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Development andValidation of a New, Simple-HPLC Method for Simultaneous Determination ofOmbitasvir, Paritaprevir, Ritonavir 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,Ombitasvir, Paritaprevir, Ritonavir and Ribavirinthat used for chronic hepatitis C virus genotype 4 infection in Egyptianpatients with or without compensated cirrhosis. The chromatographic separation achieved by isocratic elution on a reversed-phase analytical column [Magellen® C18 (10µm, 150 x 4.6 mm) column] at ambient temperature. The mobile phase was a mixture of 0.1M Phosphate buffer (pH 7)and Acetonitrile in ratio of 25:75 (v/v), injection volume was 20µl, flow rate was 1ml/ minute and detectionwavelength was 243nm. The developed method was validated as per ICH guidelines; it was precise, accurate and robust. The calibration curves of the four drugswere linear in range:5-150µg/ml for Ribavirin, 1.8-60 µg/ml forParitaprevir, and 2.5–50 µg/ml for Ritonavir,2.25–36µg/ml for Ombitasvirwith a correlation coefficient ≥0.999. The validated method was helpful for rapid routine analysis as the run time was less than 6 minutes; theretention time was 1.298, 2.82,4.115 and 5.786 minute and LOD was found to be 1.2, 0.8, 0.7 and 0.06 µg/ml and LOQ 3.6, 2.4, 2.1 and 0.21 µg/ml for Ribavirin, Paritaprevir, Ritonavir and Ombitasvir respectively. The method was successfully applied to analysis ofthese drugsin their tablet dosage formswith accepted % recovery for each one.*

Keywords:*HPLC,Ombitasvir, Paritaprevir, Ribavirin, Ritonavir,Tablets*

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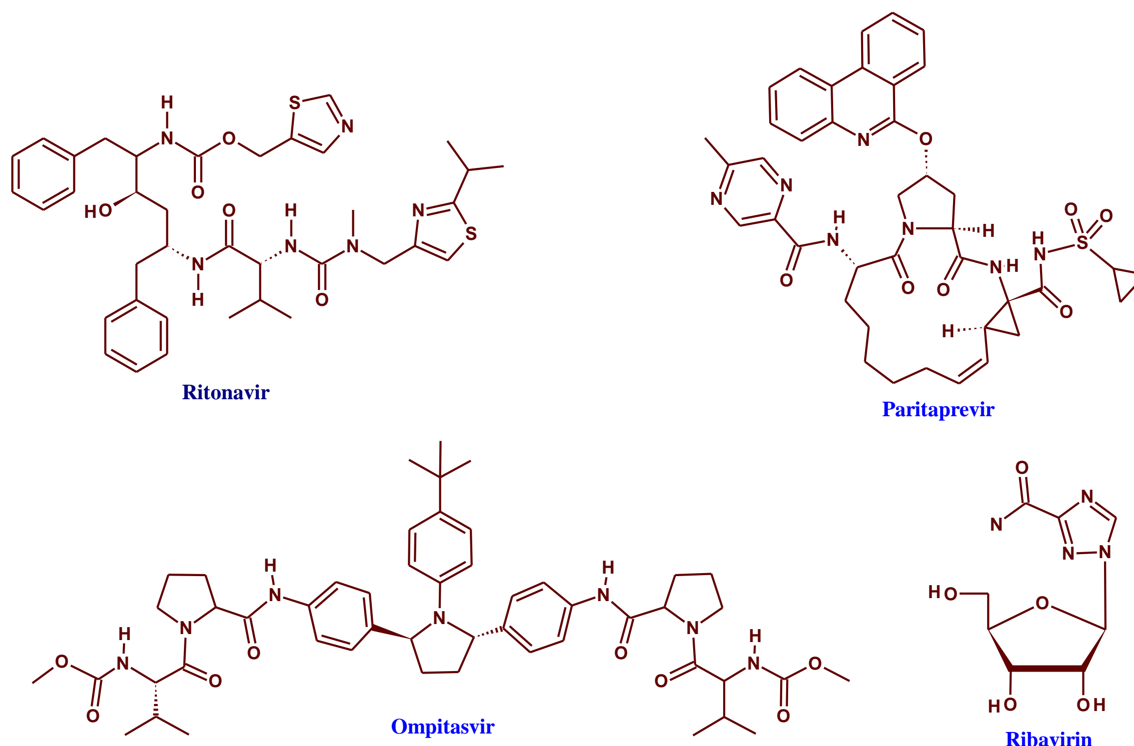
I. Introduction

Chronic infection with hepatitis C virus (HCV) occurs inroughly 180 million people worldwide, and althoughgenotype 1 accounts for roughly 48% of infections,distribution of the seven genotypes differs geographically.[1] Genotype 4 infections account for 13–20% of all HCV infections worldwide, but make up about 93% of all HCV cases in Egypt.[2,3]

The standard of care fortreatment of genotype 4 infection in Egyptwas the combination of sofosbuvir plus ribavirin,either with or without pegylated interferon, according tointerferon eligibility.[4]

The direct-actingantiviral combination of ombitasvir, paritaprevir, andritonavir plus ribavirin has achieved SVR12 in all 91 HCVtreatment-naive patients or pegylated interferon plusribavirin treatment-experienced patients with genotype 4infection. [5]

1.1. Ombitasvir [OMP],*(fig.1.)*is a potent NS5A inhibitor with broadantiviral activity against HCV genotypes 1a, 1b, 2a, 2b, 3a,4a, and 6a.[6]
1.2. Paritaprevir [PAR], *(fig.1.)*an NS3/4A protease inhibitor(with the pharmacokinetic enhancer **Ritonavir**), hassimilarly broad genotypic activity against HCV genotypes1a, 1b, 2a, 3a, 4a, and 6a.[7]
1.3. Ritonavir [RIT], an antiretroviral medication used along with othermedications to treat HIV/AIDS [8]
1.4. Ribavirin [RBV],*(fig.1.)*synthetic nucleoside analogrelated to guanine. It inhibits the replication of awide range of RNA and DNA viruses.[9]
There are several reported methods based on HPLC for analysis of RBV alone [10, 11, 12], one report for mixture of OMP, PAR, RIT [13] and one report for mixture of OMP, PAR, RIT with Dasabuvir [14]



***(fig.1.)** chemical structures of RIT, PAR, OMP, RBV*

II. Experimental

2.1. Instrumentation:

HPLC apparatus is equipped with a G1311A Quaternary pump with Micro vacuum degasser (Agilent technologies 1200 series, USA), High performance auto-sampler plus (Agilent technologies 1200 series, USA), Diode array detector (DAD) (Agilent technologies 1200 series, USA). Computer with a software AgilentChemstation® (Agilent technologies 1200 series, USA) for data collection and analysis auto-sampler vials 1.8ml screw cap (Agilent technologies 1200 series, USA).The separation and quantitation were made on Magellen®C18 (5 µm, 150x4.6mm) column (Agilent technologies 1200 series,USA).

2.2. Material and chemical reagents:

All chemicals and reagents usedwere of HPLC grade. Thedrugs used in present study wereobtained from Hetero drugs pvt.Ltd. Hyderabad.Commercially availableQurevo® tablets claimed tocontain 12.5 mg Ombitasvir; 75 mg Paritaprevir and 50 mgRitonavir and Copegus®200 mg of Ribavirin film coated tablets, have been utilized in the present work.

2.3. Preparation of solutions:

2.3.1. Preparation of mobile phase:

The mobilephase was a mixture of acetonitrile and 0.1 M potassium dihydrogen orthophosphate (75:25, v/v; pH adjusted to 7 with sodium hydroxide)**.**The mobile phase was filtered through 0.45-µm Nylon membranefilter and sonicated for 20 min.

2.3.2. Preparation of stock and working standards:

Stock standard solutions were prepared separately to give a final concentration of 1000µg/ml foreach through dissolving an accurately weighed amount (10 mg) in a totalof 10 ml of the mobile phase

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 5-150µg/ml for RBV, 1.8-60 µg/ml for PAR, and 2.5–50 µg/ml for RIT, and 2.25–36µg/ml for OMB. Three replicates 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 pharmaceutical dosage forms samples:

The content of 20 tablets of Qurevo®and Copegus®was weighed and separately grinded to gethomogenous powder. A portion of each finely powdered drug equal to one tablet (according to the labelclaimed), equivalent to 12.5 mg OMP; 75 mg PAR, 50 mg RITand 200mg RBV was accurately weighedand transferred to a 100 ml capacity volumetric flask. Thirty milliliters mobile phase 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 tosuit the concentration domain covered by the calibration graphs. The solutions were chromatographed using theHPLC conditions described above and the concentrations of OMP, PAR, RIT and RBV werecalculated**.**

2.4. Chromatographic conditions:

The analysis was achieved on a reversed-phase analytical column [Magellen®C18 (5 μm, 150x4.6mm) column (Agilent technologies 1200 series, USA).] at ambient temperature. The mobile phase was a mixture of Acetonitrile and 0.1 M potassium dihydrogen orthophosphate (75:25, v/v; pHadjusted to 7 with sodium hydroxide). 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 OMP, PAR, RIT,was found to be higher in acetonitrile and for RBV in water but mix of Acetonitrile and phosphate buffer gave better symmetric peaks so this solventmixture 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 four drugs.

Several mobile phase ratios were tried through the change ofmobile phase composition. In initial trials, Acetonitrile, water and phosphate buffer and/or methanol were tried but it was observed that peak sharpness and theoretical plates numbers were not adequate so Acetonitrile and phosphate buffer 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 Acetonitrile and 0.1 M potassium dihydrogen orthophosphate (75:25, v/v; pH adjusted to 7 with sodium hydroxide). The flow rate was 1 ml/ min.The injectionvolume was 20μl and UV detector was set at243 nm. A reversed-phase analytical column [Magellen®C18 (5 μm, 150x4.6mm) column] at ambient temperature was selected as optimum for the best peak symmetry, theoretical plates and retention time fig.2

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, PAR, RIT and OMP was *1.298, 2.82, 4.115 and 5.786* minutes, respectively.The obtained peaks were sharp and had clear baseline separation.

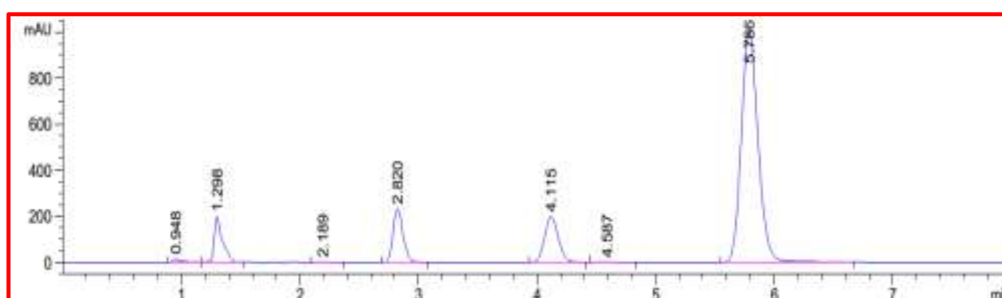


*Fig. 2:*HPLC chromatogram of Ribavirin, Paritaprevir, Ritonavir andOmbitasvir.

IV. Method Validation

Validation of the method was carried out according to ICH guidelines [15] 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[16]

Linearity of the method was tested for six concentrations of *RBV, PAR, RIT and OMP* in a range from 5-150µg/ml for RBV, 1.8-60 µg/ml for PAR, 2.5–50 µg/ml for RIT, and 2.25–36µg/ml for OMB(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 = 0.9993 –0.9999), this, indicating a good linearityof the calibration graphs*Fig.3*.

Table 1.Characteristic parameters for the calibration equationsof the proposed HPLC method for thesimultaneous determination of*RBV, PAR, RIT and OMP*

	RBV		PAR		RIT		OMP	
	Conc. µg/ml	Peak area	Conc. µg/ml	Peak area	Conc. µg/ml	Peak area	Conc. µg/ml	Peak area
	5	94.82	**2**	90.7356	**2.5**	216.4568	**2.25**	833.42
	10	191.12	**5**	226.5897	**5**	539.2244	**4.5**	2338.62
	25	465.23	**15**	684.684	**10**	1076.126	**9**	4891.11
	50	970.22	**30**	1371.37	**15**	1607.975	**18**	9901.42
	100	1949.87	**45**	2115.002	**30**	3236.55	**27**	14965.32
	150	2811.12	**60**	2745.88	**50**	5344.477	**36**	19766.33
Slope (a)	**18.919**		**46.187**		**107.51**		**552.96**	
Intercept (b)	**8.3234**		**2.8436**		**12.262**		**67.038**	
Correlation coefficient (r^2)	**0.9993**		**0.9996**		**0.9998**		**0.9999**	

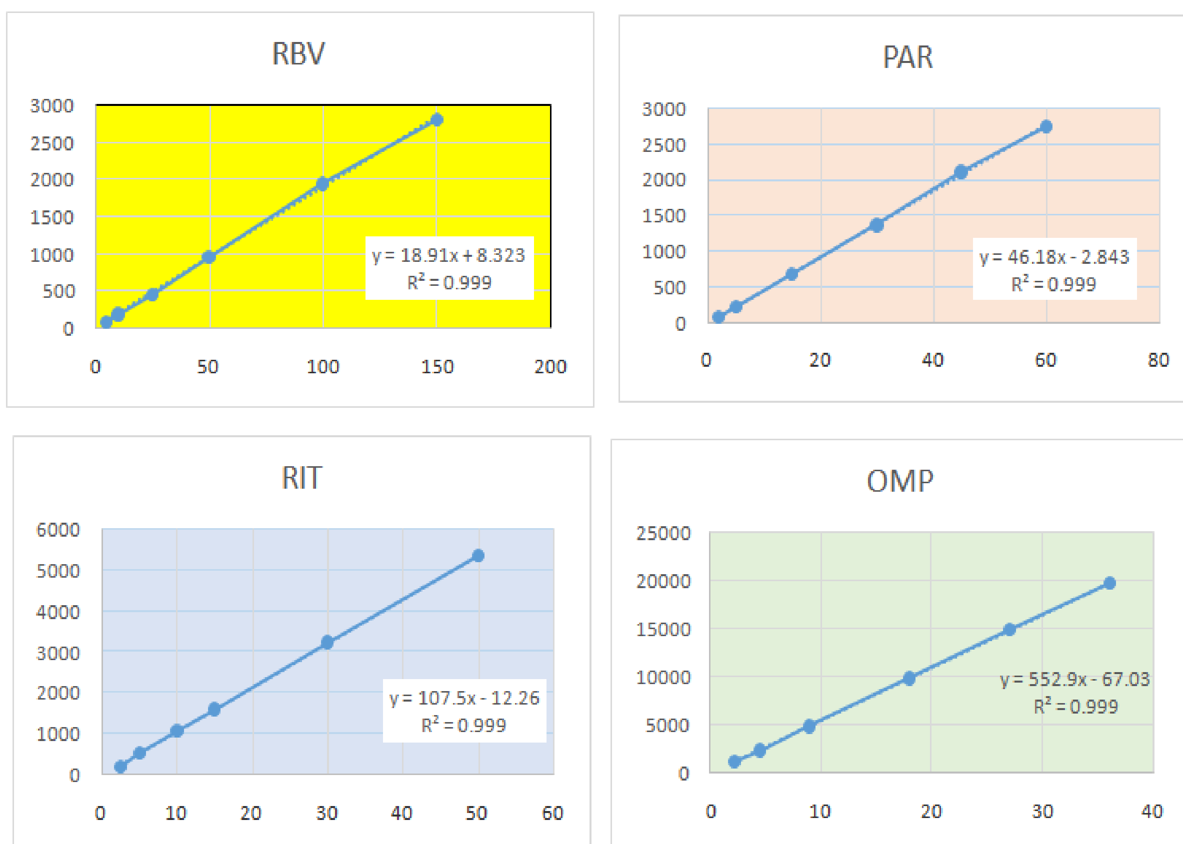


Fig. 3: Calibration curve of RBV (5-150µg/ml), PAR (1.8-60µg/ml), RIT(2.5-50 µg/ml), OMP (2.25-36µg/ml) using the proposed HPLC method with UV detection at 243 nm

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;[15]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 0.36–2.09 and 0.29–1.5, respectively.

Table 2.Results of the intra-day and inter-day precision in the assay of RBV, PAR, RIT and OMP 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] %
RBV	***25***	24.58	98.35±0.24;1.00	25.06	100.24±0.12; 0.50
	50	51.13	102.01± 0.26; 0.51	50.7	101.42± 0.4; 0.8
	100	101.43	101.43± 0.24; 0.23	100.69	100.69±0.8; 0.79
PAR	***5***	4.91	98.33±0.07;1.5	4.98	99.66±0.06;1.3
	15	14.9	99.36±0.15; 1.5	15.01	100.05±0.11; 0.74
	30	30.01	100.02±0.17; 0.59	30.15	100.52±0.08; 0.29
RIT	***10***	9.95	99.5±0.06;0.66	10.02	100.24±0.09; 0.92
	15	15.05	100.33±0.18;1.19	15.11	100.79±0.14;0.96
	30	30.41	101.38±0.37;1.2	30.36	101.2± 0.40; 1.5
OMP	***3***	3.02	100.55±0.06;2.09	3.03	101.2± 0.03; 1.14
	6	6.06	101.08±0.09;1.5	6.08	101.4± 0.06; 1.14
	9	9.11	101.26±0.05;0.36	9.12	101.34± 0.04; 0.49

[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, 5, 5 and 3 $\mu g.mL^{-1}$ of RBV, PAR, RIT and OMPrespectively**.** 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 variationsintroduced 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), in mobile phase composition (± 1%) and in pH value (±0.1). The relative standard deviation (RSD) resultswere shown in (Table 5, Table 6 and Table 7)

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 noise level. 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 were found to be 1.2, 0.8, 0.7 and 0.06 μg/ml and LOQ 3.6, 2.4, 2.1 and 0.21 μg/mlfor RBV, PAR, RIT and OMP respectively.

4.7 System suitability test: System suitability tests (SST) are based on the concept that the equipment, electronics, analyticaloperations 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 8). 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 RBV analytical solution exhibited nochanges for at least 10 days when stored refrigerated at 4˚C and for 24 hours when kept at room temperature.PAR, RIT and OMP analytical solutions in acetonitrile exhibited no changes for 14 days when stored refrigerated at 4˚C andfor 36 hours when kept at room temperature. Solutions of the studied compounds in the mobile phase exhibitedno changes for 10 hours when kept at room temperature.

4.9. Analysis of pharmaceutical products:

The validated HPLC method was applied for the determination of RBV, PAR, RIT and OMP in pharmaceuticalpreparation using Copegus®, Qurevo® tablets respectively. Three replicatedeterminations were performed at each concentration level. Satisfactory results were obtained for eachcompound in good agreement with label claims (Table 9)The obtained results were compared statistically byStudent's *t*-test (for accuracy) and variance ratio F-test (for repeatability) with USP official method[17] forRBV&the reported method [13, 14]for PAR, RIT and OMP. 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 9)

V. Conclusion

This study described a simple, specific and reliable HPLC UV method for the assay of antiviral drugs (RBV, PAR, RIT and OMP) 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.

Table 3.Results of the accuracy studies by standard addition technique in the assay ofRBV, PAR, RIT and OMP 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			
RBV	20	5	25	25.06± 0.12	100.24	0.50	0.0024
	20	30	50	50.71± 0.45	101.42	0.88	0.0142
	20	80	100	100.69±0.8	100.69	0.79	0.0069
PAR	5	10	15	15.01± 0.11	100.05	0.74	0.0005
	5	25	30	30.15± 0.08	100.5	0.29	0.0052
	5	40	45	44.79± 1.03	99.53	3.02	-0.0046
RIT	5	5	10	10.02 ± 0.09	100.24	0.92	0.0024
	5	10	15	14.96± 0.2	99.75	1.33	-0.0024
	5	25	30	29.68±1.6	98.93	5.5	-0.0106
OMP	3	3	6	5.96±0.03	99.36	0.58	-0.0060
	3	6	9	8.85± 0.2	98.42	3.03	-0.0157
	3	9	12	11.86± 0.08	98.89	0.68	-0.0110

Table 4: Determination of RBV, PAR, RIT and OMP in laboratory prepared mixtures using the proposed HPLC method

C	*RBV**		C	*PAR**		C	*RIT**		C	*OMP**	
	Peak area	*% recovery*		Peak area	*% recovery*		Peak area	*% recovery*		Peak area	*% recovery*
10	195.04	98.69	**5**	232.09	99.27	**5**	574.98	99.66	**2**	1173.01	100.01
15	295.47	101.18	**15**	691.39	99.38	**8**	866.94	99.37	**4**	2306.97	101.27
20	383.27	99.09	**30**	1384.61	99.72	**10**	1090.11	100.24	**6**	3388.85	100.12
30	574.29	99.71	**45**	2087.67	100.3	**16**	1737.66	100.3	**8**	4471.10	99.55
40	768.57	100.46	**60**	2761.33	99.54	**20**	2157.78	99.78	**12**	6678.33	99.63
mean		**99.75**			**99.85**			**100.1**			**99.77**

	RBV	PAR	RIT	OMP
SD	0.68	0.39	0.28	0.31
RSD	0.68	0.39	0.28	0.31
Variance	0.47	0.15	0.08	0.10

C = Conc. Taken µg/ml and * Average of five independent procedures.

Table 5. Robustness (Flow rate)in the assay of RBV, PAR, RIT and OMP using the proposed HPLC method

	RBV			**PAR**			**RIT**			**OMP**		
Flow rate	**1.05**	**1**	**0.95**	**1.05**	**1**	**0.95**	**1.05**	**1**	**0.95**	**1.05**	**1**	**0.95**
Determination	**Peak area**											
1	951.7	970.2	989.1	1353.4	1371.3	1422.2	1593.4	1607.9	1627.2	9723.2	9901.5	10010.4
2	953.1	971.5	990.8	1382.1	1401.1	1452.9	1583.1	1598.3	1617.5	10058.1	10243.1	10355.1
3	950.9	968.5	986.6	1367.6	1385.6	1436.0	1598.3	1612.8	1632.5	9812.8	9992.7	10102.6
4	943.0	961.3	979.5	1360.9	1377.9	1428.9	1595.5	1610.0	1629.3	9807.3	9987.7	10097.1
5	954.8	972.8	991.3	1358.3	1376.2	1427.1	1573.8	1587.4	1606.9	9936.9	10119.1	10230.4
Mean	**968.91**			**1393.51**			**994643.9**			**10025.37**		
SD	**16.12**			**32.19**			**11178.96**			**175.10**		
RSD	**1.66**			**2.31**			**1.123916**			**1.74**		

Table 6. Robustness (Mobile phase)in the assay of RBV, PAR, RIT and OMP using the proposed HPLC method

	RBV			**PAR**			**RIT**			**OMP**		
Mobile phase	**M_1**	**M**	**M_2**	**M_1**	**M**	**M_2**	**M_1**	**M**	**M_2**	**M_1**	**M**	**M_2**
Determination	**Peak area**											
1	973.0	970.2	1024.5	1422.0	1371.3	1433.0	1627.1	1608.0	1661.0	10089.6	9901.5	10119.3
2	974.4	971.6	1026.0	1453.3	1401.1	1464.2	1617.4	1598.4	1651.1	10437.3	10243.1	10468.4
3	971.7	968.6	1022.8	1436.9	1385.7	1448.1	1632.1	1612.9	1666.2	10182.6	9992.7	10212.6
4	964.1	961.3	1015.7	1428.4	1378.0	1440.0	1629.2	1610.0	1663.1	10177.8	9987.7	10207.4
5	975.1	972.9	1027.4	1427.2	1376.3	1438.2	1606.4	1587.5	1639.8	10311.4	10119.1	10341.8
Mean	**987.93**			**1420.24**			**1627.33**			**10186.15**		
SD	**26.17**			**30.15**			**24.67**			**161.12**		
RSD	**2.65**			**2.12**			**1.52**			**1.58**		

M: Phosphate buffer (P^H=7) :Acetonitrile 25:75 (v/v), M1: 24:76 (v/v) and M2: 26:74 (v/v)

Table 7. Robustness (P^H) in the assay of RBV, PAR, RIT and OMP using the proposed HPLC method

	RBV			**PAR**			**RIT**			**OMP**		
P^H	**6.9**	**7**	**7.1**	**6.9**	**7**	**7.1**	**6.9**	**7**	**7.1**	**6.9**	**7**	**7.1**
Determination	**Peak area**											
1	995.8	970.2	968.4	1383.6	1371.3	1342.4	1625.1	1608.0	1586.9	10129.9	9901.5	9891.6
2	996.1	971.6	969.1	1413.1	1401.1	1371.9	1616.7	1598.4	1577.0	10478.7	10243.1	10232.2
3	993.3	968.6	966.8	1398.9	1385.7	1356.5	1630.8	1612.9	1591.3	10222.5	9992.7	9982.7
4	986.2	961.3	959.5	1390.4	1378.0	1348.5	1627.5	1610.0	1588.4	10217.1	9987.7	9977.1
5	998.5	972.9	971.9	1388.2	1376.3	1347.3	1605.1	1587.5	1566.2	10351.8	10119.1	10109.0
Mean	**976.67**			**1376.90**			**1602.13**			**10122.51**		
SD	**13.41**			**20.99**			**19.21**			**169.69**		
RSD	**1.37**			**1.52**			**1.20**			**1.68**		

Table 8: system suitability testing using the proposedHPLC method

	RBV	**PAR**	**RIT**	**OMP**	**Recommended values**
Retention time (*t*R)(min)	*1.298*	*2.820*	*4.115*	5.786	-
Theoreticalplates(*N*)	2226	5575	6101	8366	The more plates, the better separation efficiency
Capacity factor (*k'*)	0.68	1.17	2.17	3.45	0.5 <*k'* < 10
Tailing factor (*Tf*)	1.5	0.84	0.96	0.81	0.8 <*Tf*≤1.5

Table 9: Statistical comparison between the proposed HPLC method and reported methods for thedetermination of *RBV, PAR, RIT and OMP* in pharmaceutical formulation

Analyte	**Amount taken µg /ml**	**Proposed method**		**Reportedmethods**		**t-test (2.31)***	***F*-test (6.39)***
		Recovery (%)	**RSD%**	**Recovery (%)**	**RSD%**		
RBV	5	101.42	1.2	98.26	0.97	0.24	0.99
	10	100.78		100.64			
	20	99.09		99.58			
PAR	5	99.07	0.84	100.53	1.14	0.30	0.70
	25	100.65		99.11			
	50	99.33		101.39			
RIT	3.3	100.12	0.57	101.19	0.25	0.17	0.34
	16.7	101.03		100.67			
	33.3	99.97		100.92			

OMP	2.25	101.52	0.68	99.23	1.22	0.39	0.74
	4.17	10.1.22		100.85			
	8.33	100.54		101.64			

*Tabulated t and F values at 95 % confidence limit

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