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Development andValidation of a New, Simple-HPLC Method for Simultaneous Determination ofSofosbuvir, Daclatasvir and Ribavirin in Tablet Dosage Form

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Abstract:*A sensitive, simple, selective and accurate HPLC method was developed and validated for Simultaneousanalysis of antiviral drugs,*Sofosbuvir, Daclatasvir and Ribavirin*that allowreduction in treatment duration for HCV patients that in turn decrease the cost of the treatment. The chromatographic separation achieved by isocratic elution on a reversed-phase analytical column [Hypersilgold® C18 (10µm, 150 x 4.6 mm) column] at ambient temperature. The mobile phase was a mixture of Methanol, Waterand Acetonitrile in ratio of 25:30:45 (v/v/v), injection volume was 20µl. and flow rate was 1ml/ minute, detectionwavelength was 243nm. The developed method was validated as per ICH guidelines; it was precise, accurate and robust. The calibration curves of the three drugswere linear in range:5-150µg/ml for Ribavirin, 25-300 µg/ml forSofosbuvir, and 1-100µg/ml for Daclatasvir,with a correlation coefficient ≥0.999. The validated method was helpful for rapid routine analysis as the run time was less than 7 minute; theretention time was 2.007, 3.632 and 6.922 minute and LOD 0.2, 1, 0.3 µg/ml and LOQ 0.5, 3, 0.9 µg/mlfor Ribavirin, Sofosbuvir, and Daclatasvir, respectively. The method was successfully applied to analysis of Ribavirin, Sofosbuvir, and Daclatasvir in tablet dosage form with accepted % recovery for each one.*

Keywords*:Daclatasvir, HPLC, Ribavirin, Sofosbuvir, Tablets*

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I. Introduction

Hepatitis C virus (HCV) is chronically infect about 150-200 million peopleworldwide and up to 350000 people die every year from hepatitis related diseases.HCV prevalence varies greatly, but thehighestprevalence (15–20%) has beenreported from Egypt.The conventional treatments are consisted of a combinationof pegylated interferon alpha and antiviral drug,ribavirin(RBV), but this medication effect was very lowparticularly for genotype-1 (GT-1) HCV, the cure rate wasabout 45%[1, 2]

The discovery of the new RNA polymerase inhibitor, sofosbuvir provides a high cure rate and gives an option for patient who has previously failing therapy. Two firstgeneration direct-acting antiviral (DAA), telaprevir and boceprevir, have been approved in the US and EU in 2011 for the treatment of GT-1 chronic hepatitis C (CHC). However, both of these agents must be co administered with interferon (IFN) and RBV, and are therefore associated with the known adverse effects of the IFN/RBV backbone,potentially limiting theiroverall effectiveness. Developing of a new generation IFN/RBV-free DAAs that can improve efficacy and safety in a broader GT population of CHC-infected subjects remains a high priority [3, 4]

DAAs haverevolutionized the treatment of HCV infection over the last 5 years. As a result of our better understandingof the HCV life cycle, specific DAAs have been developed for HCV that are ableto target the viral proteins implicated in replication of the virus, i.e., the NS3/4Aprotease, NS5B polymerase, and multifunctional NS5Areplication complex.

1.1. Daclatasvir*(fig.1.A.)*is methyl N-[(2S)-1-[(2S)-2-[5-[4-[4-[2-[(2S)-1-[(2S)-2-(methoxycarbonylamino)-3-methylbutanoyl] pyrrolidin-2-yl]-1H-imidazol-5-yl]phenyl]phenyl]-1H-imidazol-2-yl]pyrrolidin-1-yl]-3-methyl-1-oxobutan-2-yl]carbamate

Daclatasvir is a first-in-class HCV NS5A replication complex inhibitor with pangenotypic activity and a pharmacokinetic profile allowing once-daily dosing. Reaching in vitro 50% effective concentrations (EC50) in the picomolar range against HCV replicons representing six major HCV genotypes (1a, 1b, 2a, 3a, 4a, 5a), daclatasvir is one of the most potentHCV replication inhibitors reported todate

1.2. Sofosbuvir*(fig.1.B.)*is propan-2-yl (2S)-2-[[[(2R,3R,4R,5R)-5-(2,4-dioxopyrimidin-1-yl)-4-fluoro-3-hydroxy-4-methyloxolan-2-yl]methoxy-phenoxyphosphoryl]amino]propanoate. Sofosbuvir is an orally administered HCVnucleotide polymerase NS5B inhibitor. It is given once daily, and has a goodsafety profile. [5,6] It has a high barrier to resistance, a pangenotypic antiviral effect, and few drug–drug interactions.[7] Combination of sofosbuvir and daclatasvir with or without ribavirin has been well tolerated in previously treated or untreated HCV patients. [8]

1.3. RBV*(fig.1.C.)is* 1-[(2R,3R,4S,5R)-3,4-dihydroxy-5-(hydroxymethyl)oxolan-2-yl]-1,2,4-triazole-3-carboxamide

Ribavirin is a synthetic nucleoside analogrelated to guanine. It inhibits the replication of awide range of RNA and DNA viruses.[9] It is used orally with an IFN alfa or PEG- IFN alfa in the treatment ofchronic HCV, including HIV coinfection. [9, 10].

1.4. Sofosbuvir + daclatasvir ± RBV:a pangenotypic combination

Ribavirin-sparingregimens are desirable,considering the risks of anemiaand teratogenicity, but their role from a cost-effectivenessperspective (i.e., allowing a reduction in treatment duration)cannot be excluded.[11]

The sofosbuvir + daclatasvircombination is associated with a high rate of SVR4 in difficult-to-treat patients infected with genotype 1 or 4. Combination withribavirin increases the SVR rate in cirrhotic and treatment experienced patients with no additive effect of extension of treatment from 12 to 24 weeks. [12], [13].

Since patient compliance is an important point in the treatment so taking the three drugs in one tablet will be a better choice.On another hand, the combined therapy is economically reduced the cost of the treatment and this will give a chance for many companies to formulate the three drugs in one tablet sooner. Additionally, the co-administered drugs might affect each other and there is no sufficient information about drug-drug interaction and thus the establishment of separation method is of great importance. There are several reported methods based on HPLC for analysis of RBV[14, 15, 16], Sofosbuvir[17, 18, 19], and Daclatasvir[20, 21, 22]however, no reported HPLC method is reported for simultaneous determination of *RBV, Sofosbuvir, and Daclatasvir* mixture. Herein, a simple, sensitive and direct analysis without complicated sample preparation HPLC-UV method is optimized and validated for RBV, Sofosbuvir, and Daclatasvir determination in bulk and pharmaceutical formulation.

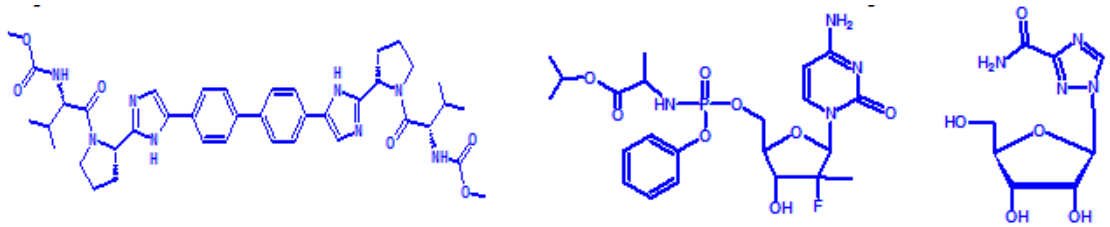

Fig.1 A. Daclatasvir **B.** Sofosbuvir **C.** Ribavirin

II. Experimental

2.1. Instrumentation:

HPLC apparatus is equipped with a Surveyor®quaternary pump with Intel vacuum degasser (Thermo Scientific Co., USA), Surveyor®auto-sampler plus (Thermo Scientific Co., USA), Surveyor®photodiode array detector (PAD) (Thermo Scientific Co., USA). Computer with a software chromo quest 5 (Thermo Scientific Co., USA) for data collection and analysis auto-sampler vials 1.8 ml screw cap (Thermo Scientific Co., USA).The separation and quantitation were made on Hypersil gold®C18 (10um, 150x4.6mm) column (Thermo Scientific Co., USA).

2.2. Material and chemical reagents:

2.2.1. Pure samples:

Pure samples of Sofosbuvir was kindly supplied by the Egyptian Pharmaceutical and Chemical Industry (EPCI); EPCI pharmaceutical company which is a part of Hikma group, Beni-Suef, Egypt with claimed purity of 99.8% according to manufacturer certificates of analysis.

Pure samples of Daclatasvir dihydrochloride was obtained from Topharman Shanghai Co., ltd, china.

Pure samples of Ribavirin was kindly supplied by sigma pharm company, Quisna, Egypt with claimed purity of 99.8% according to manufacturer certificates of analysis.

2.2.2. Pharmaceutical dosage form:

Daklanork® 60 mg of Daclatasvir film coated tablets, Mash for pharmaceuticals, Egypt.Batch No: M116116.

Hopforhep® 400 mg of Sofosbuvir film coated tablets, GLOBAL NAPI for pharmaceuticals, Egypt. Batch No.: 028403.
Copegus®200 mg of Ribavirinfilm coated tablets, La Roche Ltd, by Patheoninc., Mississauga, Canada. Batch No.: N0236.
2.2.3.Solvents: Acetonitrile, Methanol, water were of HPLC-grade.
2.3. Preparation of solutions:
2.3.1. Preparation of mobile phase:
A mixture of Methanol, Water, Acetonitrile in a ratio of *25:30:45 (v/v/v)*was prepared**.**

2.3.2. Preparation of stock and working standards:
Stock standard solutions were prepared separately to give a final concentration of 500µg/ml forDaclatasvir, Sofosbuvir and Ribavirin through dissolving an accurately weighed amount (50 mg) in a totalof 100 ml of 50% methanol

Working solutions for the standard calibration graphs were prepared immediately before analysis by furtherdilutions of the stock solutions with the mobile phase to cover the concentration ranges of 5–150, 25–300, and 1–100 µg/ml for Daclatasvir, Sofosbuvir and Ribavirin respectively. Three replicate each of 20 µl injectionsfor each drug concentration level (simultaneously prepared) were made and directly chromatographed under thespecified chromatographic conditions.

2.3.3. Preparation of sample solution:
The content of 20 tablets of Daklanork® and Hopforhep®and Copegus®was weighed and separately grounded to gethomogenous powder. A portion of each finely powdered drug equal to one tablet (according to the labelclaimed), equivalent to 60 mg Daclatasvir, 400 mg Sofosbuvir and 200mg Ribavirin was accurately weighedand transferred to a 100 ml capacity volumetric flask. Thirty milliliters 50% methanol were added to the mixture; the mixture was dissolved via ultra-sonication for 30 min at ambient temperature andthen diluted to the mark with the mobile phase. The solutions were filtered through 0.45 µm nylon membranefilter discs [MilliporeTM, Milford, MA] before use. Further dilution was carried out using the mobile phase to suit the concentration domain covered by the calibration graphs. The solutions were chromatographed using theHPLC conditions described above and the concentrations of Daclatasvir, Sofosbuvir and Ribavirin werecalculated**.**

2.4. Chromatographic conditions:
The analysis was achieved on a reversed-phase analytical column [Hypersilgold® C18 (10µm, 150 x 4.6 mm) column] at ambient temperature. The mobile phase was a mixture of Methanol, Water, Acetonitrile in a ratio of *25:30:45 (v/v/v).* The flow rate was 1 ml/ min.The injectionvolume was 20 µl. The UV detectionwavelength was243 nm. A freshly prepared mobile phase was passedon the column for 15 min before injection.

III. Results And Discussion:

3.1. Method development and optimization:
Before development of HPLC method,important information wascollected. The solubility of the three drugs was found to be higher in 50%methanol than in water so this solvent was selected forpreparation of all solutions.

The wavelength of detection was set regarding the drugs UV absorption spectra and theirrelative concentrations within the pharmaceutical formulations, the detection atλ 243 nm was the optimal wavelength for the three drugs.

Several mobile phase ratios were tried through the change ofmobile phase composition. In initial trials, water and phosphate buffer and/or methanol were tried but it was observed that peak sharpness and theoretical plates numbers were not adequate so Methanol, water, Acetonitrile mobile phase was selected for the best peak sharpness and plates and gave the best results with a reasonable retention times

Finally, among these mobile phases a mixture of Methanol, Water, Acetonitrile in a ratio of *25:30:45 (v/v/v)*was used. The flow rate was 1 ml/ min.The injectionvolume was 20µl and UV detector was set at243 nm. A reversed-phase analytical column [Hypersilgold® C18 (10µm, 150 x 4.6 mm) column] at ambient temperature was selected as optimum for the best peak symmetry, theoretical plates and retention time fig.4, table 7

The specificity of this HPLC method is illustrated at the typical chromatograms (Fig.2), where complete separation of the drugs was noticed.The retention time for RBV, Sofosbuvir, and Daclatasvir was 2.007, 3.632 and 6.922 minute, respectively.The obtained peaks were sharp and had clear baseline separation.

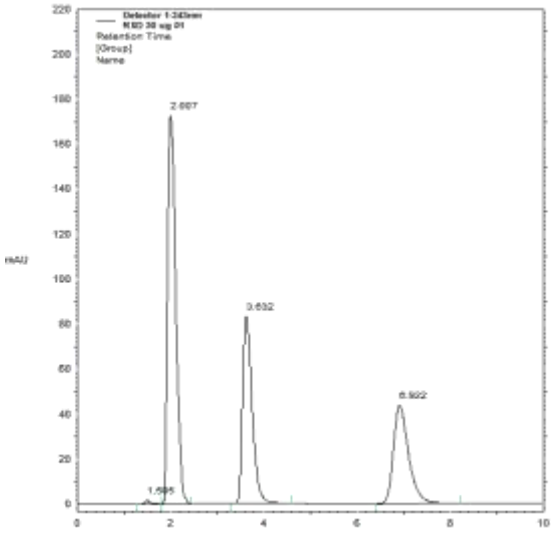

Fig. 2: Ribavirin, Sofosbuvir, Daclatasvir HPLC chromatogram

IV. Method Validation

Validation of the method was carried out according to ICH guidelines [23] to ensure that the method is suitable for its intendeduse. Linearity, accuracy, precision, ruggedness and robustness, all these parameters were tested and were found in acceptablelimits

4.1. Linearityand range (calibration curve):

The linearity of an analytical procedure is its ability (within a given range) to obtain test results whichare either, directly or through mathematical transformation proportional to the concentration of the analyte. Thisproposed HPLC method was assessed by least-squares linear regression analysis of the calibration curve[24]

Linearity of the method was tested for six concentrations of *RBV, Sofosbuvir, Daclatasvir* in a range from 5-150µg/ml for RBV, 25-300 µg/ml for Sofosbuvir, and 1-100 µg/ml for Daclatasvir(Table1). Eachconcentration was injected in triplicate and the mean value of the peak areas was imputed into a MicrosoftExcel® spreadsheet program for the calibration curve plotting. The repeated runs were genuine repeats and notjust repetitions at the same reading in which three replicate samples of each concentration level were prepared;this in order to provide information on the variation of the peak area between samples of the same concentration.The regression analyses revealed satisfactory correlations (r = 0.9996 –0.9998), this, indicating a good linearityof the calibration graphs*Fig.3a, 3b, 3c*.

Table 1.Characteristic parameters for the calibration equationsof the proposed HPLC method for thesimultaneous determination ofRibavirin, Sofosbuvir, Daclatasvir

	Ribavirin		**Sofosbuvir**		**Daclatasvir**	
	Conc. µg/ml	Peak area	Conc. µg/ml	Peak area	Conc. µg/ml	Peak area
	5	407802	**25**	616570	**1**	70430
	10	595807	**50**	1187754	**5**	144314
	25	1149058	**100**	2396240	**10**	236669
	50	2133711	**150**	3397959	**25**	515459
	100	3954871	**200**	4562717	**50**	1002743
	150	5932307	**300**	6805317	**100**	1885514
Slope (a)		**37952**		**22426**		**18410**
Intercept (b)		**211651**		**77518**		**56483**
Correlation coefficient (r^2)		**0.9998**		**0.9997**		**0.9996**

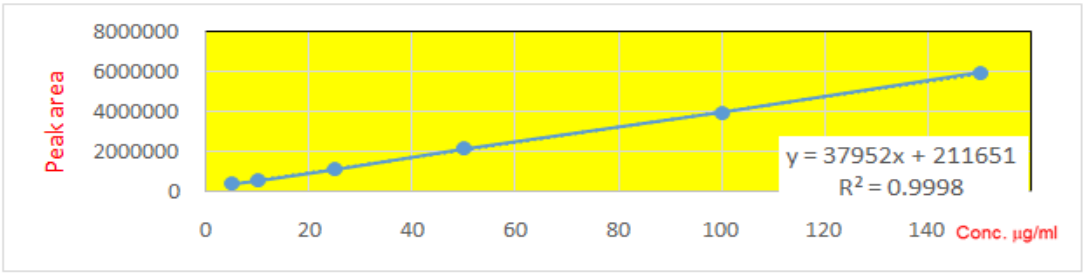

Fig. 3a: Calibration curve of Ribavirin (5-15 µg/ml) using the proposed HPLC method with UV detection at 243 nm

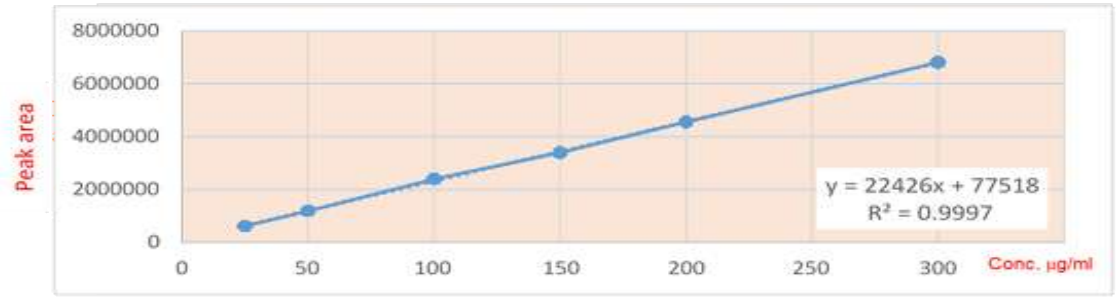

Fig. 3b: Calibration curve of Sofosbuvir(25-300µg/ml) using the proposed HPLC method with UV detection at 243 nm

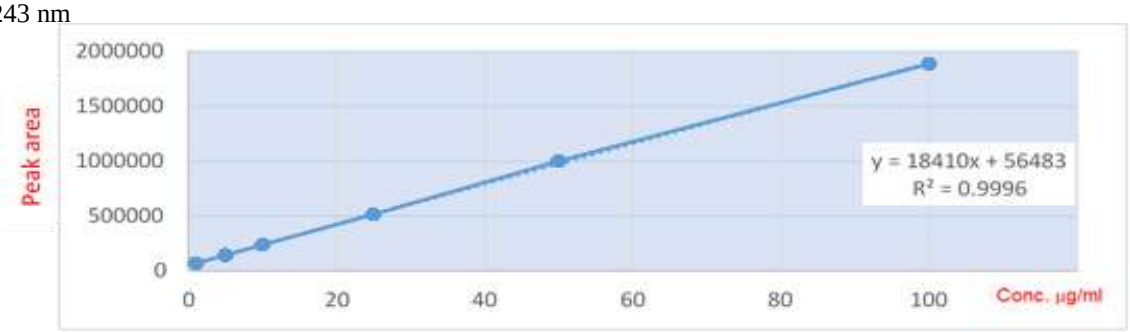

Fig. 3c: Calibration curve of Daclatasvir (1-100µg/ml) using the proposed HPLC method with UV detection at 243 nm

Regression equation: Y=aX+b, *where X is the concentration of the reference standard (µg/ml) and Yis the peak area*

4.2. Precision: The precision of the proposed HPLC analysis was evaluated as repeatability and reproducibility levels;[23] using three independent concentrations of each drug. The repeatability (intra-day precision) studies wereperformed on the same day, whereas, that of the intermediate precision (inter-day precision) were checked byrepeating these studies on three consecutive days. Every sample was injected in triplicates and both the retentiontimes (*t*R) and peak areas were determined.Within the examined time range, the peak area results presented in(Table 2) and show excellent precision for the method both during one analytical run and between different runs,with an intra-day and inter-day RSD (%), the range was 0.07–1.72 and 0.20–1.79, respectively.

Table 2.Results of the intra-day and inter-day precision in the assay of Ribavirin, Sofosbuvir, Daclatasvir using the proposed HPLC method

Drug	Conc. Taken µg/ml	Intra-day precision		Inter-day precision	
		Found µg/ml	% recovery ± SD; RSD[a] %	Found µg/ml	% recovery ± SD; RSD[b] %
Ribavirin	***25***	24.71	98.85 ±0.20; 0.81	24.83	99.32±0.20; 0.80
	50	50.90	101.80 ± 0.03; 0.07	50.24	100.48± 0.39; 0.77
	100	99.95	99.95± 0.22; 0.22	99.94	99.23 ±1.42; 1.42
Sofosbuvir	***25***	25.43	101.73±0.43;1.72	24.81	99.25±0.27;1.09
	50	50.78	101.56±0.12; 0.23	49.72	99.44±0.17; 0.34
	100	98.43	98.43±0.26; 0.26	100.61	100.61± 1.80; 1.79
Daclatasvir	***25***	25.09	100.37±0.21;0.84	25.10	100.41±0.32; 1.28
	50	50.87	101.75±0.08;0.16	50.84	101.68±0.10;0.20
	100	100.67	100.67±0.84;0.84	100.61	100.61± 0.80; 0.79

[a] Means, SD. and RSD (%), of three replicates on same day. [b] Means, SD and RSD (%), of three replicates on three consecutive days.

4.3. Accuracy: The accuracy of the proposed method, which is defined as the closeness or the nearness of the true andfound values, was evaluated by measuring the drug recoveries by using the standard addition technique. Thestandard addition analysis involves the addition of three concentration levels of each drug standard solution(covering the linearity range and higher than LOQ*)* to pre-analyzed pharmaceutical samples containing; 20, 40and 6 µg.mL^{-1} of Ribavirin, Sofosbuvir, Daclatasvir respectively**.** Each set of addition was repeated fivetimes, and the results obtained were compared with those expected from the calibration curve, (Table 3).

4.4 Selectivity: The selectivity of the proposed method was checked by preparing five laboratory-prepared mixtures ofthe studied drugs at various concentrations within their linearity range. The laboratory-prepared mixtures wereanalyzed according to the previous procedure described under the proposed method. Satisfactory results wereobtained as listed in (Table4) indicating the high selectivity of the proposed method for simultaneousdetermination of the studied drugs

4.5. Robustness: Robustness relates to the capacity of the method to remain unaffected by small but deliberate variations
introduced into the method critical parameters. Sothe method was evaluated within small variation in its parameter and was found to be robust. Robustness was examined by small change in the flow rate (±0.05ml/min), and in mobile phase composition (± 1%). The relative standard deviation (RSD) resultswere shown in (Table 5, Table 6)

4.6. LOD& LOQ: The limit of detection (LOD) for an HPLC method is the lowest drug concentration that produces aresponse detectable above the noise level of the system, typically taken as three times. The limit ofquantification (LOQ) is the lowest level of the drug that can be accurately measured, and it is often evaluated asten times the noise level. Both quantities were evaluated regarding the International Conference onHarmonization (ICH) guidelines.LOD and LOQ were found to be 0.2, 1, 0.3 µg/ml and LOQ 0.5, 3, 0.9µg/ml for Ribavirin, Sofosbuvir, Daclatasvir respectively

4.7 System suitability test: System suitability tests (SST) are based on the concept that the equipment, electronics, analytical
operations and samples to be analyzed constitute an integral system that can be evaluated as such. These testswere performed in accordance with the BP guidelines to ensure adequate performance of both thechromatographic system and the equipment, for the analysis to be performed.The observed R.S.D. (%), of the retention times regarding these repetitive injections, wasconsidered satisfactory, meeting the BP recommendation (R.S.D. (%) < 1.0). Other chromatographic parameterswere calculated from experimental data, such as; tailing factor (*T*f) also known as peak asymmetry factor (*A*s) and the apparent number of theoretical plates (*N*) and Capacity factor (*k'*) of the peak.All of these parameters are usually employed in assessing the performance of the column.Results obtained fromsystem suitability tests are presented in (Table 7). Good agreement was found when results were comparedwithrecommended values.

4.8 Analytical solutions stability:

The solutions were stored in tightly capped volumetric flasks and wrapped with aluminum foil underreduced light conditions. It was found that Ribavirin analytical solution exhibited nochanges for at least 10 days when stored refrigerated at 4˚C and for 24 hours when kept at room temperature.Sofosbuvir and Daclatasvir analytical solutions in methanol exhibited no changes for 7 days when stored refrigerated at 4˚C andfor 18 hours when kept at room temperature. Solutions of the studied compounds in the mobile phase exhibitedno changes for 8 hours when kept at room temperature.

4.9. Analysis of pharmaceutical products:

The validated HPLC method was applied for the determination of Ribavirin, Sofosbuvir andDaclatasvir in pharmaceuticalpreparation using Copegus®, Hopforhep® and Daklanork® tablets respectively. Three replicatedeterminations were performed at each concentration level. Satisfactory results were obtained for eachcompound in good agreement with label claims (Table 8)

The obtained results were compared statistically byStudent's *t*-test (for accuracy) and variance ratio F-test (for repeatability) with USP official method[25] forRibavirin &the reported method [19, 20] for Sofosbuvir and Daclatasvir. The results showed that the calculated t and F values weresmaller than the critical values at 95% confidence limit indicating that there is no significant difference betweenthe proposed and reported methods, (Table 8)

V. Conclusion

This study described a simple, specific and reliable HPLC UV method for the assay of antiviral drugs (RBV, Sofosbuvir, Daclatasvir) in bulk and tablets dosage form. The method is rapid and helpful routine work for quick analysis of a large number of samples in short time.Reliability wasguaranteed by testing various validation parameters of themethod and the successful application tocommercial tabletdosage form. The success of our method in separation of the commonly administered drugs allow the application of our method to study pharmacokinetic and pharmacodynamic parameters in various matrices.

Acknowledgement

The authors sincerely thank to Dr. EslamHamed (central lab., Faculty of veterinary medicine/ Zagazig university) for his contributions.

Table 3.Results of the accuracy studies by standard addition technique in the assay ofRibavirin, Sofosbuvir, Daclatasvir using the proposed HPLC method

Drug	Concentration (µg/ml)				% recovery	RSD %	relative error (Er)
	Initial tablet sample	Authentic amount added	Claimed total amount	Total amount found± SD			
Ribavirin	20	5	25	24.84 ± 0.20	99.40	0.80	-0.0006
	20	30	50	50.49 ± 0.41	100.99	0.78	0.0099
	20	80	100	99.95 ± 1.43	99.95	1.43	-0.0005
Sofosbuvir	40	10	50	50.91 ± 0.09	101.81	1.82	0.0181
	40	40	80	80.29 ± 0.92	100.36	1.15	0.0036
	40	60	100	100.06 ± 1.3	100.06	1.31	0.0006
Daclatasvir	6	4	10	10.12 ± 0.07	101.2	0.71	0.012
	6	14	20	19.82 ± 0.16	99.1	0.80	-0.009
	6	19	25	25.09 ± 0.43	100.36	1.72	0.0036

Table 4: Determination of Ribavirin, Sofosbuvir and Daclatasvir in laboratory prepared mixtures using the proposed method

Conc. Taken µg/ml	***Ribavirin****		Conc. Taken µg/ml	***Sofosbuvir****		Conc. Taken µg/ml	***Daclatasvir****	
	Peak area	*% recovery*		Peak area	*% recovery*		Peak area	*% recovery*
5	397889	98.14	**25**	629914	98.52	**5**	149025	100.53
8	518988	100.72	**40**	969135	99.39	**8**	210065	99.57
10	583945	98.09	**50**	1203324	100.40	**10**	239663	101.38
16	821365	100.30	**80**	1889658	101.01	**16**	358989	101.50
20	982895	101.60	**100**	2366045	102.04	**20**	427854	99.81
mean		**99.89**			**100.27**			**100.56**
SD		**1.67**			**1.37**			**0.88**
RSD		**1.68**			**1.36**			**0.87**
Variance		**2.82**			**1.86**			**0.76**

* Average of five independent procedures.

Table 5. Robustness (Flow rate)in the assay of Ribavirin, Sofosbuvir, Daclatasvir using the proposed HPLC method

	Ribavirin			**Sofosbuvir**			**Daclatasvir**		
Flow rate	**1.05**	**1**	**0.95**	**1.05**	**1**	**0.95**	**1.05**	**1**	**0.95**
Determination	**Peak area**								
1	2102210	2133711	2154798	1155942	1177754	1200698	970845	994144	1004985
2	2094575	2137297	2156426	1150217	1199739	1201282	983424	1003875	1008379
3	2091774	2125851	2160319	1151038	1187974	1207047	989235	996079	1004168
4	2096665	2132376	2158175	1151309	1186332	1205777	988198	997125	1004428
5	2098791	2133073	2167909	1151840	1190601	1218993	976491	994625	1003658
Mean	**2129597**			**1182436**			**994643.9**		
SD	**26904.57**			**24260.87**			**11178.96**		
RSD	**1.263365**			**2.05177**			**1.123916**		

Table 6. Robustness (Mobile phase)in the assay of Ribavirin, Sofosbuvir, Daclatasvir using the proposed HPLC method

	Ribavirin			Sofosbuvir			Daclatasvir		
Mobile phase	**M_1**	**M**	**M_2**	**M_1**	**M**	**M_2**	**M_1**	**M**	**M_2**
Determination	**Peak area**								
1	2156634	2133711	2146592	1205757	1177754	1196292	1002881	994144	994456
2	2147332	2137297	2141490	1201949	1199739	1199107	1000498	1003875	999802
3	2152102	2125851	2142548	1201422	1187974	1198802	994627	996079	995674
4	2153622	2132376	2151011	1204523	1186332	1191021	1002765	997125	989862
5	2148881	2133073	2143434	1205566	1190601	1210113	996548	994625	999711
Mean	**2143064**			**1197130**			**997511.5**		
SD	**9019.483**			**8785.009**			**3948.577**		
RSD	**0.420869**			**0.733839**			**0.395843**		

M: mobile phase of Methanol, Water, Acetonitrile in a ratio of 25:30:45 (v/v)
M1: mobile phase of Methanol, Water, Acetonitrile in a ratio of 26:30:44 (v/v)
M2: mobile phase of Methanol, Water, Acetonitrile in a ratio of 24:30:46 (v/v)

Table 7: system suitability testing using the proposedHPLC method

	Ribavirin	Sofosbuvir	Daclatasvir	Recommended values
Retention time (*t*R)(min)	*2.007*	*3.632*	*6.922*	-
Theoreticalplates(*N*)	650	1528	2219	The more plates, the better separation efficiency
Capacity factor (*k'*)	1.9	4.7	6.8	$0.5 < k' < 10$
Tailing factor (*Tf*)	1.5	1.5	1.38	$0.8 < Tf \leq 1.5$

Table 8: Statistical comparison between the proposed HPLC method and reported methods for thedetermination of Ribavirin, Sofosbuvir, Daclatasvir in pharmaceutical formulation

Analyte	Amount taken µg /ml	Proposed method		Reportedmethods		t-test (2.31)*	*F*-test (6.39)*
		Recovery (%)	RSD%	Recovery (%)	RSD%		
Ribavirin	5	98.14	2.01	98.26	0.97	0.44	0.52
	10	98.09		100.64			
	20	101.60		99.58			
Sofosbuvir	80	98.06	0.99	99.39	0.33	0.18	0.20
	100	99.65		100.05			
	120	99.87		99.67			
Daclatasvir	1.5	99.71	0.53	100.50	0.35	0.04	0.59
	3	100.01		100.25			
	6	98.97		99.81			

*Tabulated t and F values at 95 % confidence limit

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