

Original Research Article

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

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 F₂₅₄ 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 $\mu\text{g mL}^{-1}$ for UPLC and 2-12 $\mu\text{g / spot}$ 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.

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13 *Keywords: Sofosbuvir; UPLC; HPTLC; Degradates, Tablet dosage form.*

14 **1. INTRODUCTION**

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16 Sofosbuvir, chemically described as (S)-isopropyl-2-(S)-(2R, 3R, 4R, 5R)-5-(2,4-dioxo-

17 3,4-dihydropyrimidin-1(2H)-yl)-4-fluoro-3-hydroxy-4-methyltetrahydrofuran

18 2-yl)methoxy)phenoxy)phosphoryl amino)propanoate (1), Fig.(1). Sofosbuvir is a direct-

19 acting antiviral agent against HCV. The HCV polymerase (NS5B protein) is an RNA-

20 dependent RNA polymerase (RdRp). It is the essential initiating and catalytic subunit of this

21 replication complex and is critical for the viral replication cycle (2). Literature survey revealed

22 that sofosbuvir has been determined by spectrophotometry and HPLC methods (3-6). It was

23 also assayed in combination with other drugs by RP- HPLC (7, 8), LC – MS/MS method (9)

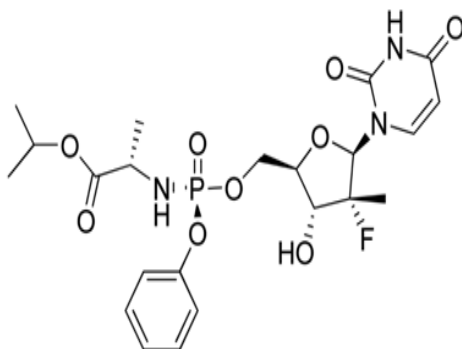
24 and bio-analytical UPLC-ESI-MS/MS methods (10-12). Moreover, few stability HPLC

25 indicating methods were reported for its analysis (13-15). The primary goal of this work was

26 to develop a simple, rapid and sensitive methods for the determination of sofosbuvir in

27 presence of its alkaline, acidic and oxidative degradates as well as in its tablet dosage

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Fig. 1: Structure of Sofosbuvir

34 **2. EXPERIMENTAL**

35 **2.1. Instrumentation**

36 - Agelint 1290 Ultra HPLC with binary pump and UV detector (USA).

37 - Camag TLC scanner 3, with WINCATS computer software (Switzerland).

38 - Precoated TLC plates, silica gel 60 GF254 (20 × 20 cm), (Fluka chemie, Switzerland).

39 Using Hamilton 50- μ L micro syringe (Germany).

40 - UV lamp with short wavelength (254 nm), (Desega, Germany).

41 - pH meter combined plus electrode (Adwa model AD1030 pH mv), (UK).

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43 **2.2. Material and Reagents**

44 **2.2.1. Pure and Market samples**

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

49 **2.2.2. Degraded samples**

50 Ten mg of pure sofosbuvir was accurately weighed and refluxed separately with 25 mL
51 of 5N NaOH for 3.5 h, or with 25 mL 5N HCL for 7 h. For oxidative degradation 10 mg of
52 pure drug was set aside with 25 mL of 3% H₂O₂ for one week at room temperature. The
53 alkaline and acidic hydrolysed solutions were cooled and neutralized to about pH 7 with 5N
54 HCL or 5N NaOH and evaporated to dryness under vacuum. The obtained residues were
55 extracted three times each with 25 mL methanol then filtered separately into 100- mL
56 volumetric flask and diluted to the volume with methanol to obtain a stock solution claimed to
57 contain degradates derived from 0.1mg mL⁻¹ intact drug which was used for UPLC and
58 HPTLC methods. Also oxidative degradate was exposed to dryness at room temperature
59 then the residue was extracted three times each with 25 mL methanol then filtered into 100-
60 mL volumetric flask and diluted to the volume with methanol to obtain a stock solution
61 claimed to contain degradates derived from 0.1mg mL⁻¹ intact drug which was used for the
62 proposed methods.

63 **2.3. Chemicals and reagents**

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

66 - Methanol; HPLC grade (Sigma-Aldrich, USA).

67 - Ortho phosphoric acid (Fisher, UK)

68 - Sodium hydroxide, hydrochloric acid, chloroform and ammonia (El-Nasr Co., Egypt).

69 **2.4. Standard solutions**

70 Stock solution of the drug (1 mg mL⁻¹) was prepared in methanol. This solution was
71 stable for 1 week in refrigerator or at room temperature. It was diluted with methanol to
72 obtain working standard solution of 0.1mg mL⁻¹.

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74 **2.5. Procedures**

75 **2.5.1. Chromatographic conditions**

76 **i. UPLC method**

77 At ambient temperature, isocratic separation was carried out on Kinetex 2.6 μ C₁₈ 100A
78 (4.6-mm \times 100-mm) using a mobile phase composed of mixture of 0.1% ortho-phosphoric
79 acid in water and methanol with the ratio of (40:60% v/v) pH adjusted at 3.5 with ortho
80 phosphoric acid. The mobile phase was pumped at flow rate 1 mL min⁻¹; the injection volume
81 was 10 μ L and the detection at 260 nm.

82 **ii. HPTLC method**

83 Analysis was performed on precoated (20 \times 20 cm) TLC aluminum silica gel 60 GF254
84 plates. Samples were applied to the plates using Hamilton micro syringe (50 μ L). Plates
85 were spotted 2 cm apart from each other and 1.5 cm apart from the bottom edge. The
86 chromatographic tank was pre-saturated with the mobile phase consisting of methanol -
87 chloroform - conc. ammonia (2.5: 6: 1.5 v/v/v) for 20 minutes, and then developed by
88 ascending chromatography. The plates were air dried, and detected under UV- lamp 254 nm.
89 Densitometry was performed at 261 nm in absorption mode. The slit dimension was 6.0 \times
90 0.3 μ m and the scanning speed was 20 mm/s with data resolution: 100 nm / step.

91 **2.5.2. Linearity**

92 **i. UPLC method**

93 Aliquots of working standard drug solution (0.1mg mL⁻¹) containing (0.01-0.2 mg) were
94 transferred into a series of 10- mL volumetric flasks and diluted to the volume with methanol.
95 Triplicate 10 μ L of each solution were injected into the UPLC system and chromatographed
96 under the above mentioned conditions. Calibration curve was obtained by plotting the peak
97 area against concentration of the drug.

98 **ii. HPTLC method**

99 Aliquots of stock standard drug solution (1 mg mL⁻¹) equivalent to (2- 12 μ g mL⁻¹) were
100 introduced into a series of 10 - mL volumetric flasks diluted to volume with methanol. 10 μ L
101 were spotted on a TLC plate following the above mentioned specific chromatographic
102 conditions and scanned at 261nm. Calibration curve was constructed by plotting area under
103 the peak versus the corresponding drug concentrations in μ g / spot.

104 **2.5.3. Assay of laboratory prepared mixtures**

105 **i. UPLC method**

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

112 **ii. HPTLC method**

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

119 **2.5.4. Application to tablet dosage form**

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

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128 **3. RESULTS AND DISCUSSION**

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

131 **3.1. Degradation**

132 Stressed degradation of sofosbuvir was studied by refluxing the drug using different
133 conditions. Complete degradation was attained upon refluxing the drug with 5N NaOH and
134 5N HCL for 3.5 h and 7 h respectively at 100°C . Also, oxidative degradation was carried out
135 by keeping of the drug with 3% H_2O_2 for one week at room temperature. The degradation
136 was confirmed with IR using KBr disc and Mass as follow.

137 The pure drug showed appearance of broad band of (OH) group at 3352 cm^{-1} , sharp
138 band of two (NH) group at 3248 cm^{-1} , band of aromatic (CH) group at 3089 cm^{-1} and band of
139 ester (COO) group at 1716 cm^{-1} in its IR spectrum; EI mass showed molecular ion peak at
140 $m/z = 529$ with high intensity (30 %); Fig. (4-a),(5-1). While the alkaline degradate showed
141 appearance of broad band of phosphoric (OH) group at 3421 cm^{-1} in its IR spectrum and
142 disappearance of CH aromatic band at 3009 cm^{-1} , EI mass showed molecular ion peak at
143 $m/z = 453$ with high intensity (19 %), this indicate decreasing in molecular ion peak by 77
144 unit. this means loss of phenyl group. From IR and EI mass analyses, It was concluded that
145 the proposed degradate formed by removal of phenyl group to afford the free phosphoryle
146 group; Fig. (4-b),(5-b).The acidic degradate showed disappearance of the band of C=O
147 group of ester moiety at 1720 cm^{-1} and appearance of broad band of phosphoric (OH) group
148 at 3421 cm^{-1} in its IR spectrum, EI mass showed molecular ion peak at $m/z = 416$ with high

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

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

156 **3.2. Method Development**

157 **3.2.1. UPLC Method**

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

169 **3.2.2. HPTLC Method**

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

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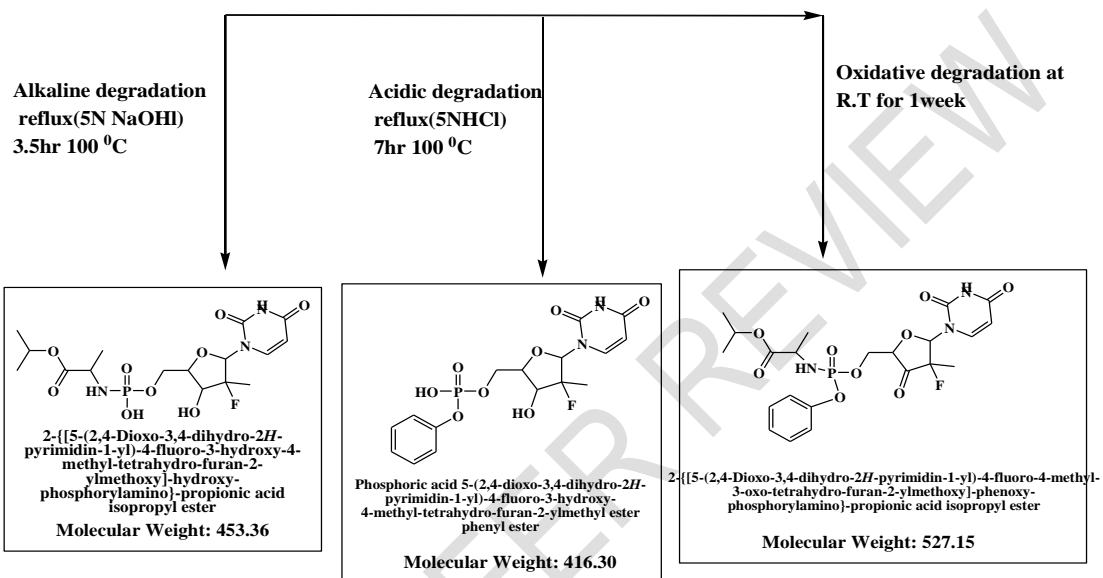
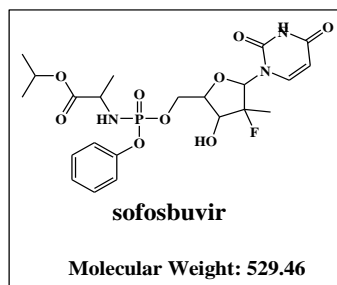
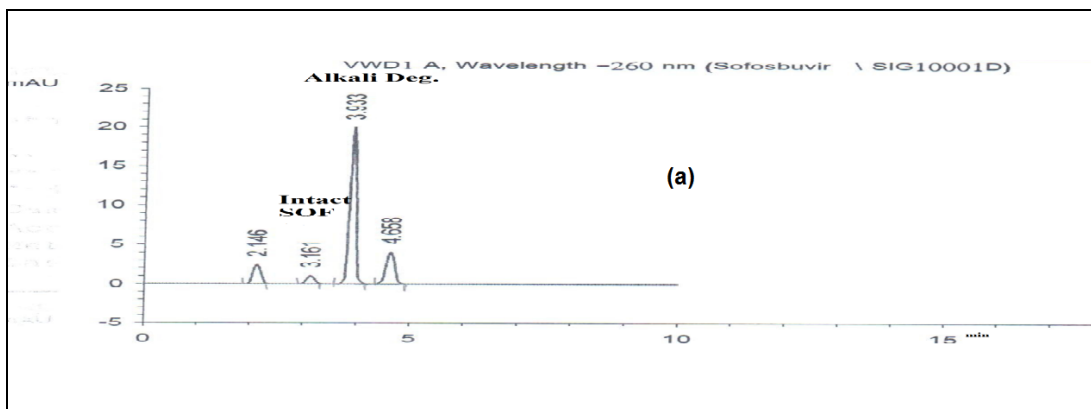


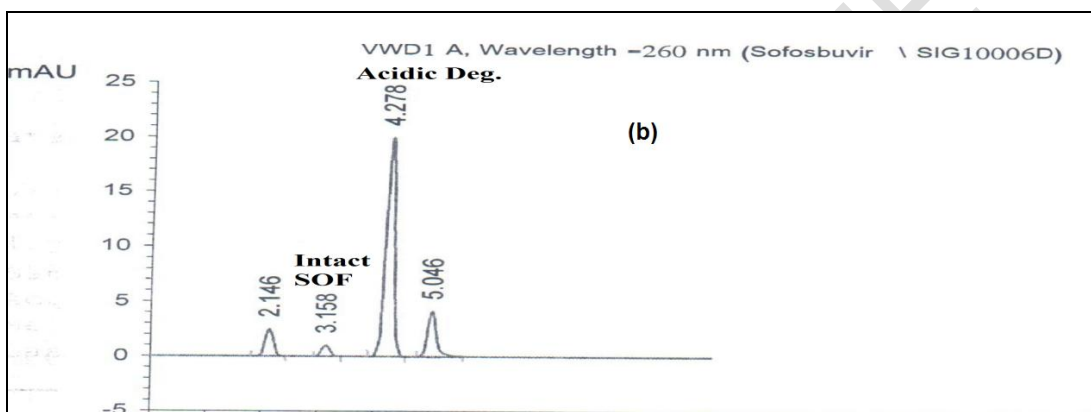
Chart: 1: Suggested degradation pathway of Sofosbuvir

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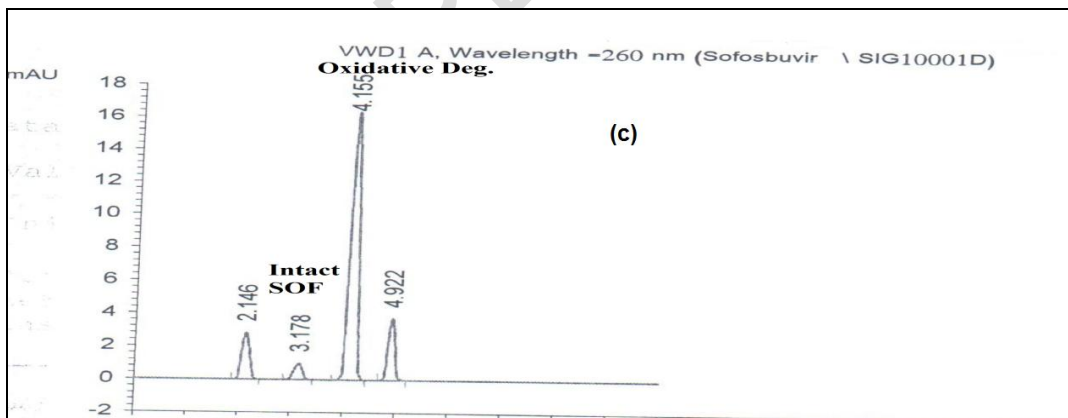
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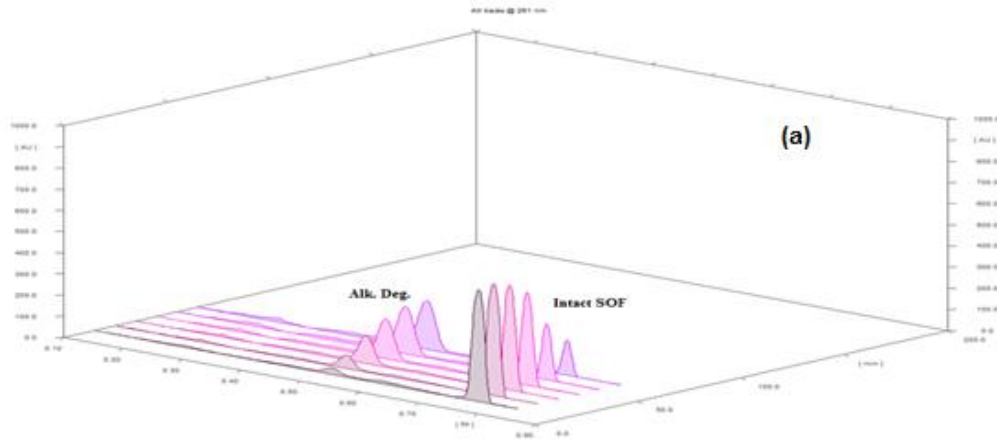
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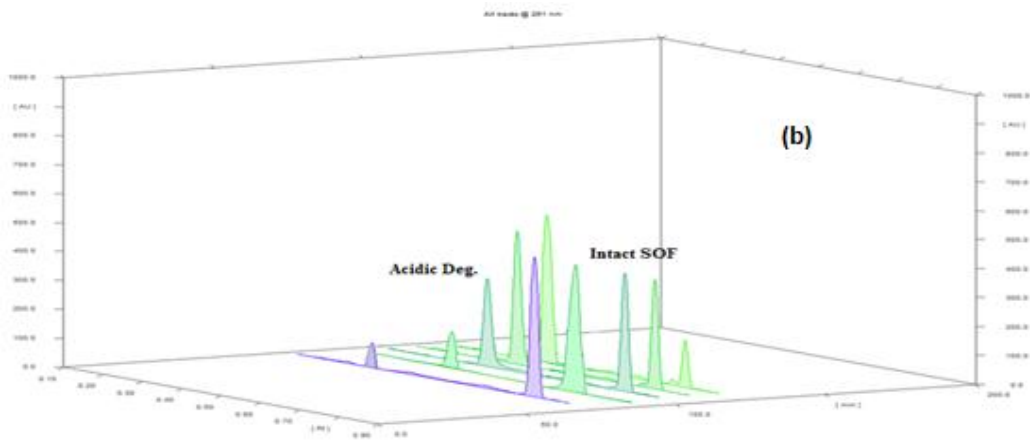
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202 Fig. (2): UPLC chromatograms of mixture of ($1 \mu\text{g mL}^{-1}$) of intact Sofosbuvir and its
203 alkaline (a), its acidic (b) and its oxidative (c) degradates at 260 nm.



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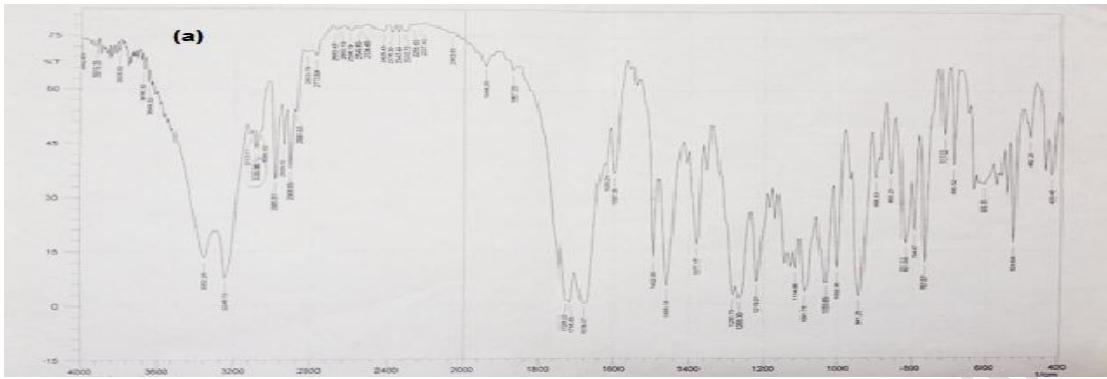
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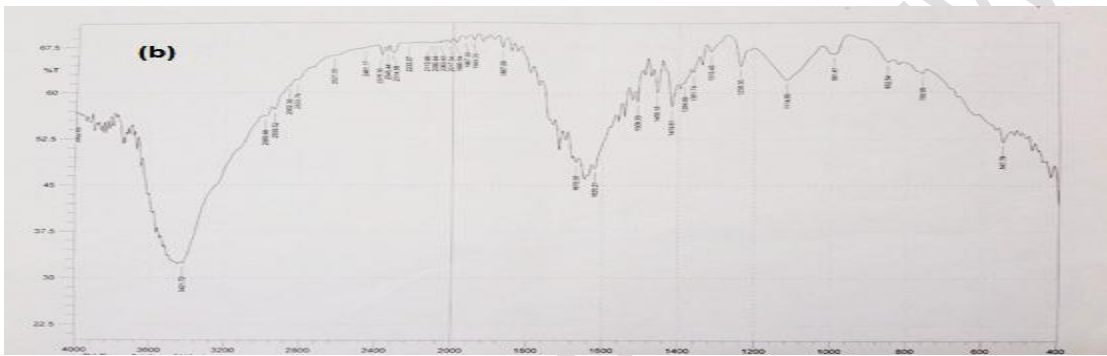
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Fig. (3): Densitometric chromatograms of mixture of intact Sofosbuvir and its alkaline (a), its acidic (b) and its oxidative (c) degradates at 261 nm.

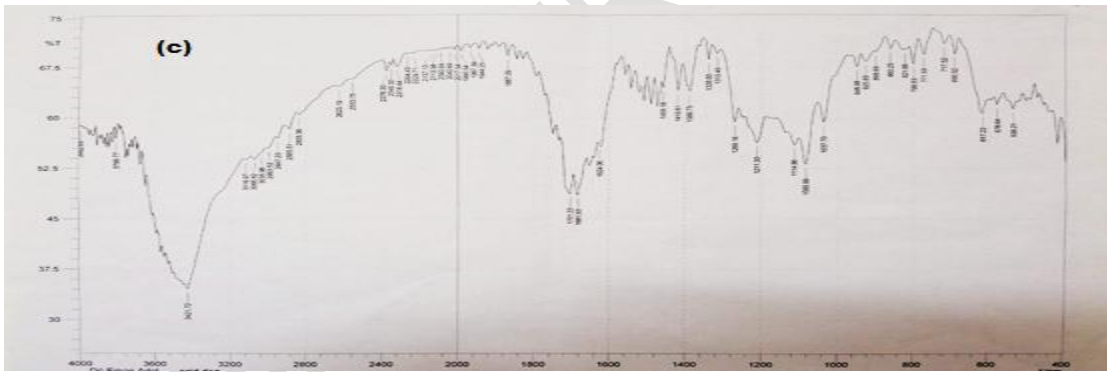
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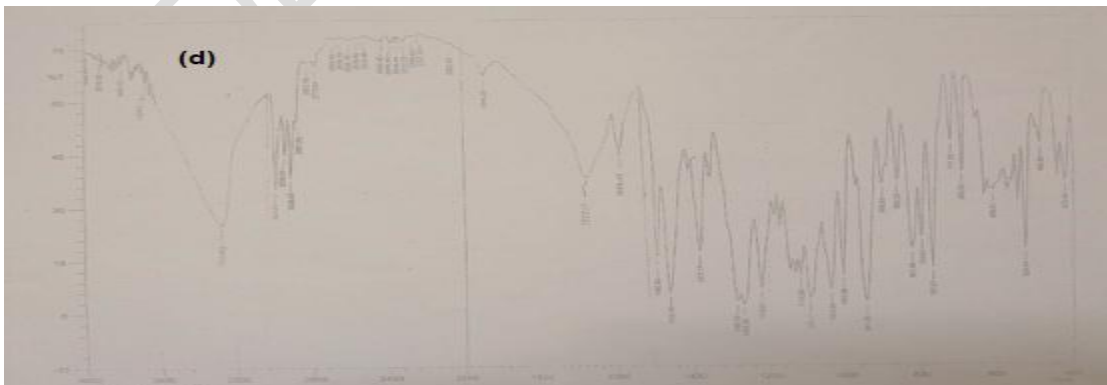
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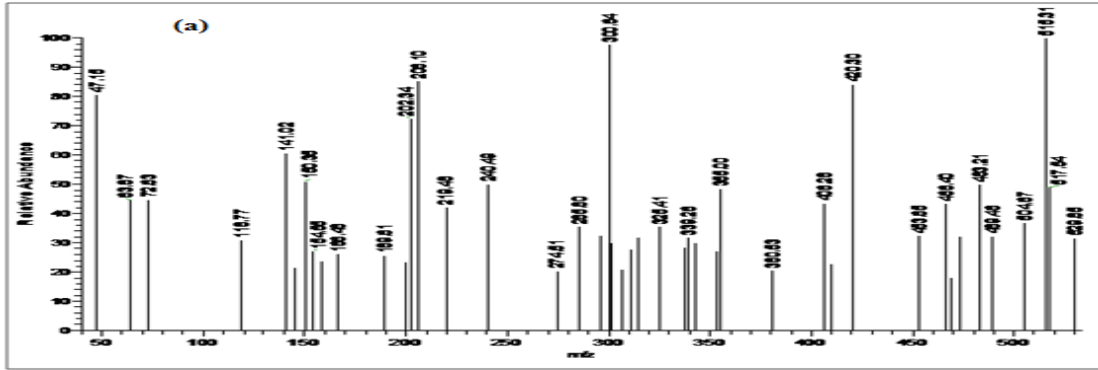


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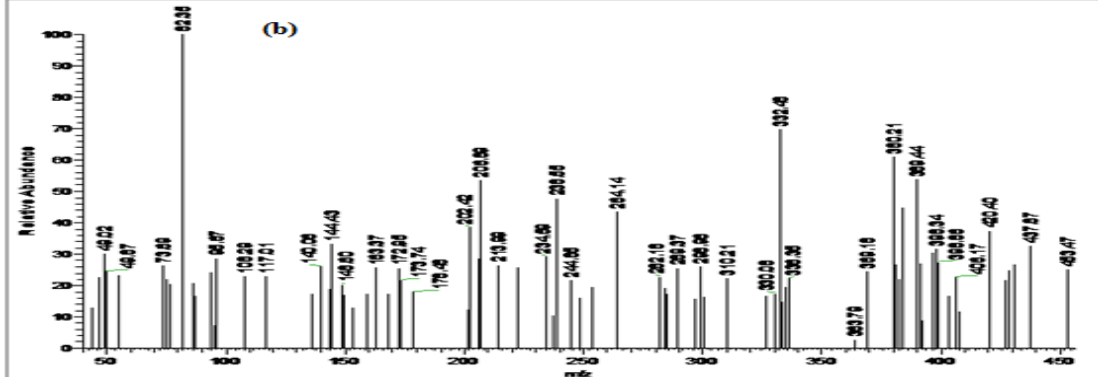


213 Fig. (4): IR Spectra of intact Sofosbuvir (a), Its alkaline degradate (b), its acidic
214 degradate(c),and its oxidative degradate(d) on KBr disc.

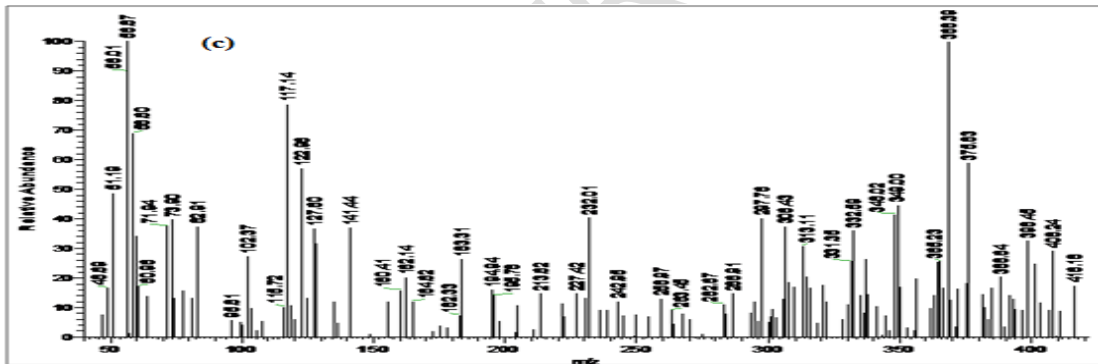
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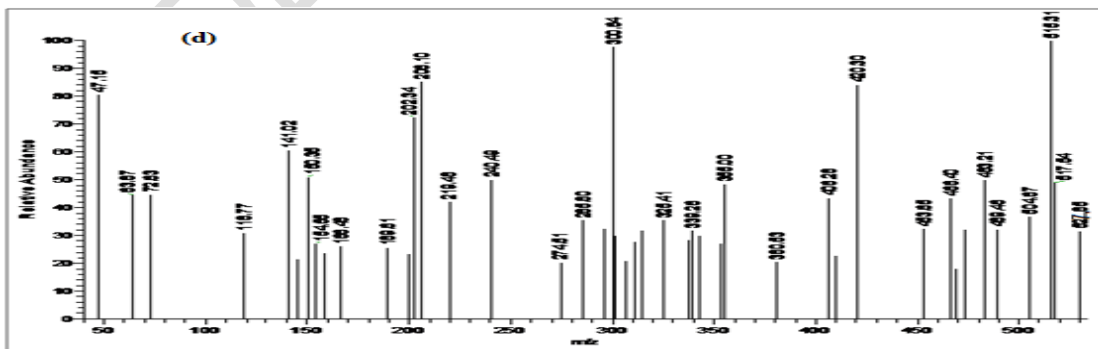
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219 Fig. (5): Mass spectra of intact Sofosbuvir (a), Its alkaline degradate (b), its acidic
220 degradate(c),and its oxidative degradate(d).

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223 **3.3. Method validation**

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

- 225 • **System suitability-** System suitability test was performed for UPLC method in
226 accordance with USP. The results shown in Table (1) indicate adequate resolution
227 and reproducibility of the UPLC method and deliberate variations did not affect the
228 system suitability parameters indicate good robustness.
- 229 • **Linearity-** Under the prescribed and experimental conditions a good linearity
230 between peak area and corresponding drug concentration in UPLC and HPTLC
231 methods were obtained over the range of 1-20 $\mu\text{g mL}^{-1}$ and 2-12 $\mu\text{g/spot}$. Regression
232 parameters were computed and presented in Table (2).
- 233 • **Accuracy and precision-** They were assessed by triplicate analysis of three
234 different concentrations covering the linearity range within one day for intraday and
235 three different days for interday analysis. Accuracy (R%) was 99.11% and 100.44%
236 for UPLC and HPTLC methods ; respectively. Intraday precision (RSD %) ranged
237 from (0.68 - 1.66) and (0.60 - 0.63) using the two proposed methods; respectively,
238 was shown in Table (2).
- 239 • **LOD and LOQ-** They were ranged between (0.182, 0.552) and (0.069, 0.210) for
240 both UPLC and HPTLC; respectively, as shown in Table (2).
- 241 • **Selectivity-** It was assured by applying the proposed methods to laboratory
242 prepared mixtures of the intact drug together with its degradation products.
243 Successful selective determination of intact sofosbuvir in presence of up to 95 % of
244 its three degradates in UPLC method as shown in Table (3), while in HPTLC method
245 the selective determination of intact sofosbuvir in presence of up to 91 % of its
246 degradates ; Table (4).
- 247 • **Robustness-**The robustness of the proposed methods was assessed by study the
248 influence of deliberate variation in the mobile phase contents ratio. It was observed
249 that no significant difference in R_f value. The RSD % did not exceed 0.85 and 1.75
250 for both methods; respectively. Also, it checked by studying the effect of different
251 sources of methanol, it was found that using methanol (Sigma – Aldrich, Germany,
252 El-Nasr Co., Egypt) gave RSD% of 0.35 whereas peak area remains acceptable
253 throughout the assay also by checking flow rate in which the produced RSD % was
254 1.32 which proved the robustness of the method.

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258 **3.4. Application to tablet dosage form**

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

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

parameter	K	N	R	α	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

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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 ($\mu\text{g mL}^{-1}$)	2-12 ($\mu\text{g/spot}$)
<u>Regression equation</u>		
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 ($\mu\text{g mL}^{-1}$)	0.069 ($\mu\text{g/band}$)
LOQ	0.552 ($\mu\text{g mL}^{-1}$)	0.210 ($\mu\text{g/band}$)

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*Average of 9 determinations.

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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 ($\mu\text{g mL}^{-1}$)	Degraded ($\mu\text{g mL}^{-1}$)	Recovery% of Intact	Intact ($\mu\text{g mL}^{-1}$)	Degradd ($\mu\text{g mL}^{-1}$)	Recover% of Intact	Intact ($\mu\text{g mL}^{-1}$)	Degradd ($\mu\text{g mL}^{-1}$)	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%±		101.07±1.27			98.67±1.18			99.24±1.44
SD								

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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 $\mu\text{g/spot}$	Degraded $\mu\text{g/spot}$	Recovery of Intact	Intact $\mu\text{g/spot}$	Degraded $\mu\text{g/spot}$	Recovery of Intact	Intact $\mu\text{g/spot}$	Degraded $\mu\text{g/spot}$	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%±		100.42±1.38			100.54±0.62			99.37±1.62
SD								

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302 **Table 5. Application of standard addition technique for the determination of**
 303 **sofosbuvir in Mpiviropack® tablets by the proposed methods.**
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Recovery ±SD%	UPLC Method			Recovery ±SD%	HPTLC Method		
	Standard addition				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
98.07±0.89	5	1	102.21	100.73±1.11	4	1	98.50
	5	5	101.99		4	3	101.02
	5	10	99.05		4	5	100.15
	5	15	100.61		4	7	100.70
Mean%±SD			100.96±1.45				100.09±1.12

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308 **Table 6. Results obtained by the proposed method compared with reported method⁽³⁾**
 309 **for determination of sofosbuvir in Mpiviropack® tablets.**
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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)	---

311

312 **4. CONCLUSION**

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

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320 **COMPETING INTRESTS**

321 Authors have declared that no competing interests exist.

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