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Research Article

Simultaneous Estimation of Ombitasvir, Paritaprevir and Ritonavir in Tablet Dosage Form by Reverse Phase High-Performance Liquid Chromatography

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Abstract: Ombitasvir/paritaprevir/ritonavir are the newest medicines approved for use in the treatment of hepatitis C virus (HCV) and are available in tablet form as an oral combination. Specifically, these agents are indicated in the treatment of HCV in patients with genotype 1 infection. Due to the therapeutic importance and increased use of Viekira Pak, proper methods for its determination in bulk and pharmaceutical formulations must be developed. The purpose of this work is to develop an accurate and precise RP-HPLC method for the determination of ombitasvir, paritaprevir and ritonavir in bulk and pharmaceutical preparations. Drugs were separated using Inertsil ODS-C18; 5µm (4.6 X 250mm) column using a mobile phase consisting of 0.02M phosphate buffer (pH-4.5): Acetonitrile: Methanol, (50:30:20) (v/v). The retention time was 2.98, 3.77 and 4.70 min for Ombitasvir, Paritaprevir, Ritonavirrespectively and total run time was 10 min. at a flow rate of 1.0 mL/ min and the detection wavelength was 262 nm. The linearity was observed in the range of 15-45µg/ mL for ombitasvir, paritaprevir and ritonavir with a correlation coefficient of 0.9903, 0.9996 and 0.9998 respectively. The proposed method was validated for its linearity, accuracy, precision and robustness. This method can be employed for routine quality control analysis of Ombitasvir, Paritaprevir and Ritonavir in tablets. The LOD and LOQ values were found to be 1.8, 0.29 and 0.69 µg/ mL and 5.7, 0.90 and 2.10 µg/ mL for Ombitasvir, Paritaprevir and Ritonavir, respectively. The proposed method was successfully applied for the determination of ombitasvir/paritaprevir/ritonavir tablets, without interference from the excipient peaks. Hence, the method can be applied for the routine quality control analysis of the studied drugs, either in bulk or dosed forms.

Keywords: Hepatitis C virus, Dosage forms, RP- HPLC; Validation, Quality control

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1. INTRODUCTION

More than 170 million people worldwide are infected with Hepatitis-C virus (HCV). The leap forward in HCV treatment wasn't until the discovery of directly acting antiviral drugs (DAADs) few years ago. According to the American Association for the Study of Liver Diseases and Infectious Diseases Society of America (AASLD-IDSA), the estimated average cost of regimens using DAADs ranges from 26,400 to 94,500USD per patient [1]. For example, the lowest identified price for treatment course for sofosbuvir alone in a developing country was $ 900 in Egypt [1]. This can demonstrate the massive production size of HCV regimens all over the world which will require development of simple, fast and economic methods of analysis for determination of HCV drugs in pharmaceutical quality control (QC) and research laboratories.Technivie® (paritaprevir, ritonavir plus ombitasvir) was approved by FDA in 2015, as single dose combination therapy [2]. Ombitasvir (OMB) is a hepatitis C virus NS5A inhibitor, paritaprevir (PAR) is a hepatitis C virus NS3/4A protease inhibitor, while ritonavir (RIT) is a CYP3A inhibitor. This combination showed activity against HCV genotype-4 with sustained virologic response of 100% [3]. HCV genotype-4 has been considered the most difficult-to-treat genotype which accounts for more than 90% of the HCV infections in Egypt alone (about 14.1% of Egyptian population)[3]. Ombitasvir, paritaprevir and ritonavir (Figure:1.0) drugs were combined in a single dosage form (film-coated tablet) in the brand name of TECHNIVIE for the treatment of Hepatitis-C. These three drugs will act against the hepatitis-C virus (HCV) in three different mechanisms.Ombitasvir, produces its antiviral activity by inhibiting the HCV nonstructural protein (NS) 5A. OMTR chemically designated as dimethyl ([(2S,5S)-1-(4-tert-butyl phenyl) pyrrolidine-2,5diyl] bis {benzene -4, 1 diylcarbamoyl (2S) pyrrolidine -2, 1-diy l[(2S) -3-methyl -1-oxobutane -1, 2diyl]}) biscarbamate hydrate with molecular weight of 894.11 g/mole (Fig. 1)[1-4]. Paritaprevir chemically designated as (2R, 6S, 12Z, 13aS,14aR, 16aS)-N-(cyclopropylsulfonyl)-6-{[(5- methyl-2- pyrazinyl) carbonyl] amino}-5, 16 -dioxo-2-(6-phenanthridinyloxy) -1, 2, 3, 6, 7, 8, 9, 10, 11, 13a, 14, 15, 16, 16a -tetradecahydrocyclopropa[e] pyrrolo[1,2-a] [1,4] diazacyclopentadecine -14a(5H)-carboxamide with molecular weight of 765.89 g/mole (Fig. 1). TECHNIVIE inhibits the NS-3/4A serine protease of HCV. Subsequently, replication of HCV genetic components and translation into a single polypeptide, NS-3, and its activating cofactor NS-4A are accountable for splitting it into the succeeding nonstructural and structural proteins essential for assembly into a mature virus, viz., NS-3, NS-4A, NS-4B, NS-5A, and NS-5B. By inhibiting viral protease NS-3/4A, PRTR, therefore, prevents viral replication and function[1-4]. Ritonavir RTNR is an anti-retroviral medication utilized along with other medications to treat the human immunodeficiency virus. This combination treatment is known as highly active antiretroviral therapy (HAART). At low doses of RTNR, it is utilized with other protease inhibiting agents and useful in combination with other Hepatitis-C medications. It is chemically designated as 1, 3-thiazol- 5-ylmethyl N- [(2S, 3S, 5S) -3- hydroxy- 5-[(2S)- 3- methyl -2-{[methyl({[2-(propan-2-yl)-1,3-thiazol-4-yl] methyl}) carbamoyl] amino} butanamido]-1,6 diphenylhexan-2-yl] carbamate with molecular weight of 720.946 g/mole [4]. Literature review reveals that few chromatographic methods were reported for simultaneous determination of Ombitasvir, Paritaprevir and Ritonavir. As per literature review, combination of drugs was estimated by chromatographic techniques like HPLC [3-9, 12, 18, 20-25], UPLC [17], HPTLC [13, 21], LC-MS/MS[14-16, 19, 24], Capillary electrophoresis [26, 27] have been reported for the estimation of Ombitasvir, Paritaprevir and Ritonavir. To our knowledge, only four research methods were reported using HPLC. The purpose of this research reported here was to develop a new RP-HPLC for separation and quantification of three drugs in bulk and formulations.

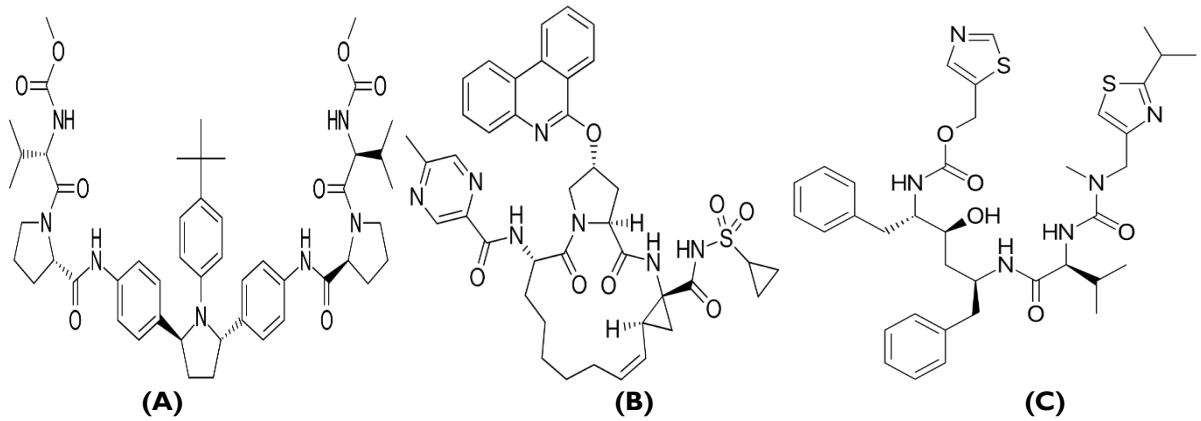

Fig.1: Chemical structures of (A) Ombitasvir (B) Paritaprevir (C) Ritonavir

2. MATERIALS AND METHODS

2.1 Chemicals and Reagents

Hetero Pharma. Pvt. Ltd., Hyderabad, India, graciously sent a gift sample of pure Working Standards of known efficacy of Ombitasvir, Paritaprevir, and Ritonavir. The marketed sample contained ombitasvir 25 mg, paritaprevir 150 mg, and ritonavir 100 mg and was obtained from a local pharmacy. Orthophosphoric Acid (OPA) from HI Media Laboratories Pvt. Ltd., Water, Methanol, Acetonitrile, Trimethylamine from Merk, and Potassium dihydrogen phosphate from Thermo Fisher Scientific India Pvt. Ltd. were used as reagents.

2.2 Instrumentation

The HPLC system (Agilent HPLC 1200 Infinity LC Specifications) had a pump (Agilent LC20AT) configured with Ezchrom Elite Software and a rheodyne injector. The

detector was made up of a UV/VIS (UV-2489) type that worked at a wavelength of 262 nm. At room temperature, an Inertsil CN-3 column was employed.

2.3 Preparationof Standard Stock Solution

Each 10mg of Ombitasvir, Paritaprevir and Ritonavir were transferred to 100 ml volumetric flask, dissolved and diluted to the mark with methanol. The stock solutions were further diluted with mobile phase to obtain a solution of 100 µg/mL.[7,8]

2.4 Test Sample Preparation

Tablet powder equivalent to 10mg of Ombitasvir, Paritaprevir and Ritonavir was weighed from a pooled powder of twenty tablets and transferred into a 10 ml volumetric flask, few ml of methanol was added and sonicated for 10 min. The volume was made up to mark with methanol and the sample solution was filtered and used for further dilution.

2.5 Optimization of HPLC Method

The HPLC procedure was optimized with a view to develop a simultaneous assay method for Ombitasvir, Paritaprevir and Ritonavir, respectively. The mixed standard stock solution (100 µg/mL) was injected. For RP-HPLC method optimization of different ratios of methanol and water were tried, but it was found that drugs were separated using Inertsil ODS-C_{18}; 5µm (4.6 X 250mm) column using a mobile phase consisting of 0.02M phosphate buffer (pH-4.5): Acetonitrile: Methanol, (50:30:20) (v/v). The retention time was 2.98, 3.77 and 4.70 min for Ombitasvir, Paritaprevir, Ritonavir, and total run time was 10 min at a flow rate of 1.0 mL/ min and the detection wavelength was 262 nm gives ideal system suitability parameters like retention time, Peak area, USP plate count, USP Tailing factor, resolution, were depicted in Table 1.

Table 1. System suitability parameters

Name of Peak	Retention time(min)	*PeakArea	*USP plate count	*USP Tailing	Resolution
Ombitasvir	2.980	787012	7533	1.019	-
Paritaprevir	3.779	11706368	11323	1.126	3.54
Ritonavir	4.701	14965799	9856	1.096	2.54
Acceptancecriteria	-	NA	More than 2000	Less than 2	More than 2

n=6 (Average of six determinations)

3. METHOD VALIDATION

After the development of RP-HPLC method for the estimation of drug in a dosage form, validation of the method was performed as per ICH guidelines.[28-30]

3.1 System Suitability Parameters

System suitability tests are used to verify that the resolution and reproducibility of the system are adequate. Several suitability parameters, including the capacity factor, selectivity, efficiency, resolution and tailing factor were calculated, as shown in Table 1. The peaks obtained were sharp and showed clear baseline separation.

3.2 Linearity

A series of standard solutions were prepared in the range of 15µg/mL-45µg/mL containing Ombitasvir, Paritaprevir and Ritonavir standards and injected. A plot of average peak area versus the concentration (r^2) in µg/mL is made and from this the correlation coefficient, y-intercept and slope of the regression line were calculated. The calibration data and calibration curve shown in Table No.2 and Fig No. 2, 3 and 4.

Table 2: Linearity data of Detector Response

Concentration range	15-45µg/mL	15-45µg/mL	15-45µg/mL
Slope (m)	27167	22409	27471
Analyte name	Ombitasvir	Paritaprevir	Ritonavir
Correlation coefficient (r^2)	0.9903	0.9996	0.9998

n=6 (Average of six determinations)

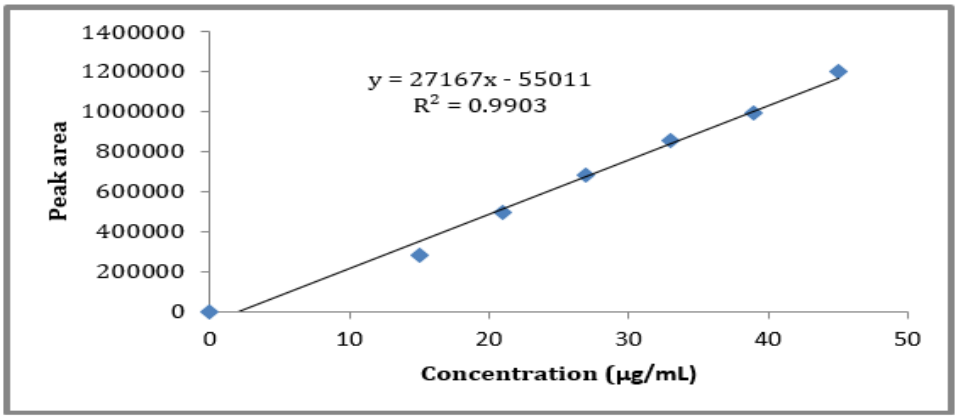

Fig 2: Linearity Plot of Ombitasvir

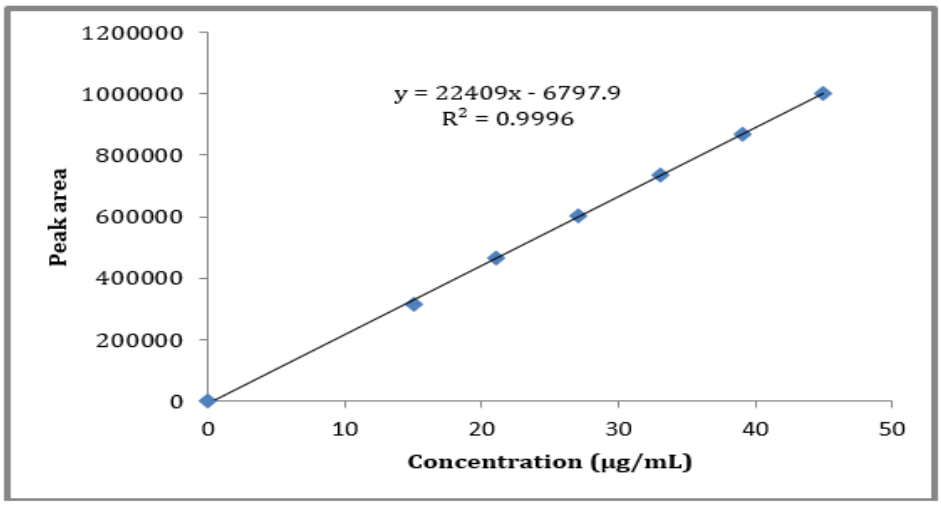

Fig 3: Linearity Plot of Paritaprevir

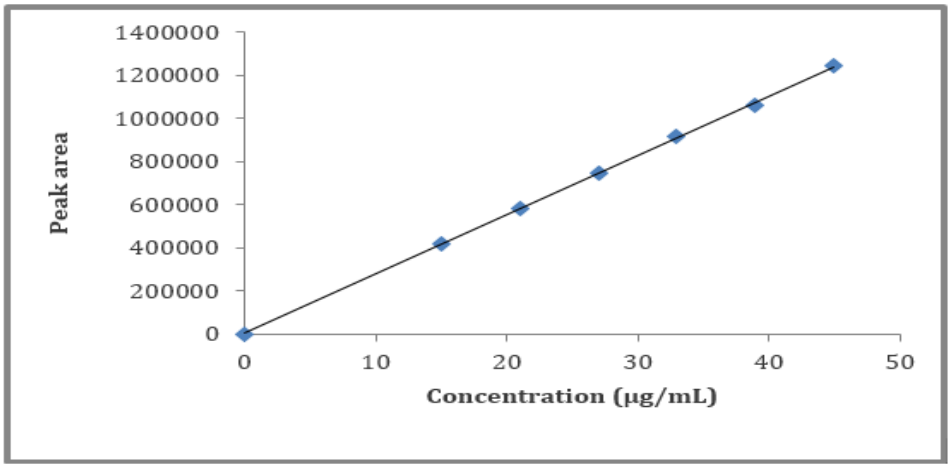

Fig 4: Linearity Plot of Ritonavir

3.3 Precision

The precision of the test procedure was evaluated by injecting the six test solutions (33µg/ml). The Relative Standard Deviation of six injections was calculated. The result of Precision studies is given in Table No.03.

Table 3 Precision for Ombitasvir, Paritaprevir and Ritonavir

	Intraday precision					
	Test conc: 33µg/mL					
Mean	838353	99.29	733562	98.21	908979	98.33
SD	11574.29	0.58	1228.86	1.11	4917.51	0.82
% RSD	1.38	0.58	0.17	1.13	0.54	0.83
Inter day precision						
Mean	840680	97.82	729415	97.82	908947.50	98
SD	15630.89	1.37	2018.62	1.06	5771.51	1.02
% RSD	1.86	1.40	0.28	1.08	0.63	1.04

SD= Standard deviation, *RSD= Relative standard deviation

3.4 Specificity

Specificity is the ability of a method to discriminate between the analyte (s) of interest and other components that are present in the sample. A study of placebo interference from excipients was conducted. Equivalent weight of placebo taken as per the test method and placebo interference was conducted in duplicate [28-30].

3.5 Accuracy

To validate whether the test method can accurately quantify Ombitasvir, Paritaprevir and Ritonavir, prepare samples in three times for higher and lower levels, in triplicate for other levels by spiking Ombitasvir, Paritaprevir and Ritonavir of active material with equivalent amount of placebo and perform CU as per test procedure. Samples were prepared at levels 80% and 120% of the target assay concentration i.e. 100% level. Table no 4 shows the results for accuracy of Ombitasvir, Paritaprevir and Ritonavir.

Table 4: Accuracy results									
	Ombitasvir			Paritaprevir			Ritonavir		
Mean	SD	%RSD	Mean	Mean	SD	%RSD	Mean	SD	%RSD
98.54	0.28	0.29	98.54	100.41	1.86	1.85	103.72	1.57	1.52
101.02	1.70	1.68	101.02	102.95	1.31	1.28	98.88	0.40	0.40
100.03	1.08	1.07	100.03	99.77	0.42	0.42	101.97	1.77	1.73

SD= Standard deviation, *RSD= Relative standard deviation

3.6 Limit of Detection (LOD) And Limit of Quantitation (LOQ)

The limit of detection and limit of quantitation were determined by diluting known concentrations of each drug until signal to noise ratios of approximately 3:1 and 10:1 were obtained, respectively. The LOD and LOQ of Ombitasvir, Paritaprevir and Ritonavir, which represent the capability of the method to detect and quantify low concentrations, were 1.8, 0.29 and 0.69 µg/ mL and 5.7, 0.90 and 2.10 µg/ mL, respectively. This result indicates the capability of the method to detect and quantify low concentrations. The results are summarized in Table 2.

Table 5: LOD and LOQ			
Analyte name	Ombitasvir	Paritaprevir	Ritonavir
LOD	1.899	0.297	0.693
LOQ	5.753	0.901	2.101

4. ASSAY

Six replicates of the sample solutions were injected for quantitative analysis. The amounts of Ombitasvir, Paritaprevir and Ritonavir estimated were found to be 99.52, 102.0 and 99.02 respectively. A good separation and resolution of both drugs indicate that there was no interference from the excipients commonly present in pharmaceutical formulations. This showed that the estimation of dosage form was accurate within a given acceptable level of 95% to 105%. The amount of Ombitasvir, Paritaprevir and Ritonavir per tablet were calculated by extrapolating the value of area from the calibration curve. Analysis procedure was repeated six times with tablet formulation. The result formulation was reported in Table 5.

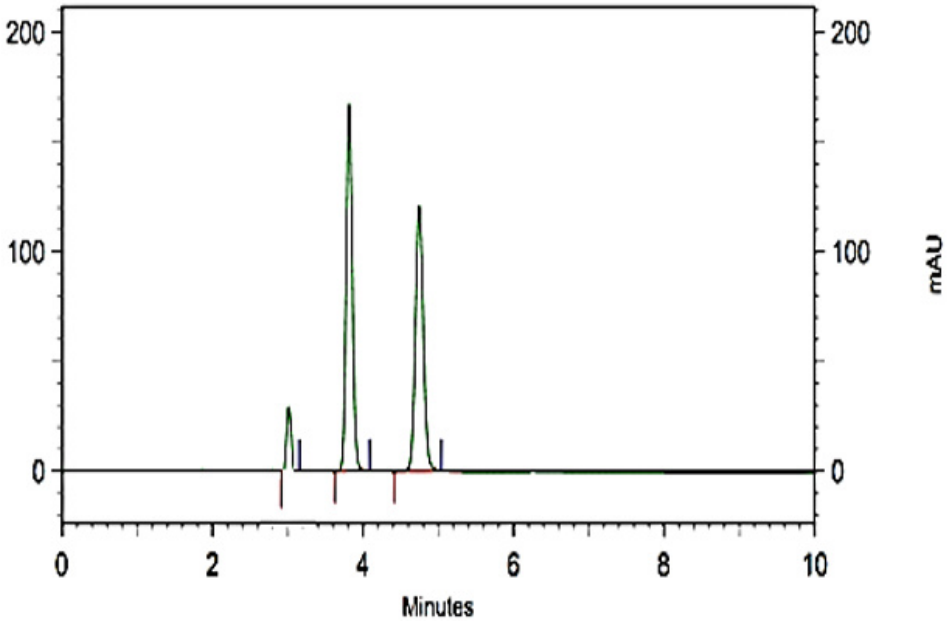

Fig 5: Chromatogram of test sample

5. DISCUSSION

When we compare the proposed HPLC method to other reported methods; HPTLC [13, 21], LC-MS/MS[14-16, 19, 24], Capillary electrophoresis [26, 27] have lower solvents and energy consumptions than the conventional HPLC methodologies. However, UPLC [17]methods have higher maintenance costs due to the shorter column life-time and requirement of special instrumentation especially those coupled with MS-MS detectors[14-16, 19, 24] and due to this UHPLC is usde in economic establishments like pharmaceutical quality control laboratories and is limited and not widespread.

5.1 Method development and optimization of chromatographic conditions

The method was developed based upon the experience obtained from the HPLC method previously developed for the analysis of Ombitasvir/paritaprevir/ritonavir. The previous experiment was performed using a mobile phase consisting of acetonitrile and phosphate buffer (pH 3) at a

ratio of 60:40, v/v. For the separation of analytes from mixtures containing Ombitasvir/paritaprevir/ritonavir, methanol and acetonitrile were used as organic modifier, peak symmetry and optimum pressure was obtained by using acetonitrile. Various ratios of acetonitrile and phosphate buffer solutions and different mobile phase pH values were tested using a C18 (150 × 4.5 mm, 3.5 µm) column, higher acetonitrile ratio resulted in shorter retention times of drugs Using this mobile phase ratio best results were obtained in terms of peak symmetry, selectivity and analysis time for drugs and the results are shown in Fig. 2. The pKa values of the studied drugs are reported in the literature as 2.8 for ritonavir, 2.5 for Ombitasvir, and 4.6 and for paritaprevir, which has two pKas. Therefore, the pH of the mobile phase was adjusted to 4.5. A wavelength of 262 nm was selected for the simultaneous determination of three analytes with high sensitivity. Moreover, the strength of the phosphate buffer solution (10–100 mM) was evaluated. Good resolution and reasonable retention times were observed for all of the drugs when 0.02M phosphate buffer (pH-4.5): Acetonitrile: Methanol, (50:30:20) (v/v) was delivered at a flow rate of 1 ml/min.

5.2 Method validation

The aim of the present work was to develop a rapid, precise, accurate and cost effective HPLC method for simultaneous estimation of Ombitasvir, Paritaprevir and Ritonavir in its pharmaceutical tablet formulation, using the reverse phase (RP) C18 column with UV detection and validate develop method as per US FDA guideline and ICH guideline [28-30]. The method was found to be specific. There was no peak found in blank sample chromatogram at Ombitasvir, Paritaprevir and Ritonavir peak retention times. The Correlation Coefficient, r^2 of Ombitasvir, Paritaprevir and Ritonavir was found to be 0.9903, 0.9996 and 0.9998 respectively. The Percentage Relative standard deviation of individual area response of six replicate injections for Ombitasvir, Paritaprevir and Ritonavir was found to be 1.38, 0.17 and 0.54 respectively. The Percentage Relative standard deviation of areas of six replicate injections for Ombitasvir, Paritaprevir and Ritonavir standard were found to be within limits. The tailing factor for Ombitasvir, Paritaprevir and Ritonavir peaks was found to be 1.01, 1.12 and 1.09 respectively. The tailing factor for Ombitasvir, Paritaprevir and Ritonavir peaks was found to be within limits (Less than 2). The number of theoretical plates for Ombitasvir, Paritaprevir and Ritonavir were found to be 7533, 11323 and 9856 respectively. The resolution was found to be 3.54 and 2.54 respectively(Table-1). Interference was not observed with the standard peaks and the chromatograms of Standard and Sample were identical with the same retention time.The mean % Recovery of Ombitasvir, Paritaprevir and Ritonavir were found to be within limits at each level. The % RSD of recovery of Ombitasvir, Paritaprevir and Ritonavir from the three sample preparations was found to be 1.01, 1.18 and 1.21. The proposed HPLC method has several advantages compare to published methods [3-9, 12, 18, 20-25]. Firstly, the same mobile phase could be stored and used several times for several elutions (i.e. Recycled). Secondly, the simultaneous processing of sample and standard under the exact same conditions gave rise to improved analytical precisions and accuracies. Additionally, the low cost of HPLC method encourages its use as an analytical tool. No interference was observed from the co-formulated substances compare to reported methods by HPLC.[3-9, 12, 18, 20-25]

Table 5: Assay of test sample

Test formulation (Tablet)	Label claimed (mg/tab)		
	OT	**RT**	**PT**
Ombitasvir, Paritaprevir, Ritonavir tablets	12.5	75	50
	Conc found (mg)		
	12.44	76.2	49.51
	%Assay		
	99.52	102	99.02

6. CONCLUSION

RP-HPLC method was developed and validated as per ICH guidelines. It can be concluded that the method is specific for the estimation of Ombitasvir, Paritaprevir, and Ritonavir in the pharmaceutical dosage form. The high accuracies and precisions of the assays obtained, taken together with the low solvent consumption and replacing hazardous solvents made this method eligible for use in different research and pharmaceutical quality control laboratories for the determination of these drugs. The method has a linear response in the stated range and is accurate and precise. Statistical analysis proves that the method is suitable for the analysis of Ombitasvir, Paritaprevir, and Ritonavir as bulk drugs drug and in the pharmaceutical formulation without any interference from the excipients.

7. ACKNOWLEDGEMENTS

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8. AUTHORS CONTRIBUTION STATEMENT

Gollapalli Nagaraju carried out the experiment and wrote the manuscript with support from Ritesh Agarwal, Rama Rao Nadendla. Prof. Rama Rao Nadendla helped supervise the project. Ritesh Agarwal, Rama Rao Nadendla conceived the original idea. Rama Rao Nadendla supervised the project.

9. CONFLICT OF INTEREST

Conflict of interest declared none.

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