Contents lists available at UGC-CARE

International Journal of Pharmaceutical Sciences and Drug Research

[ISSN: 0975-248X; CODEN (USA): IJPSPP]

journal home page : <http://ijpsdr.com/index.php/ijpsdr>

Research Article

Stability-Indicating Method Development and Validation for Simultaneous Estimation of Ombitasvir, Paritaprevir, and Ritonavir in Formulation by Ultra Performance Liquid Chromatography

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ARTICLE INFO

Article history:

Received: 15 April, 2020

Revised: 10 August, 2020

Accepted: 26 August, 2020

Published: 30 September, 2020

Keywords:

Ombitasvir, Paritaprevir, Ritonavir, Stability, Ultra performance liquid chromatography (UPLC), Validation.

DOI:

10.25004/IJPSDR.2020.120505

ABSTRACT

The present research work aimed to develop a sensitive, precise, and robust stability-indicating ultra performance liquid chromatography (UPLC) method for the simultaneous estimation of ombitasvir (OMTR), paritaprevir (PRTR), and ritonavir (RTNR) in formulations. The chromatographic separation of a mixture of OMTR, PRTR, and RTNR was attained in the isocratic method utilizing a mobile phase of 0.01N potassium dihydrogen orthophosphate (pH 5.3) and methanol in the proportion of 60:40 %v/v, utilizing a BEH C18 column which has dimensions of 100 × 3 mm, 1.7μm particle size, and the flow rate of 0.3 mL/min. The detection system was monitored at 252 nm wavelength maximum with 0.2 mL injection volume. The retaining time for OMTR, PRTR, and RTNR was achieved at 1.765, 2.192, and 1.326 minutes, respectively. OMTR, PRTR, and RTNR and their combined drug formulation were exposed to thermal, acidic, oxidative, photolytic, and alkaline conditions. The present method was validated based on the International Council for Harmonisation (ICH) guidelines for specificity, accuracy, sensitivity, linearity, and precision. The developed method was highly sensitive, rapid, precise, and accurate than the earlier reported methods. The total run time was decreased to 3 minutes; hence, the technique was more precise and economical. Stability studies were directed for the suitability of the technique for degradation studies of OMTR, PRTR, and RTNR. The projected method can be utilized for routine analysis in the quality control department in pharmaceutical trades.

INTRODUCTION

Ombitasvir (OMTR), paritaprevir (PRTR), and ritonavir (RTNR) 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. OMTR 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-2,5-diyl) bis {benzene-4, 1 diylcarbamoyl (2S) pyrrolidine-2, 1-diyl} [(2S)-3-methyl-1-oxobutane-1, 2-diyl])) biscarbamate hydrate with molecular weight of 894.11 g/mole (Fig. 1).^[1-3]

PRTR 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). PRTR

Fig. 1: Structures of a) OMTR; b) PRTR; c) RTNR

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Relevant conflicts of interest/financial disclosures: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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is powerful 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.^[3,4]

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 anti-retroviral therapy (HAART). At low doses of RTNR, it is utilized with other protease inhibiting agents and useful in combination with other hepatitis-C medicaments. It is chemically designated as 1, 3-thiazol- 5-ylmethyl *N*-[(2*S*, 3*S*, 5*S*) -3- hydroxy- 5-[(2*S*) -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 (Fig. 1).^[5,6]

The literature review discloses that a very few liquid chromatography (LC) with tandem mass spectrometry (LC-MS/MS),^[7] high performance liquid chromatographic,^[8-13] and spectrophotometric^[14] techniques have been reported for the estimation of OMTR, PRTR, and RTNR. Based on the reported high performance liquid chromatography (HPLC) methods, there is a need to develop a rapid, sensitive reversed-phase UPLC method for simultaneous estimation of OMTR, PRTR, and RTNR, in bulk and formulations.

MATERIALS AND METHODS

Chemicals and Reagents

The standard components of OMTR, PRTR, and RTNR were provided as a gift sample from Spectrum Pharma Research Solutions, Hyderabad. Technivie tablets labeled to contain OMTR 12.5 mg, PRTR 75 mg, and RTNR 50 mg were procured from the local market. HPLC grade methanol was obtained from A. B. Enterprises, Mumbai, India. Orthophosphoric acid was bought from Ranchem, Mumbai, India. HPLC grade water was processed by utilizing the Milli-Q Millipore water purification system used during the method development.

Liquid Chromatography

Chromatographic system of Waters UPLC system furnished with photodiode array detector, auto-sampler, and BEH C18 column, which have dimensions of 100 × 3 mm, 1.7 m particle size. The output signal was monitored and integrated, utilizing water Empower-2.0 software. The isocratic mobile consisting of 0.01N potassium dihydrogen orthophosphate (pH 5.3) and methanol in the proportion of 60:40 %v/v, pumped through the BEH C18 (100 × 3 mm, 1.7 m) column at a fixed flow of 0.3 mL/min. The injection volume of 0.2 mL was utilized to measure the chromatograms at 252 nm as the wavelength maximum in the detection system.

Preparation of Buffer

Accurately weighed 1.36 grams of potassium dihydrogen orthophosphate in a 1,000 mL of the volumetric flask, added about 900 mL of Milli-Q water added and degas to sonicate, and finally made up the volume with water. Then, pH adjusted to 5.3 with a dilute orthophosphoric acid solution.

Preparation of Standard Stock Solution

Accurately weighed and transferred 18.75 mg of PRTR, 12.5 mg of RTNR, and 3.125 mg of OMTR working standards into a 25 mL clean dry volumetric flasks, added 10 mL of diluent, sonicated for 10 minutes, and made up to the final volume with diluent [water: methanol (50:50)] to get 750 µg/mL of PRTR, 500 µg/mL of RTNR, and 125 µg/mL of OMTR.

Preparation of Sample Solution

Twenty tablets were weighed and calculated the average weight of tablets, and then the weight equivalent to one tablet was transferred into a 100 mL volumetric flask containing 50 mL of diluent and sonicated for 25 minutes. Further, the volume made up with diluent and subjected for filtration by HPLC filters (750 µg/mL of PRTR, 500 µg/mL of RTNR, and 125 µg/mL of OMTR). From the filtrate, 1 mL solution was pipetted out into a 10 mL volumetric flask and made up to 10 mL with diluent to get 75 µg/mL of PRTR, 50 µg/mL of RTNR, and 12.5 µg/mL of OMTR.

Analytical Method Validation

The developed method for OMTR, PRTR, and RTNR was subjected for validation for the parameters, like the limit of detection (LOD), the limit of quantification (LOQ), linearity, robustness, precision, system suitability, and accuracy as per the guidelines of ICH.^[15-18]

RESULTS

Optimized Chromatographic Conditions

After systematic trials with different mobile phase compositions and other parameters involved in the technique, the following chromatographic conditions were employed:

Mobile Phase

Buffer: methanol (60:40 %v/v)

Flow Rate

0.3 mL/min

Column

BEH C18 100 × 3 mm, 1.7 m

Detector Wavelength

252 nm

Column Temperature

30°C

Injection Volume

0.2 mL

Run Time

3 minutes

Diluent

Water:methanol (50:50)

Specificity

It is the ability of a method to unequivocally evaluate the analyte components in the presence of other components, like impurities, degradants, and excipients, etc., expected to be present. This parameter was estimated by injecting and evaluating the blank, placebo, standard and sample solutions, and chromatograms.^[15,16] Chromatograms of blank, placebo, and sample solution shown no peaks at the retaining time of OMTR, PRTR, and RTNR peaks. The chromatograms of OMTR, PRTR, and RTNR of standard, blank, formulation, and placebo are represented in Fig. 2.

Linearity

Aliquots of 0.25, 0.5, 0.75, 1, 1.25, and 1.5 mL of standard stock solution were pipetted out from the standard stock solution of concentration 750 µg/mL of PRTR, 500 µg/mL of RTNR, and 125 µg/mL of OMTR, and made up to 10 mL mark with diluent. The resulting solutions came into 18.75 to 112.5 µg/mL of PRTR, 12.5 to 75 µg/mL of RTNR, and 6.25 to 37.5 µg/mL of OMTR concentration range. The resulting linearity solutions were infused into a chromatographic system, and from the chromatograms, the linearity graph was plotted by taking the peak area on Y-axis and concentration on X-axis.^[17,18] The calibration graphs were shown in Table 1 and Figs 3 to 5, and all findings were within limits.

System Suitability

Six replicates of the standard reference solution were processed and infused to perform the system suitability parameter, and the resulting chromatograms peak area,

Fig. 2: Chromatograms of A) Blank; B) Placebo; C) Standard; D) Formulation

Fig. 3: Linearity of RTNR

Fig. 4: Linearity of OMTR

Table 1: Calibration curve data of OMTR, PRTR, and RTNR

PRTR		RTNR		OMTR	
Concentration (µg/mL)	Peak area	Concentration (µg/mL)	Peak area	Concentration (µg/mL)	Peak area
18.75	264,180	12.5	81,636	6.25	49,632
37.5	535,644	25	164,776	12.5	95,550
56.25	814,159	37.5	245,348	18.75	140,564
75	1,052,124	50	329,483	25	187,293
93.75	1,304,536	62.5	406,730	31.25	232,810
112.5	1,542,961	75	486,017	37.5	284,512
Regression equation					
y = 13,764x + 13,436		y = 6,494.1x + 1,325.8		y = 7,495.1x + 947.85	
Correlation coefficient (R2)					
0.9993		0.9999		0.9997	

Table 2: OMTR, PRTR, and RTNR system suitability results

S. No.	Peak name	Peak area	Retention time	Plate count	Resolution	Tailing
1.	RTNR	322,068	1.331	3,637	-	1.15
2.	OMTR	196,787	1.783	5,770	4.9	1.31
3.	PRTR	903,096	2.212	7,291	4.2	1.32

Table 3: Limit of detection and limit of quantification results

Parameter	Measured concentration ($\mu\text{g/mL}$)		
	OMTR	PRTR	RTNR
LOD	0.34	0.48	0.1
LOQ	1.03	1.44	0.29

Table 4: System precision data

S. No.	Peak area response of analytes		
	OMTR	PRTR	RTNR
1	195,011	900,762	326,623
2	196,240	908,579	323,604
3	198,455	908,664	322,068
4	196,312	903,595	323,068
5	199,634	903,033	320,916
6	196,787	903,096	325,455
Average	197,073	904,622	323,022
STDV	1,677.8	3,249.7	2,117.1
% RSD	0.9	0.4	0.7

STDV: Standard deviation; RSD: Relative standard deviation

Table 5: Method precision results

S. No.	Peak area response of drugs		
	OMTR	PRTR	RTNR
1	198,386	905,817	328,929
2	196,022	905,698	320,925
3	195,669	913,631	325,056
4	196,916	907,149	323,527
5	197,400	900,497	324,498
6	197,831	901,256	324,874
Average	197,037	905,675	324,635
STDV	1,048.6	4,729.3	2,595.5
% RSD	0.5	0.5	0.8

STDV: Standard deviation; RSD: Relative standard deviation

retention time, resolution, plate count, and tailing were measured. The system suitability parameter findings are shown in Table 2, and related chromatograms are given in Fig. 2(C).

Limit of Detection (LOD) and Limit of Quantification (LOQ)

The LOD and LOQ parameters for OMTR, PRTR, and RTNR were calculated to form the linear regression equation.^[16] Linearity values, graphs, and regression equations were obtained from the linearity study,

Fig. 5: Linearity of PRTR

and the LOD and LOQ values are represented in Table 3.

Precision

Analytical method precision is defined as the closeness of agreement between the replicate measurements of the analyte. It is expressed as the percentage coefficient of correlation or relative standard deviation (RSD) of the replicate measurements.

System Precision

Working standard preparation of 0.2 μL solution was infused six times into the chromatographic system, and chromatograms were obtained. % RSD of the peak area was calculated. The findings of system precision are shown in Table 4.

Method Precision

Working sample solutions of 0.2 μL were infused six times into the chromatographic system, and chromatograms were obtained. The % RSD of the assay result of six preparations was determined. The findings achieved for assay were represented in Table 5.

Intermediate Precision

Working standard preparation of 0.2 μL was infused six times test preparations into the chromatographic system, and chromatograms were obtained. The % RSD was evaluated for peak areas. The findings of the intermediate precision study are represented in Table 6.

Accuracy

A known amount of OMTR, PRTR, and RTNR at each three concentration levels of 50, 100, and 150% was added to a pre-analyzed sample solution and injected in triplicate

at each level into the chromatographic system.^[15,16] The mean percentage recovery of OMTR, PRTR, and RTNR at each level was estimated. The findings are represented in Table 7.

Robustness

Working standard solution prepared as per test method was infused into the chromatographic system at variable conditions, such as, the flow rate at ± 0.1 mL/min, mobile organic phase composition by $\pm 10\%$, and column temperature by $\pm 5^\circ\text{C}$. The robustness study parameter results, like peak area, retention time, plate count, and tailing factor, were within limits.

Table 6: Intermediate precision results

S. No.	Peak area response of drugs		
	OMTR	PRTR	RTNR
1	180,926	862,024	306,500
2	184,329	872,445	309,802
3	181,999	865,006	300,803
4	180,541	871,249	307,626
5	184,666	864,840	304,839
6	181,777	884,123	302,310
Average	182,373	869,948	305,313
STDV	1,733.7	8,027	3,361.3
% RSD	1	0.9	1.1

Forced Degradation Studies

Acid Degradation Studies

To 1 mL of stock solution OMTR, PRTR, and RTNR, 1 mL of 2N hydrochloric acid was added and refluxed for 30 minutes at 60°C .^[19-21] The resultant solution was diluted to obtain 750 $\mu\text{g/mL}$ of PRTR, 500 $\mu\text{g/mL}$ of RTNR, and 125 $\mu\text{g/mL}$ of OMTR solution, and 0.2 μL solution was injected into the chromatographic system, and the chromatograms were recorded to assess the stability of the sample (Table 8; Fig. 6).

Oxidation

To 1 mL of stock solution of VXR, SFR, and VLR, 1 mL of 20% hydrogen peroxide (H_2O_2) was added separately. The solutions were kept for 30 minutes at 60°C . For the UPLC study, the resultant solution was diluted to obtain

Fig. 6: Chromatogram for acid degradation study

Table 7: Percentage recovery results

Spiked level (%)	OMTR				PRTR				RTNR			
	Spiked ($\mu\text{g/mL}$)	Recovery ($\mu\text{g/mL}$)	% recovery	Mean % recovery	Spiked ($\mu\text{g/mL}$)	Recovery ($\mu\text{g/mL}$)	% recovery	Mean % recovery	Spiked ($\mu\text{g/mL}$)	Recovery ($\mu\text{g/mL}$)	% recovery	Mean % recovery
50	12.5	12.48	99.8	100.19	37.5	37.25	99.33	99.62	25	25.32	101.27	99.86
	12.5	12.56	100.47		37.5	37.37	99.64		25	25.17	100.69	
	12.5	12.41	99.27		37.5	37.38	99.65		25	25.11	100.43	
100	25	24.87	99.48		75	75.33	100.44		50	50.01	100.02	
	25	24.97	99.87		75	74.61	99.48		50	49.56	99.12	
	25	25.11	100.45		75	74.51	99.35		50	49.76	99.52	
150	37.5	38.07	101.53		112.5	111.95	99.51		75	74.048	98.73	
	37.5	37.72	100.6		112.5	112.11	99.65		75	74.65	99.54	
	37.5	37.59	100.24		112.5	111.95	99.51		75	74.58	99.44	

Table 8: Results of stress degradation study

S. No.	Degradation condition	PRTR		RTNR		OMTR	
		% recovery	% degraded	% recovery	% degraded	% recovery	% degraded
1	Acid hydrolysis	93.91	6.09	92.88	7.12	86.97	13.03
2	Base hydrolysis	93.99	6.01	94.7	5.3	95.92	4.08
3	Peroxide	94.94	5.06	89.55	10.45	90.59	9.41
4	Dry heat	97.34	2.66	96.44	3.56	97.32	2.68
5	Photostability	98.78	1.22	97.43	2.57	98.62	1.38
6	Water sample	99.62	0.38	99.33	0.67	99.64	0.36

750 µg/mL of PRTR, 500 µg/mL of RTNR, and 125 µg/mL of OMTR solutions, and 0.2 µL solution was injected into the chromatographic system, and the chromatograms were recorded to assess the stability of sample (Table 8; Fig. 7).

Alkali Degradation Studies

To 1 mL of stock solution OMTR, PRTR, and RTNR, 1 mL of 2N sodium hydroxide was added and refluxed for 30 minutes at 60°C.^[20] The resultant solution was diluted to obtain 750 µg/mL of PRTR, 500 µg/mL of RTNR, and 125 µg/mL of OMTR, and 0.2 µL solution was injected into the chromatographic system, and the chromatograms were recorded to assess the stability of sample (Table 8; Fig. 8).

Dry Heat Degradation Studies

The standard drug solution was placed in an oven at 105°C for 6 hours to study dry heat degradation. For the UPLC study, the resultant solution was diluted to get 750 µg/mL of PRTR, 500 µg/mL of RTNR, and 125 µg/mL of OMTR, and 0.2 µL solution was injected into the chromatographic system. The chromatograms were recorded to assess the sample's stability (Table 8; Fig. 9).

Photo Stability Studies

The photochemical stability of the drug was also studied by exposing the (100 µg/mL, 400 µg/mL, and 100 µg/mL) solution to ultra-violet (UV) light by keeping the beaker in a UV chamber for 3 days or 200 watt-hours/m² in photostability chamber.^[21] For the UPLC study, the resultant solution was diluted to obtain 750 µg/mL of PRTR, 500 µg/mL of RTNR, and 125 µg/mL of OMTR, and 0.2 µL solution was injected into the chromatographic system, and the chromatograms were recorded to assess the stability of sample (Table 8; Fig. 10).

Neutral Degradation Studies

Stress testing under neutral conditions was studied by refluxing the drug in water for 6 hours at 60°C. For the UPLC study, the resultant solution was diluted to get 750 µg/mL of PRTR, 500 µg/mL of RTNR, and 125 µg/mL of OMTR, and 0.2 µL solution was injected into the chromatographic system. The chromatograms were recorded to assess the sample's stability (Table 8; Fig. 11).

Assay of Marketed Formulation

The marketed formulation of Technivie (film-coated tablet) was evaluated by infusing 0.2 µL of reference and analyte solutions six times into the chromatographic system, and the resulting chromatograms of analytes were documented. The quantity of analytes that existed in the marketed formulation was estimated by equating the peak

Fig. 8: Chromatogram for alkali degradation study

Fig. 9: Chromatogram for dry heat degradation study

Fig. 10: Chromatogram for photostability study

Fig. 7: Chromatogram for oxidation degradation study

Fig. 11: Chromatogram for neutral degradation study

area of reference and analyte. The % assay of OMTR, PRTR, and RTNR was found to be 98.6 to 101.2%.

DISCUSSION

All the methods were reported on the HPLC techniques with more retention time and run times in the literature. In the present work, we selected UPLC to reduce the total run time. Method development was executed with different columns and mobile phases. Finally, the method was optimized with a mobile phase of 0.01N potassium dihydrogen orthophosphate (pH 5.3) and methanol in the proportion of 60:40 %v/v utilizing a BEH C18 column, which has dimensions of 100 × 3 mm, 1.7 μm particle size, and the flow rate of 0.3 mL/min. Further, the developed method was subjected to validation and forced degradation studies. Validation was executed as per the ICH Q2R1 guidelines for the parameters specificity, linearity, system suitability, LOD, LOQ, precision, accuracy, and robustness. All the parameters were within limits. The developed method was subjected to forced degradation studies as per the ICH, like neutral degradation, photostability, dry heat degradation, alkali degradation, oxidation, and acid degradation. The degradation results are also produced in the results section.

CONCLUSION

A sensitive, rapid, and accurate, stability-indicating RP-UPLC method for the simultaneous estimation of OMTR, PRTR, and RTNR in formulations was developed and validated as per the ICH guidelines. Retention times for OMTR, PRTR, and RTNR were achieved at 1.765, 2.192, and 1.326 minutes, respectively. The mean percentage recovery of OMTR, PRTR, and RTNR were 100.19, 99.62, and 99.86%, respectively. LOD/LOQ values obtained from regression equations of OMTR, PRTR, and RTNR and were found to be 0.34/1.03, 0.48/1.44, and 0.1/0.29 μg/mL, respectively. The regression equation of OMTR, PRTR, and RTNR were: $y = 7,495.1x + 947.85$, $y = 13,764x + 13,436$, and $y = 6,494.1x + 1,325.8$, respectively. Stability studies of these drugs proved that the percentage of analytes' degradation was between 0.36 and 13.03%. Retention time and total run times of analytes were decreased. Hence, the developed method was rapid and economical that can be applied in the routine analysis of these drugs in the quality control department of pharmaceutical trades.

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HOW TO CITE THIS ARTICLE: Maneka SL, Saravanakumar RT, Male A. Stability-indicating method development and validation for simultaneous estimation of ombitasvir, paritaprevir, and ritonavir in formulation by ultra performance liquid chromatography. *Int. J. Pharm. Sci. Drug Res*. 2020;12(5):457-463. DOI: 10.25004/IJPSDR.2020.120505