied on TLC plate following the procedure under "Linearity"; Fig. (3). Peak areas of the obtained chromatograms were measured and the concentration of the drug was calculated from the corresponding regression parameters.

2.5.4. Application to tablet dosage form

Ten tablets of Mpiviropack® containing 400 mg of sofosbuvir were powdered and mixed well. An accurately weighed quantity of the powder equvelent to 100 mg was introduced into 100 mL volumetric flask, diluted to volume with methanol and filtered. The obtained solution labeled to contain 1 mg mL^{-1} of the drug was analyzed by HPTLC method. Ten mL of the above solution were diluted to 100 mL with methanol to obtain a solution labeled to contain (0.1 mg mL^{-1}) of the drug analyzed by the proposed UPLC method. The drug concentrations were calculated from the appropriate regression parameters.

3. RESULTS AND DISCUSSION

Two chromatographic methods were developed for the selective determination of sofosbuvir in presence of its degradation products by UPLC and HPTLC methods.

3.1. Degradation

Stressed degradation of sofosbuvir was studied by refluxing the drug using different conditions. Complete degradation was attained upon refluxing the drug with 5N NaOH and 5N HCL for 3 h and 7 h respectively at 100^{o} C. Also, oxidative degradation was carried out by keeping of the drug with 3% H_2O_2 for one week at room temperature. The degradation was confirmed with IR using KBr disc and Mass as follow.

The pure drug showed appearance of broad band of (OH) group at 3352 cm^{-1}, sharp band of two (NH) group at 3248 cm^{-1}, band of aromatic (CH) group at 3089 cm^{-1} and band of ester (COO) group at 1716 cm^{-1} in its IR spectrum; EI mass showed molecular ion peak at *m/z* = 529 with high intensity (30 %); Fig. (4-a),(5-1). While the alkaline degradate showed appearance of broad band of phosphoric (OH) group at 3421 cm^{-1} in its IR spectrum and disappearance of CH aromatic band at 3009 cm^{-1}, EI mass showed molecular ion peak at *m/z* = 453 with high intensity (19 %), this indicate decreasing in molecular ion peak by 77 unit. this means loss of phenyl group. From IR and EI mass analyses, It was concluded that the proposed degradate formed by removal of phenyl group to afford the free phosphoryle group; Fig. (4-b),(5-b).The acidic degradate showed disappearance of the band of C=O group of ester moiety at 1720cm^{-1} and appearance of broad band of phosphoric (OH) group

at 3421 cm^{-1} in its IR spectrum, EI mass showed molecular ion peak at *m/z* = 416 with high intensity (25%), this indicate decreasing in molecular ion peak equal 113 unit It was concluded that the proposed degradate compound formed by removal of isopropyl alaninate moiety to afford the free phosphate group; Fig. (4-c), (5-c). The appearance of ketonic band of (CO) at 1727 cm^{-1} group confirms the oxidation of (OH) group and EI mass showed molecular ion peak at *m/z* = 527.15 with high intensity (32%), this indicate decreasing in molecular ion peak by 2 units; Fig. (4-d),(5-d).

Thus a degradation pathway was illustrated in scheme (1).

3.2. Method Development

3.2.1. UPLC Method

Different mobile phases such as ortho-phosphoric acid- acetonitrile -methanol, methanol - water - potassium hydrogen phosphate, acetonitrile - ortho-phosphoric acid, methanol – water and potassium hydrogen phosphate – methanol in different ratios at different pH values were evaluated to obtain optimum resolution. Good resolution with good peak shape and purity were obtained on Kinetex 2.6µ C18 100A (4.6-mm × 100-mm) column using mobile phase of 0.1% ortho-phosphoric acid in water and methanol (40:60% v/v) adjusted to pH 3.5 with ortho phosphoric . Different flow rates (0.5 -2 mL min^{-1}) at different wavelengths (250,254, 255, 260, 261, 262 and 265 nm) were tried where optimal flow rate was found to be 1 mL min^{-1} with detection at 260 nm. Intact sofosbuvir was well resolved from the peak of its degradates where Rt was 3.92, 4.27 and 4.15 for alkaline, acidic and oxidative degradates: respectively.

3.2.2. HPTLC Method

Initial studies on the cited drug and its degradation products were carried out to achieve good separation in which different mobile phases in different ratios were tested such as chloroform - methanol- ammonia, chloroform - methanol - acetonitrile, methanol - toluene- acetonitrile, ethyl acetate - methanol - acetonitrile and methanol - water- ammonia and different scanning wavelengths were tried including 250, 245, 255, 260, 262 and 265 nm. It was found that best separation was achieved using a mobile phase of methanol - chloroform - conc. ammonia (2.5: 6: 1.5 % v/v/v). The plates were visualized under UV lamp at 254 nm, where bands appear at R_f 0.73 for intact sofosbuvir and 0.49, 0.38 and 0.64 for its alkaline, acidic and oxidative degradates: respectively. The separated bands of the drug were scanned densitometrically at 261 nm without any interference from its degradates.

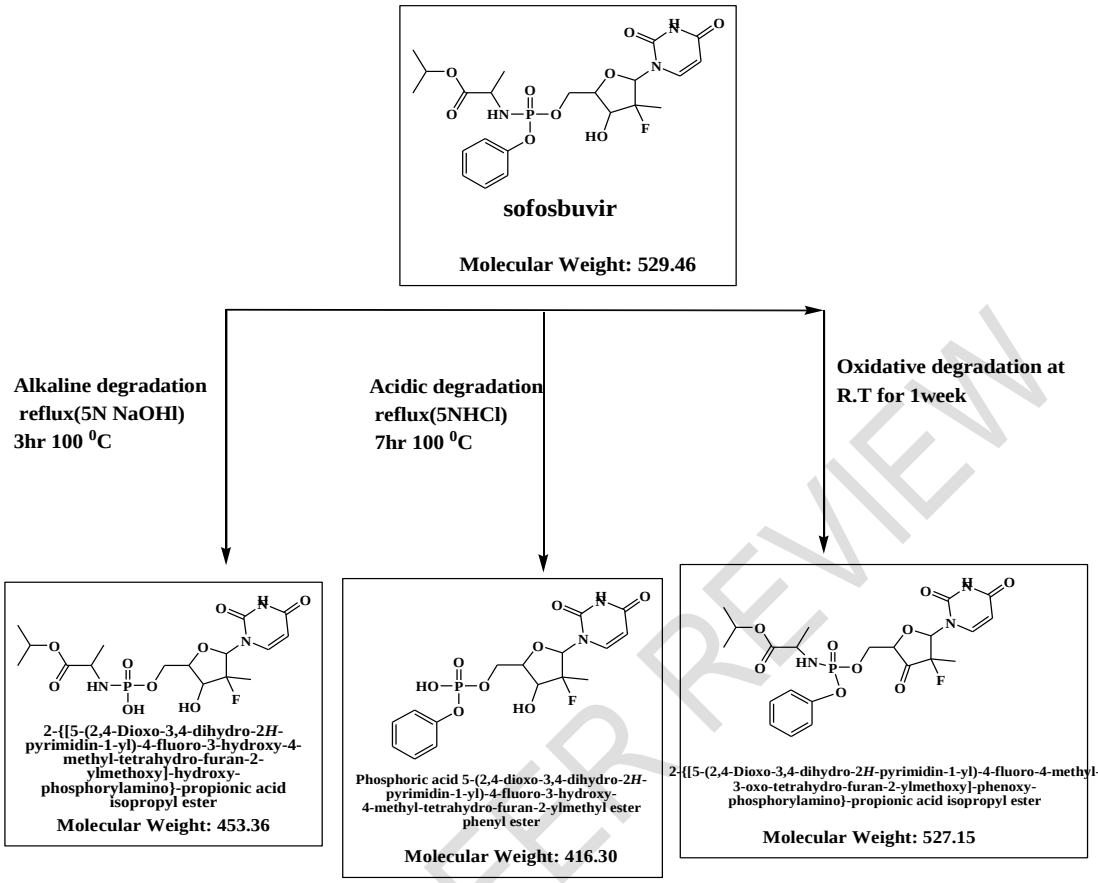
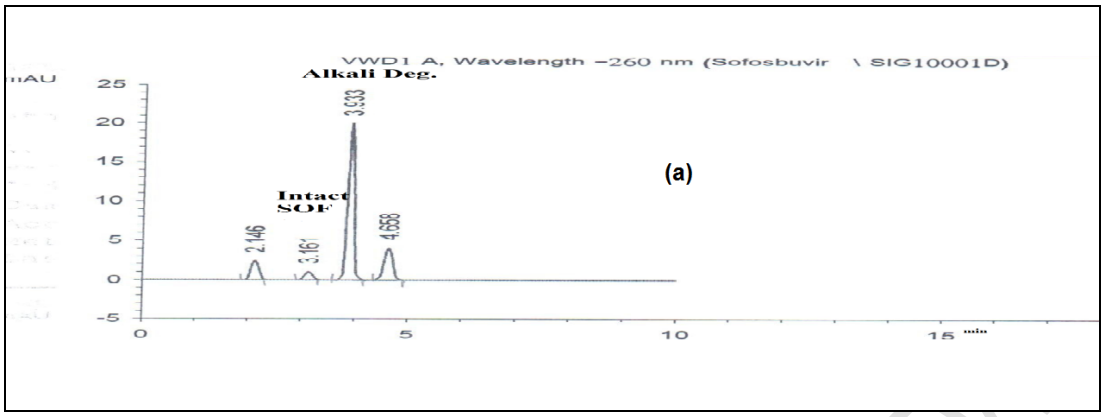
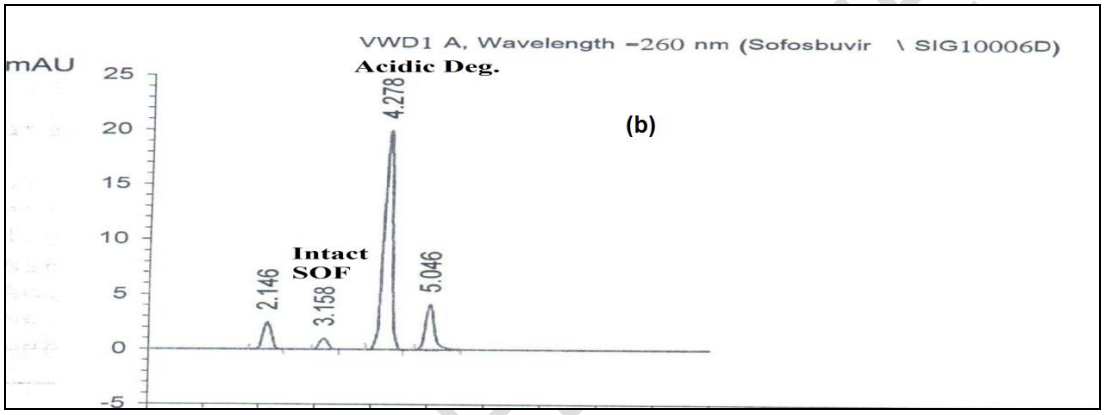


Chart: 1: Suggested degradation pathway of Sofosbuvir





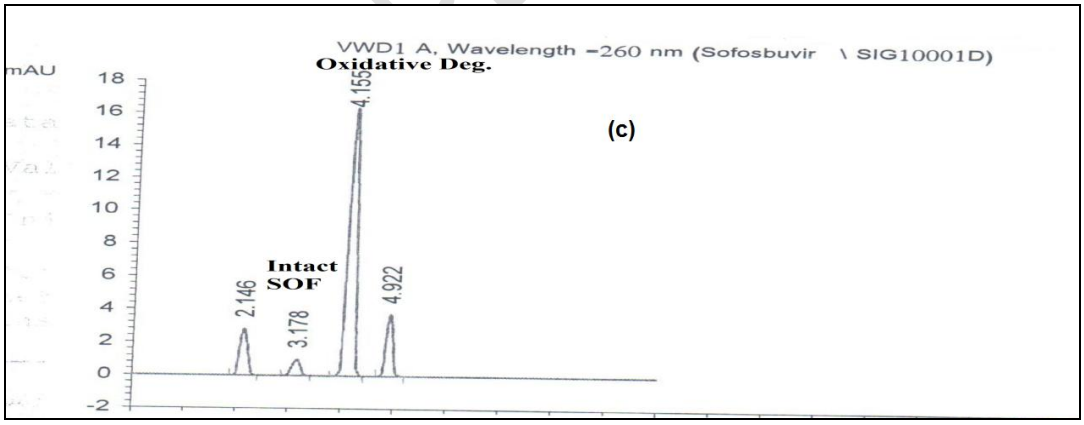
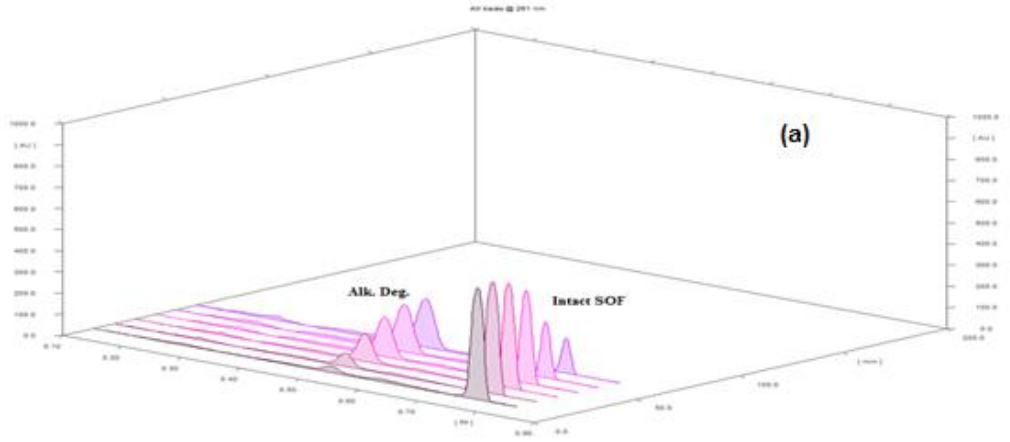


Fig. (2): UPLC chromatograms of mixture of (1 µgmL^{-1}) of intact Sososbuvir and its alkaline (a), its acidic (b) and its oxidative (c) degradates at 260 nm.



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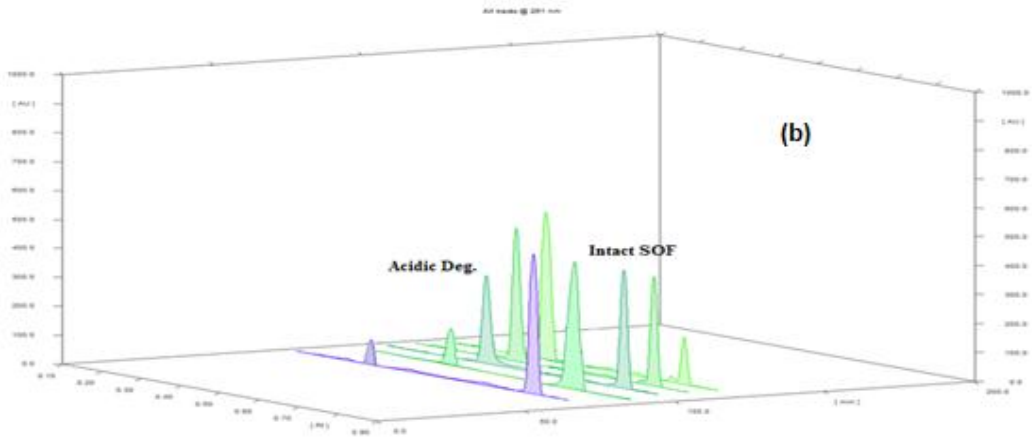

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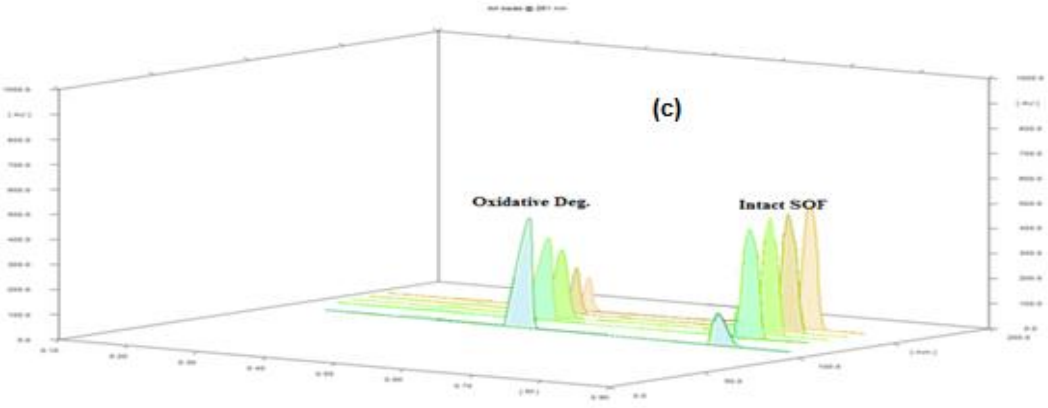

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207 **Fig. (3): Densitometric chromatograms of mixture of intact Sofosbuvir and its alkaline**
208 **(a), its acidic (b) and its oxidative (c) degradates at 261 nm.**

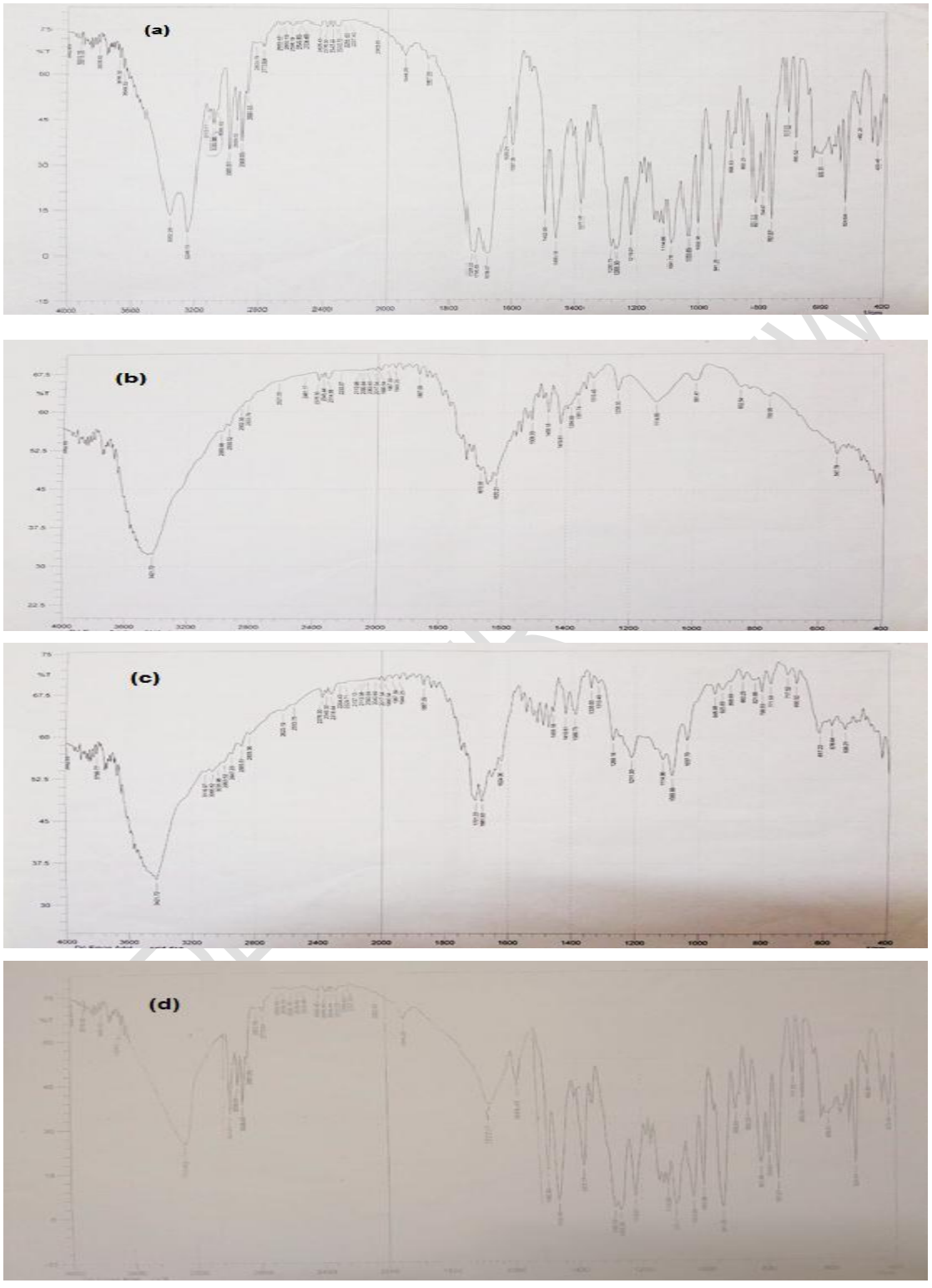

Fig. (4): IR Spectra of intact Sofosubuvir (a), Its alkaline degradate (b), its acidic degradate(c),and its oxidative degradate(d) on KBr disc.

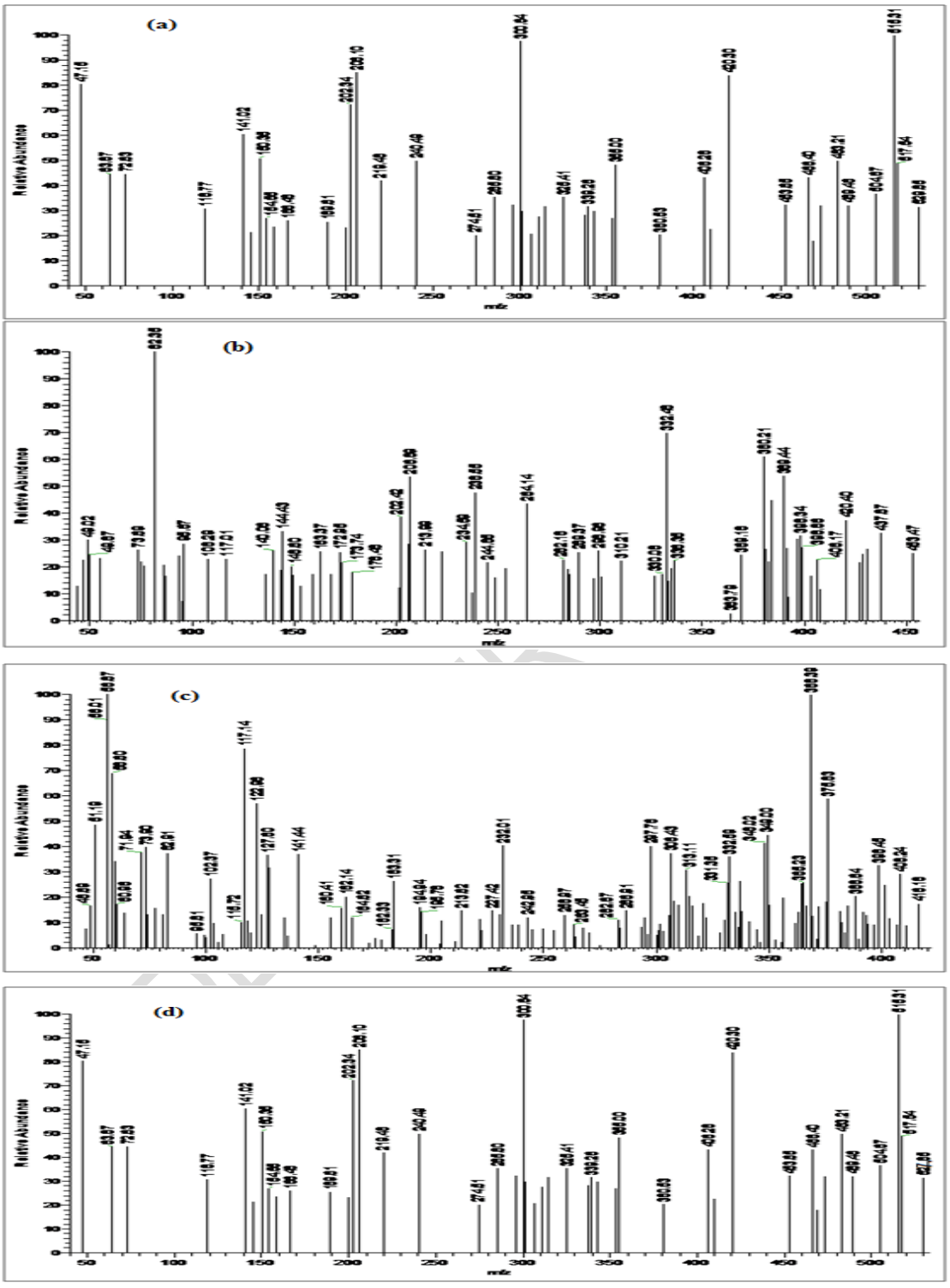

Fig. (5): Mass spectra of intact Sofosubuvir (a), Its alkaline degradate (b), its acidic degradate(c),and its oxidative degradate(d).

3.3. Method validation

The methods were validated as per ICH guidelines (16).

- **System suitability-** System suitability test was performed for UPLC method in accordance with USP. The results shown in Table (1) indicate adequate resolution and reproducibility of the UPLC method and deliberate variations did not affect the system suitability parameters indicate good robustness.
- **Linearity**- Under the prescribed and experimental conditions a good linearity between peak area and corresponding drug concentration in UPLC and HPTLC methods were obtained over the range of 1-20 $\mu g mL^{-1}$ and 2-12 µg/spot. Regression parameters were computed and presented in Table (2).
- **Accuracy and precision**- They were assessed by triplicate analysis of three different concentrations covering the linearity range within one day for intraday and three different days for interday analysis. Accuracy (R%) was 99.11% and 100.44% for UPLC and HPTLC methods ; respectively. Intraday precision (RSD %) ranged from (0.68 - 1.66) and (0.60 - 0.63) using the two proposed methods; respectively, was shown in Table (2).
- **LOD and LOQ**- They were ranged between (0.182, 0.552) and (0.069, 0.210) for both UPLC and HPTLC; respectively, as shown in Table (2).
- **Selectivity**- It was assured by applying the proposed methods to laboratory prepared mixtures of the intact drug together with its degradation products. Successful selective determination of intact sofosbuvir in presence of up to 95 % of its three degradates in UPLC method as shown in Table (3), while in HPTLC method the selective determination of intact sofosbuvir in presence of up to 91 % of its degradates ; Table (4).
- **Robustness**-The robustness of the proposed methods was assessed by study the influence of deliberate variation in the mobile phase contents ratio. It was observed that no significant difference in R_f value. The RSD % did not exceed 0.85 and 1.75 for both methods; respectively. Also, it checked by studying the effect of different sources of methanol, it was found that using methanol (Sigma – Aldrich, Germany, El-Nasr Co., Egypt) gave RSD% of 0.35 whereas peak area remains acceptable throughout the assay also by checking flow rate in which the produced RSD % was 1.32 which proved the robustness of the method.

3.4. Application to tablet dosage form

The proposed methods were applied for determination of Mpiviropack® Tablets where no interference from excipients and additives were observed. The results presented in Table (5) revealed mean recoveries of 98.07% ± 0.89 and 100.73% ± 1.11 for the two proposed methods; respectively. The recovery of the proposed methods was also validated by applying the standard addition technique, Table (5). Statistical comparison between results obtained from the proposed methods and the reported HPLC method (3) for determination of sofosbuvir in its tablets showed less calculated t and F values than the tabulated ones revealing no significant difference in accuracy and precision at 95% confidence limit (17); Table (6). However the proposed methods are much more sensitive and are stability indicating; determining the intact drug in presence of its three degradates.

Table 1. Robustness results of system suitability parameters for the determination of sofosbuvir by the proposed UPLC method.

parameter	Capacity factor **(K)**	Number of theoretical Plates **(N)**	Resolution factor **(R)**	Selectivity factor **(ά)**	Tailing factor **(T)**
Mobile phase Buffer: MeOH					
mp 39/61	2.75	6712	16.57	9.22	0.86
mp 40/60	2.77	6785	16.24	9.10	0.89
mp 41/59	2.69	6681	16.43	9.14	0.79
pH					
pH 3.6	2.80	5985	16.27	8.79	0.90
pH 3.5	2.73	5879	16.08	8.87	0.84
pH 3.4	2.70	5822	16.16	8.83	0.87

Table 2. Regression analysis and validation parameters for the determination of sofosbuvir by the proposed methods.

parameter	UPLC method	HPTLC method
λ_{max} (nm).	260	261
Linearity range	1-20 (µg mL^{-1})	2-12 (µg/spot)
Regression equation		
slope± SD(S_Y)	8.13±0.03	1364.06±29.78
Intercept± SD(S_X)	-0.21±0.44	-79.07± 23.19
SD of residual(S_{YX})	0.930	248.36
Correlation coefficient (r^2)	0.9999	0.9990
Accuracy (Mean%± SD)	99.11±0.34	100.44± 0.54
Precision (RSD %)		
Intraday precision*	1.66	0.63
Interday precision*	0.8	0.60
LOD	0.182 (µgmL^{-1})	0.069 (µg/band)
LOQ	0.552 (µgmL^{-1})	0.210 (µg/band)

*Average of 9 determinations.

Table 3. Determination of sofosbuvir in laboratory prepared mixtures with its degradation products by the proposed UPLC method.

Alkaline degradate			Acidic degradate			Oxidative degradate		
Intact (µgmL^{-1})	**Degraded (µgmL^{-1})**	**Recovery% of Intact**	**Intact (µgmL^{-1})**	**Degradd (µgmL^{-1})**	**Recover% of Intact**	**Intact (µgmL^{-1})**	**Degradd (µgmL^{-1})**	**Recovery% of Intact**
1	19	100.80	1	19	97.90	1	19	98.30
5	15	101.62	5	15	97.40	3	17	99.01
7	13	102.85	7	13	99.82	9	11	98.05
13	7	101.05	13	7	100.18	11	9	98.50
15	5	98.95	15	5	99.06	17	3	101.95
19	1	101.15	19	1	97.68	19	1	99.63
Mean%± SD		101.07±1.27			98.67±1.18			99.24±1.44

Table 4. Determination of sofosbuvir in laboratory prepared mixtures with its degradation products by the proposed HPTLC method.

Alkaline degradate			Acidic degradate			Oxidative degradate		
Intact µg/spot	**Degraded µg/spot**	**Recovery of Intact**	**Intact µg/spot**	**Degraded µg/spot**	**Recovery of Intact**	**Intact µg/spot**	**Degraded µg/spot**	**Recovery % of Intact**
10	2	99.56	10	2	99.92	10	2	97.69
9	3	100.43	9	3	100.00	9	3	99.53
8	4	101.90	7	5	101.34	8	4	98.51
7	5	102.23	4	8	100.41	7	5	99.12
6	6	98.69	1	11	101.01	1	11	101.99
1	11	101.20						
Mean%± SD		100.42±1.38			100.54±0.62			99.37±1.62

Table 5. Application of standard addition technique for the determination of sofosbuvir in Mpiviropack® tablets by the proposed methods.

UPLC Method				HPTLC Method			
Recovery ±SD%	**Standard addition**			**Recovery ±SD%**	**Standard addition**		
	Claimed taken (µg mL^{-1})	**Pure added (µg mL^{-1})**	**Recovery % of pure added**		**Claimed taken (µg mL^{-1})**	**Pure added (µg mL^{-1})**	**Recovery % of pure added**
	5	1	102.21		4	1	98.50
	5	5	101.99		4	3	101.02
98.07±0.89	5	10	99.05	100.73±1.11	4	5	100.15
	5	15	100.61		4	7	100.70
Mean%±SD			100.96±1.45				100.09±1.12

Table 6. Results obtained by the proposed method compared with reported method [3] for determination of sofosbuvir in Mpiviropack® tablets.

Parameters	Mpiviropack® tablets		
	UPLC method	HPTLC method	Reported method[3]
Linearity range	1-20 (µg mL^{-1})	2-12 (µg/spot)	2-60 (µgmL^{-1})
N	5	6	5
Mean%	98.07	100.73	99.87
SD	0.89	1.11	1.60
Variance	0.79	1.23	2.58
t-	2.94(3.18)	0.66 (3.18)	---
F-	3.19(9.27)	2.08(9.27)	---

4. CONCLUSION

The proposed study describes UPLC and HPTLC methods for estimation of sofosbuvir in presence of its alkaline, acidic and oxidative degradation products. The methods were validated and found to be simple, accurate, precise and selective. The two methods proved their ability to be used for stability indication of the drug. Therefore, they can be conveniently adopted for estimation, stability studies and routine quality control analysis of sofosbuvir.

COMPETING INTRESTS

Authors have declared that no competing interests exist.

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