CHAPTER - 5

OMBITASVIR, PARITAPREVIR AND RITONAVIR

DRUG PROFILE

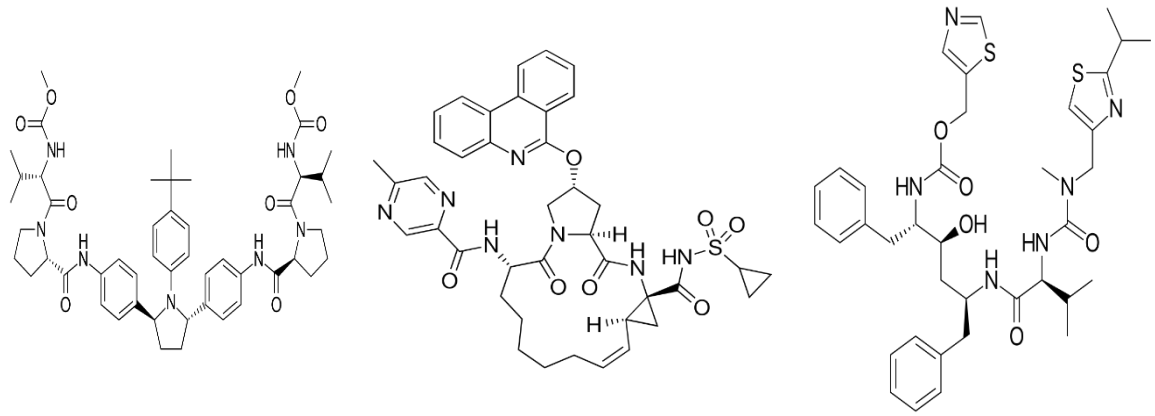
Ombitasvir, paritaprevir and ritonavir (Figure: 1.0) drugs were combined in a single dosage form (film-coated tablet) in the brand name of TECHNIVIE for the treatment of hepatitis-C. These three drugs will acts against the hepatitis-C virus (HCV) in three different mechanisms.

Ombitasvir, produces its antiviral activity by inhibiting the HCV non-structural protein (NS) 5A. Ombitasvir 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).

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). It 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, Paritaprevir, therefore, prevents viral replication and function.

Ritonavir 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 ritonavir, 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-[(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.



(A) **(B)** **(C)**

Fig. 5.1: Chemical structures of (A) Ombitasvir (B) Paritaprevir (C) Ritonavir

MATERILAS AND METHODS

Instrumentation:

The HPLC system (Agilent HPLC 1200 Infinity LC Specifications) consisted of a pump (Agilent LC20AT) programmed with Ezchrom Elite Software and rheodyne Injector was used. The detector consisted of UV/VIS (UV-2489) model was operated at a wavelength of 262 nm. The column used was Inertsil CN- 3 column at ambient temperature

Chemicals and Reagents:

Hetero Aurobindo Pharma Pvt. Ltd, Hyderabad, India kindly supplied the pure working standards of known potency of ombitasvir, paritaprevir and ritonavir as a gift sample. The marketed sample with strength of ombitasvir 25 mg, paritaprevir 150 mg, and ritonavir 100 mg purchased from the local Pharmacy. The reagents like orthophosphoric acid (OPA) of Hi-Media Laboratories Pvt. Ltd, water, methanol, acetonitrile, triethylamine of Merk, potassium dihydrogen phosphate of Thermo Fisher Scientific India Pvt. Ltd were used**.**

Preparation of Standard Stock Solution:

Each 10mg of ombitasvir, paritaprevir and ritonavir were transferred to 100 ml volumetric flask and 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.

Test sample preparation:

Tablet powder equivalent to 10 mg 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.

METHOD DEVELOPMENT

For developing the method, a systematic study of the effect of various factors were undertaken by varying one parameter at a time and keeping all other conditions constant. Method development consists of selecting the appropriate mass parameters and choice of stationary and mobile phases. The following studies were conducted for this purpose.

Trail -1:

Chromatographic conditions	:	
Mobile Phase	:	Water: Methanol (60:40, %V/V)
Column	:	C18, HPLC column, Emerald, 3 µm, 30.0×3.0 mm
Detection Wavelength	:	254 nm

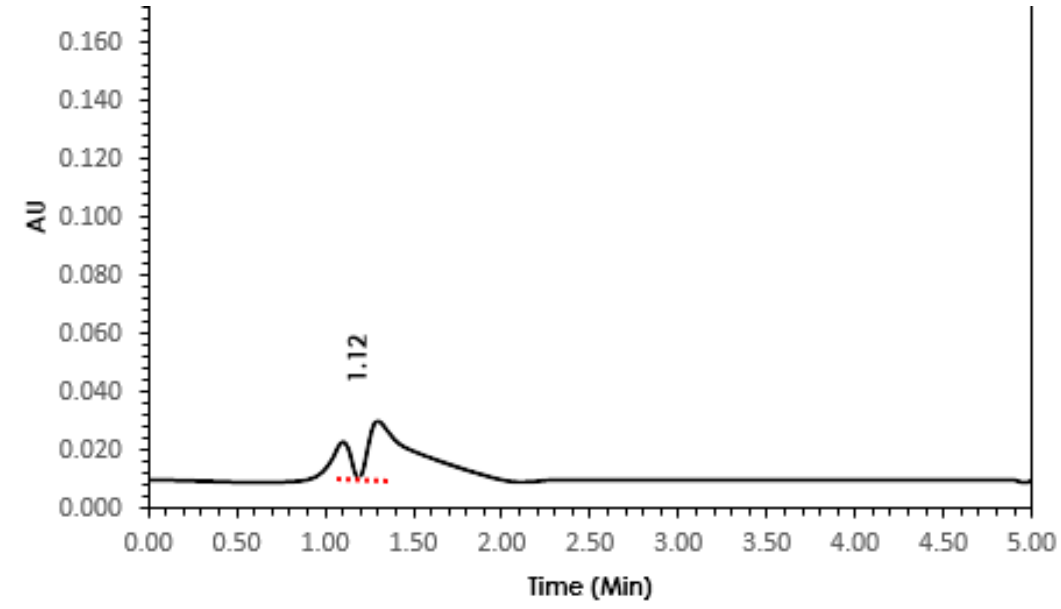


Fig. 5.2: Chromatogram Showing Trial – 1

Trail - 2:

Chromatographic conditions	:	
Mobile Phase	:	Water: Acetonitrile: Methanol (80:10: 10, %V/V)
Column	:	Kinetex 2.6u XB-C18 150x4.60mm
Detection Wavelength	:	260 nm

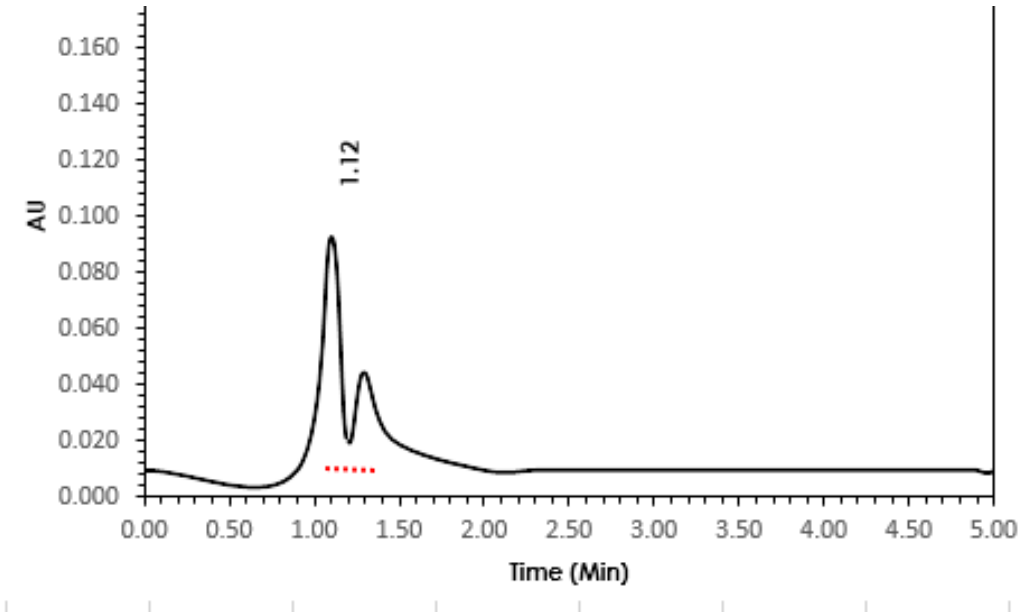


Fig. 5.3: Chromatogram Showing Trial – 2

Conclusion: The components were not separated completely.

Selection of Wavelength (λ_{max}): 10 mg of the ombitasvir, paritaprevir and ritonavir standard drug is taken in a 10 ml volumetric flask and dissolved in acetonitrile and volume made up to the mark, from this solution 0.1ml is pipetted into 10 ml volumetric flask and made up to the mark with the acetonitrile to give a concentration of 10 µg/ml. The above prepared solution is scanned in UV between 200-400 nm using acetonitrile as blank. The λ_{max} was found to be 262nm.

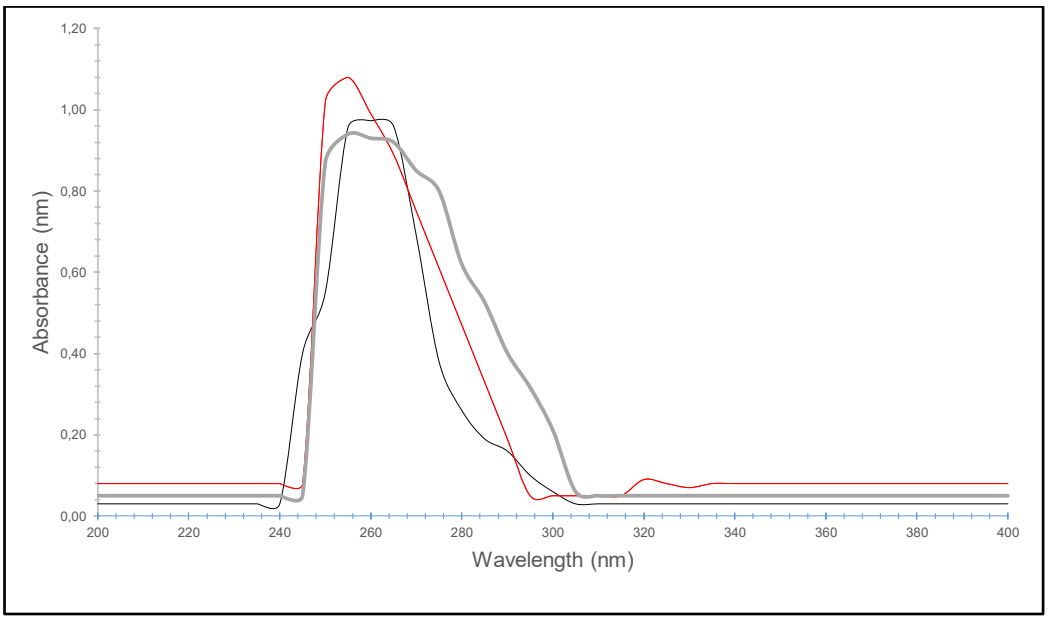


Fig. 5.4: Over lay spectrum UV spectrum of Ombitasvir, Paritaprevir and Ritonavir

Choice of stationary phase

Initially the separation was tried with different columns having different dimensions like diameter and length and pore size. Finally good separation with finest peak shape was achieved with the analytical column Inertsil ODS-C18; 5µm (4.6 X 250mm).

Selection of mobile phase

Several systematic test plans were performed to optimize the mobile phase. Different solvents like methanol, water and acetonitrile in different ratios and different pH values of the mobile phase ratios, by using different buffer solutions in order to get sharp peak and base line separation of the components and without interference of the excipients. Satisfactory peak symmetry, resolved and free from tailing was obtained in mobile phase 0.02M phosphate buffer (pH-4.5): acetonitrile: methanol, (50:30:20) (v/v).

Selection of the mobile phase flow rate

Flow rates of the mobile phase were changed from 0.5-1.0 ml/min for optimum separation. A minimum flow rate as well as minimum run time gives the maximum saving on the usage of solvents. It was found from the experiments that 1.0 mL/min flow rate was ideal for the successful elution of the analyte.

Optimized Chromatographic conditions

After series of trials, the chromatographic conditions was accomplished with following parameters.

Buffer	:	0.02M phosphate buffer (pH-4.5)
Mobile Phase	:	0.02M phosphate buffer (pH-4.5): Acetonitrile: Methanol, (50:30:20) (v/v)
Column	:	Inertsil ODS-C18; 5µm (4.6 X 250mm)
Flow Rate	:	1.0ml/min
Temperature	:	Ambient
Injection Volume	:	20µl
Detector	:	262nm
Diluent	:	Water: Acetonitrile (50:50) column with a mixture of as mobile phase.

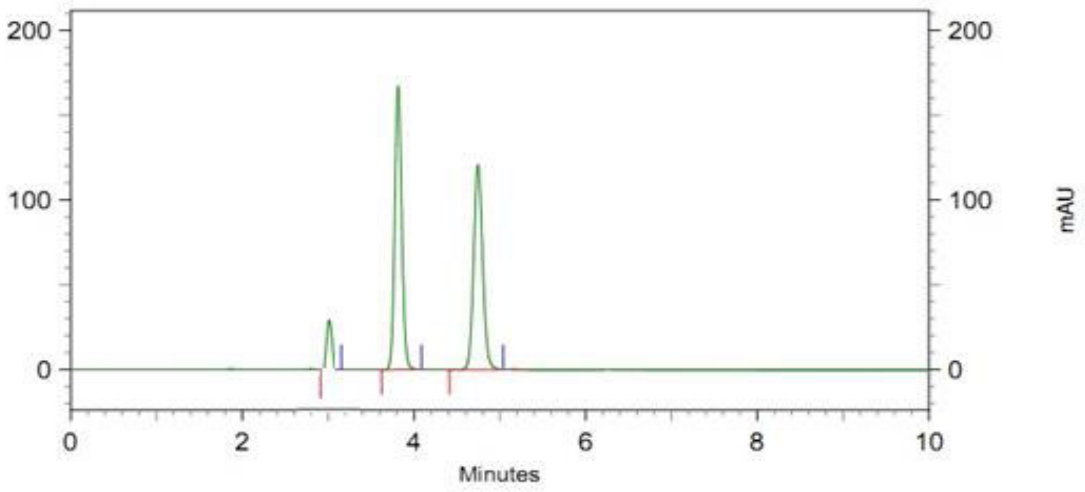


Fig. 5.5: Chromatogram of Ombitasvir, Paritaprevir and Ritonavir

METHOD VALIDATION

After the development of RP-HPLC method for the estimation of drug in a dosage form, validation of the method was performed. This part describes the procedure followed for validation of the developed method.

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.

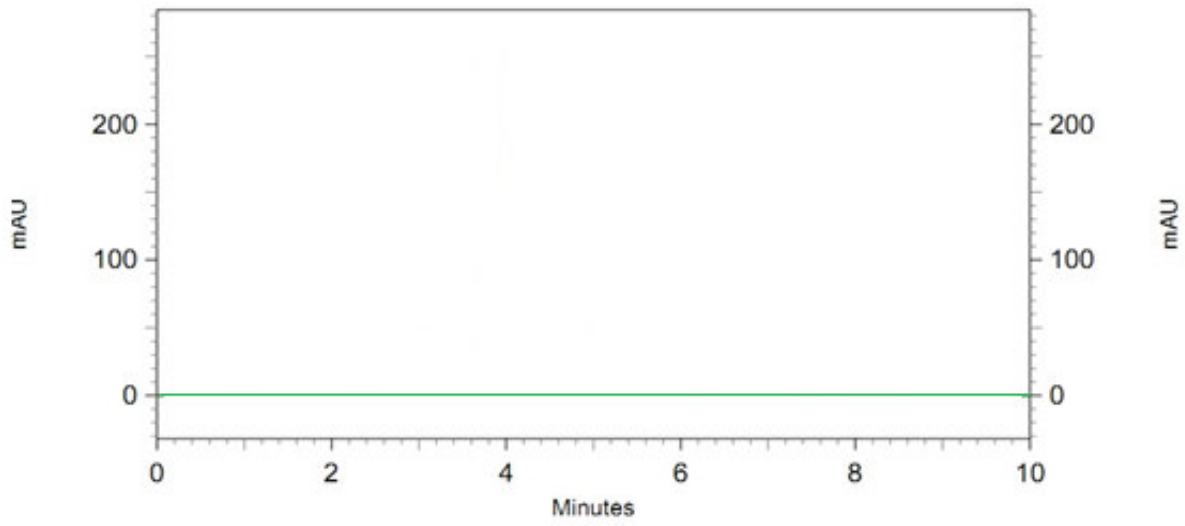


Fig. 5.6: Chromatogram of Blank

System suitability

To verify the system producing the consistent results with the optimized method injected the standard for six times with the criteria of % RSD for retention time and area NMT 2.0%, theoretical plates NLT 3000 plates, tailing factor NMT 1.5 and resolution NLT 4.

Table. 5.1: System suitability parameters

Parameter	Compound	Result
Retention Time	Ombitasvir	3.14 min
	Paritaprevir	3.92 min
	Ritonavir	4.91 min
Peak Area	Ombitasvir	254622
	Paritaprevir	565892
	Ritonavir	431312
Theoretical plates	Ombitasvir	4231
	Paritaprevir	3452
	Ritonavir	1342
Tailing Factor	Ombitasvir	0.12
	Paritaprevir	0.34
	Ritonavir	0.35
Resolution	Ombitasvir	-
	Paritaprevir	3.34
	Ritonavir	4.34

Linearity:

A series of standard solutions (not less than 5 is recommended) 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 in µg/ml or mg/ml is made and from this the correlation coefficient, y-intercept (constant of regression) and slope (coefficient of regression) of the regression line were calculated. The calibration data and calibration curve shown in Table No.02 and Fig No. 2, 3 and 4.

Table 5.2: Linearity data

S.NO	Concentration µg/mL	Area of OT	Concentration µg/mL	Area of PT	Concentration µg/mL	Area of RT
1	15	279425	15	313823	15	414740
2	21	496653	21	467371	21	580637
3	27	684443	27	600918	27	746533
4	33	853368	33	734456	33	912430
5	39	991189	39	867991	39	1059512
6	45	1199969	45	1001530	45	1244222
Concentration range	15-45µg/mL		15-45µg/mL		15-45µg/mL	
Slope (m)	27167		22409		27471	
Correlation coefficient (r^2)	0.9903		0.9996		0.9998	

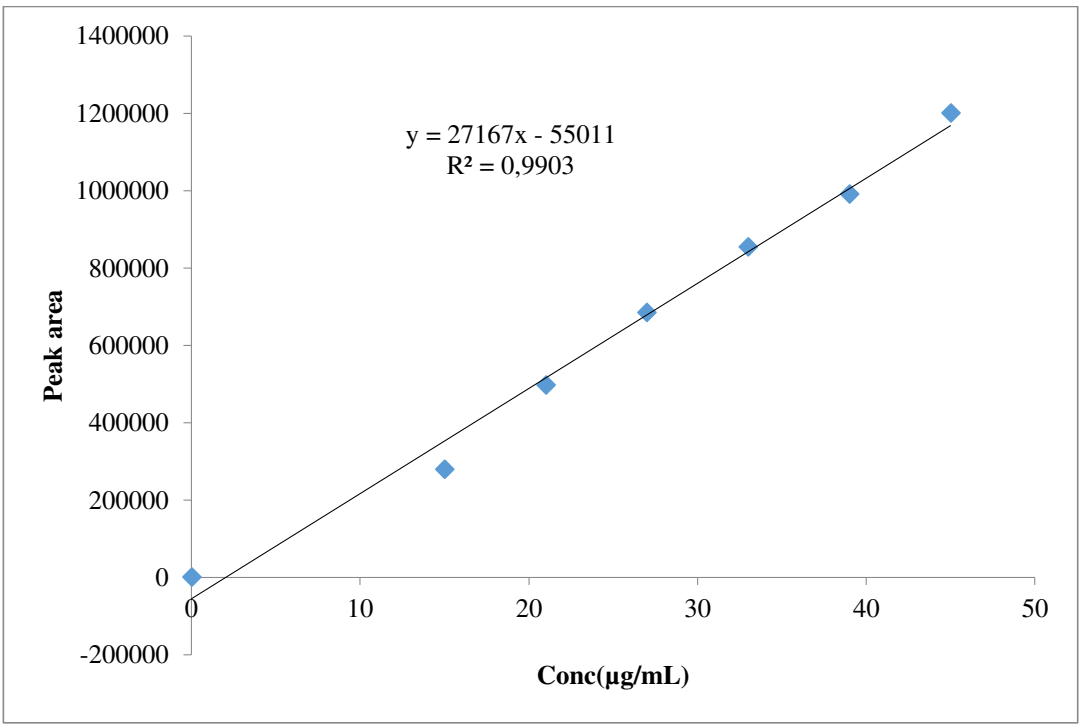


Fig. 5.7: Linearity Plot of Ombitasvir

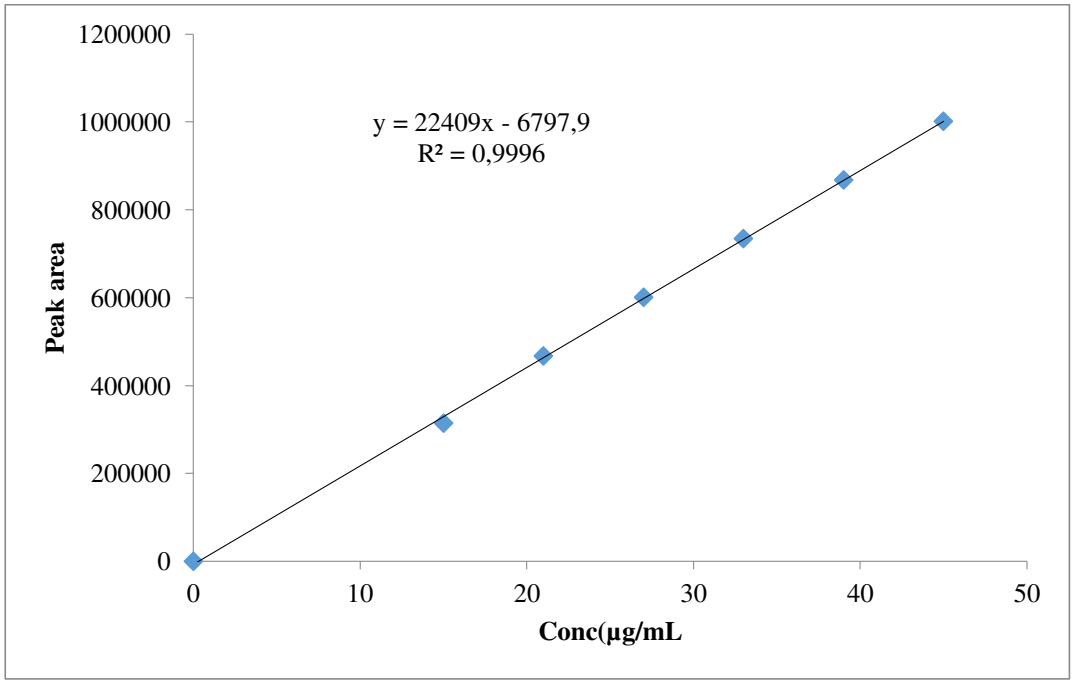


Fig. 5.8: Linearity Plot of Paritaprevir

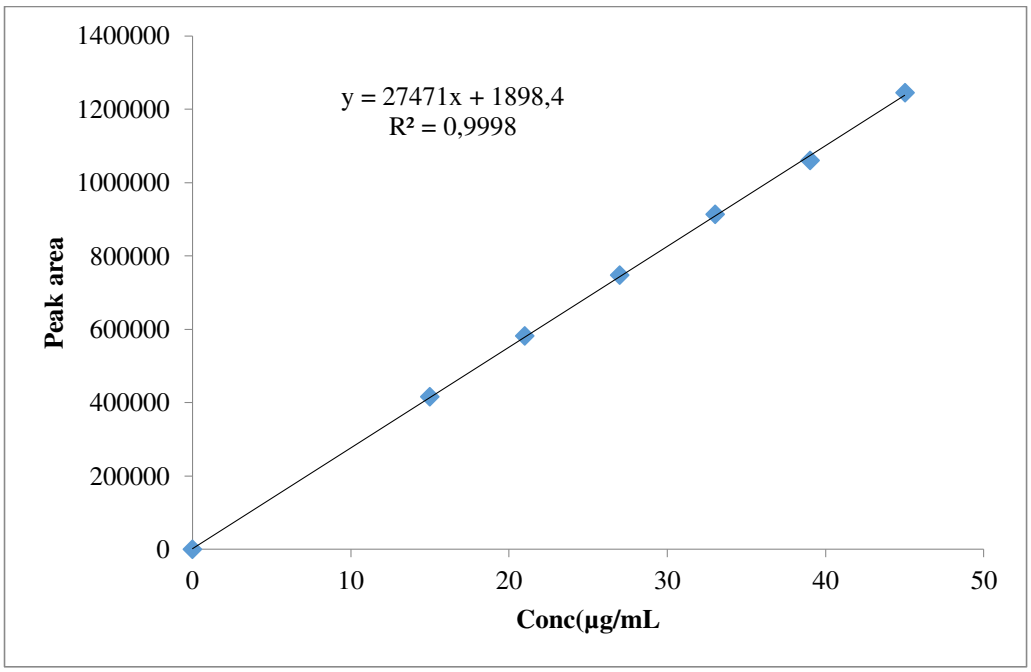


Fig. 5.9: Linearity Plot of Ritonavir

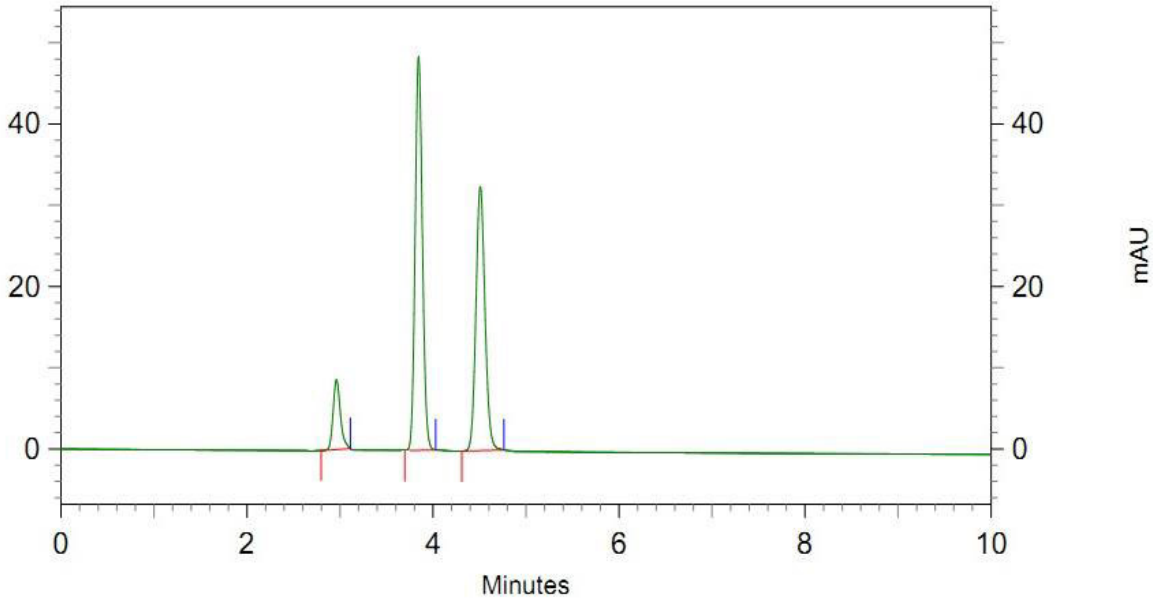


Fig. 5.10: Chromatogram of 5 mcg/ml

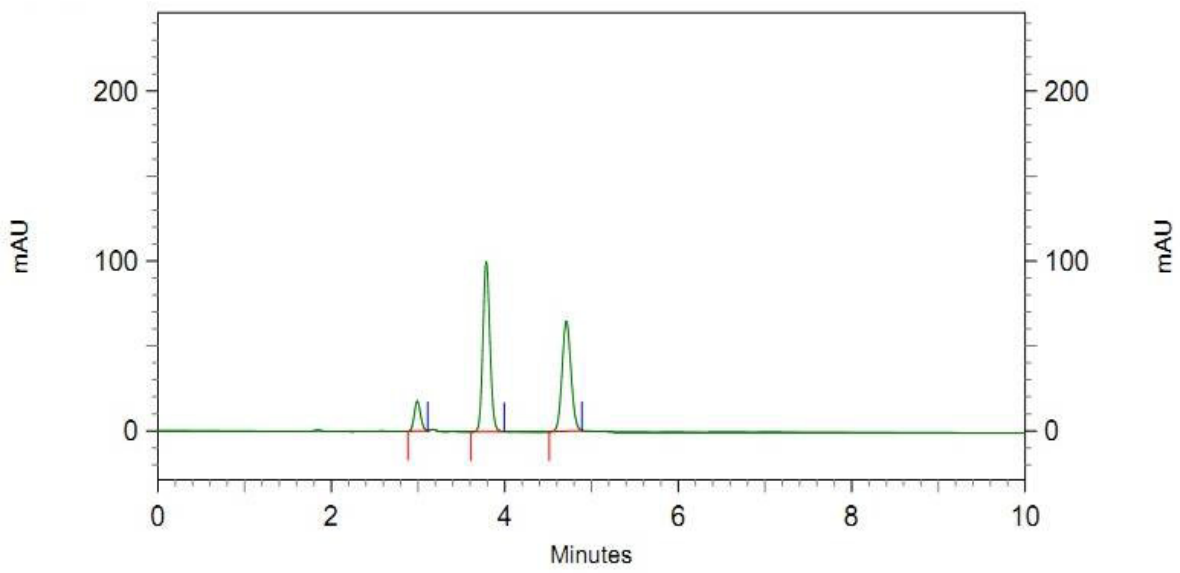


Fig. 5.11: Chromatogram of 10mcg/ml

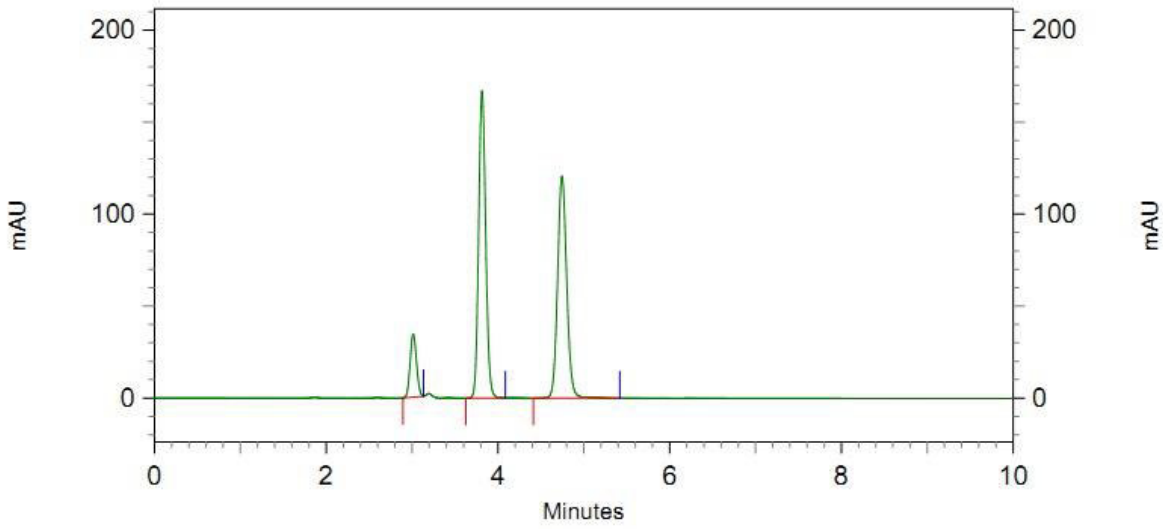


Fig. 5.12: Chromatogram of 15 mcg/ml

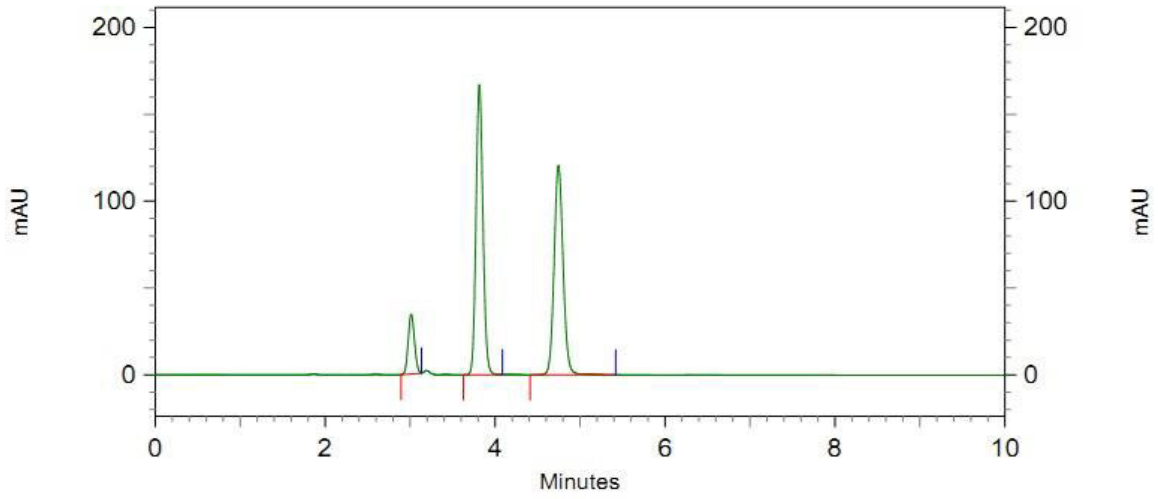


Fig. 5.13: Chromatogram of 20mcg/ml

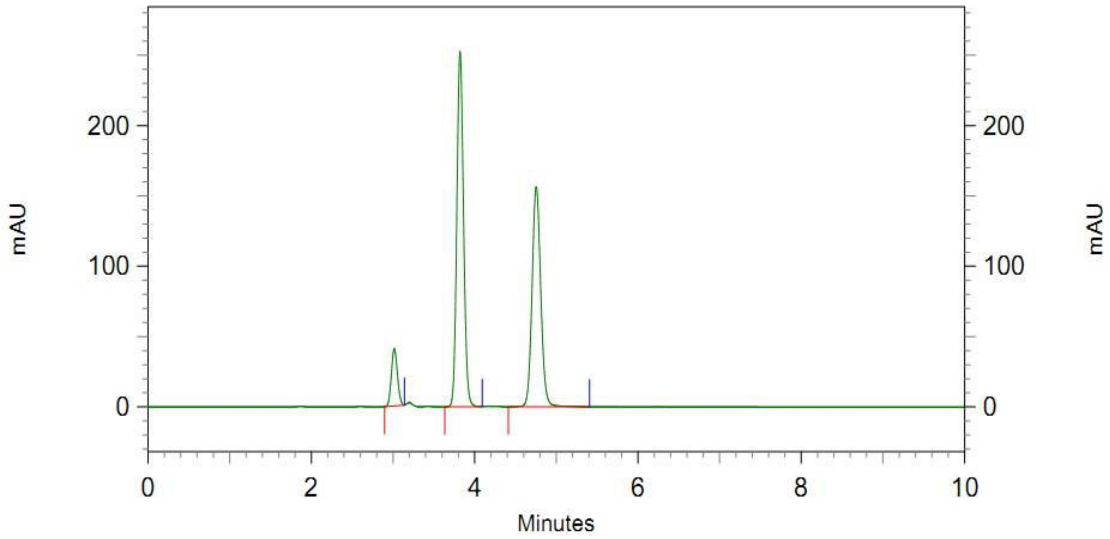


Fig. 5.14: Chromatogram of 25mcg/ml

Precision

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

Table 5.3: Precision for Ombitasvir, Paritaprevir and Ritonavir

	Intraday precision					
	Test conc: 33µg/ml					
Sample. No	**OT**	**%Assay**	**PT**	**%Assay**	**RT**	**%Assay**
1	853368	99.45	734451	99.67	912310	98.56
2	821362	98.32	734121	99.54	911321	99.06
3	829356	99.16	734123	97.95	901235	99.16
4	837350	99.94	731243	97.43	904320	97.35
5	845344	99.76	734312	97.43	912349	97.29
6	843338	99.11	733123	97.23	912340	98.54
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					
	Test conc: 33µg/ml					
Sample. No	**OT**	**%Assay**	**PT**	**%Assay**	**RT**	**%Assay**
1	822371	98.34	732112	98.20	901235	98.56
2	824345	95.91	731033	96.38	904320	96.74
3	837350	98.20	729954	98.56	907405	97.92
4	845344	96.38	728875	96.74	910490	96.10
5	853338	98.61	727796	97.92	913575	98.28
6	861332	99.45	726717	99.10	916660	98.46
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**	**% RSD**	**1.04**

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.04 shows the results for accuracy of ombitasvir, paritaprevir and ritonavir.

Table 5.4: Accuracy results of Ombitasvir

Level of % recovery	Target Conc. (µg/mL)	Amount of drug spiked(µg/mL)	Nominal conc (µg/mL)	Drug recovered (µg/mL)	% Recovery	Mean	SD	%RSD
80	33	27.00	60.00	59.24	98.73	98.54	0.28	0.29
				58.93	98.22			
				59.21	98.68			
100	33	33.00	66.00	66.25	99.62	101.02	1.70	1.68
				66.34	100.52			
				67.92	102.91			
120	33	40.00	73.00	73.25	100.34	100.03	1.08	1.07
				72.15	98.84			
				73.67	100.92			

Table 5.5: Accuracy results of Paritaprevir

Level of % recovery	Target Conc. (µg/mL)	Amount of drug spiked (µg/mL)	Nominal conc (µg/mL)	Drug recovered (µg/mL)	% Recovery	Mean	SD	%RSD
80	33	27.00	60.00	61.34	102.23	100.41	1.86	1.85
				60.28	100.47			
				59.11	98.52			
100	33	33.00	66.00	64.92	101.66	102.95	1.31	1.28
				68.83	104.29			
				67.92	102.91			
120	33	40.00	73.00	73.13	100.18	99.77	0.42	0.42
				72.84	99.78			
				72.52	99.34			

Table 5.6: Accuracy results of Ritonavir

Level of % recovery	Target Conc. (µg/mL)	Amount of drug spiked (µg/mL)	Nominal conc (µg/mL)	Drug recovered (µg/mL)	% Recovery	Mean	SD	%RSD
80	33	27.00	60.00	63.22	105.37	103.72	1.57	1.52
				62.13	103.55			
				61.34	102.23			
100	33	33.00	66.00	67.04	98.45	98.88	0.40	0.40
				65.32	98.97			
				65.49	99.23			
120	33	40.00	73.00	73.28	100.38	101.97	1.77	1.73
				74.2	101.64			
				75.83	103.88			

Robustness

Robustness of the method is performed by altering the chromatographic conditions such as pH of the buffer, Wavelength, Mobile phase composition and observed the variation of the results which should be within the acceptance criteria.

Table 5.7: Robustness results

S. No.	Parameter	Condition	OT		PT		RT	
			Area (n=3)	% change	Area (n=3)	% change	Area (n=3)	% change
1	Standard	Standard conditions	837350	0	729954	0	907405	0
2	Mobile Phase composition (±2%)	0.02M phosphate buffer (pH - 4.5): Acetonitrile: Methanol, (v/v), (52:28:20)	834512	0.339	728847	#REF!	917341	-1.095
		0.02M phosphate buffer (pH - 4.5): Acetonitrile: Methanol, (v/v), (48:32:20)	831674	0.340	727740	0.152	927277	-1.083
3	Mobile	2.9	838836	-0.861	726633	0.152	917213	1.085
	phase pH	3.1	835998	0.338	725526	0.152	917149	0.007
4	Wavelength (nm)	248	833160	0.339	724419	0.153	914065	0.336
		252	830322	0.341	723312	0.153	907021	0.771
5	Flow rate (mL) ±0.2mL	1.2	837484	-0.863	722205	0.153	916957	-1.095
		0.8	834646	0.339	721098	0.153	906893	1.098

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

The detection limit of an individual analytical procedure is the lowest amount of analyte in a sample which can be detected but not necessarily quantitated as an exact value.

1. Based on Signal-to-Noise for LOD (3:1), LOQ (10:1)
2. Based on the Standard Deviation of the Response and the Slope

The quantitation limit of an individual analytical procedure is the lowest amount of analyte in a sample which can be quantitatively determined with suitable precision and accuracy.

From the linearity data, the limit of detection and quantitation were calculated using the following formula.

$$LOD = \frac{3.3\,\sigma}{S}, \quad LOQ = \frac{10\,\sigma}{S}$$

σ = standard deviation of the response, S = slope of the calibration curve

LOD and LOQ of Amlodipine, Hydrochlorothiazide and Olmesartan are performed by spiking of known concentrations of the sample into the placebo of formulation and inject the sample.

Table 5.8: Results of LOD and LOQ

Sample	LOD	LOQ
Ombitasvir	1.89859	5.753304
Paritaprevir	0.297175	0.900531
Ritonavir	0.693251	2.100761

ASSAY

Six replicates of the samples solutions were injected for quantitative analysis. The amounts of ombitasvir, paritaprevir and ritonavir estimated were found to 99.52%, 102.00% and 99.02% respectively. A good separation and resolution of both drugs indicate that there were no interference from the excipients commonly present in pharmaceutical formulations. This showed that the estimation of dosage form was accurate within 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 No. 05.

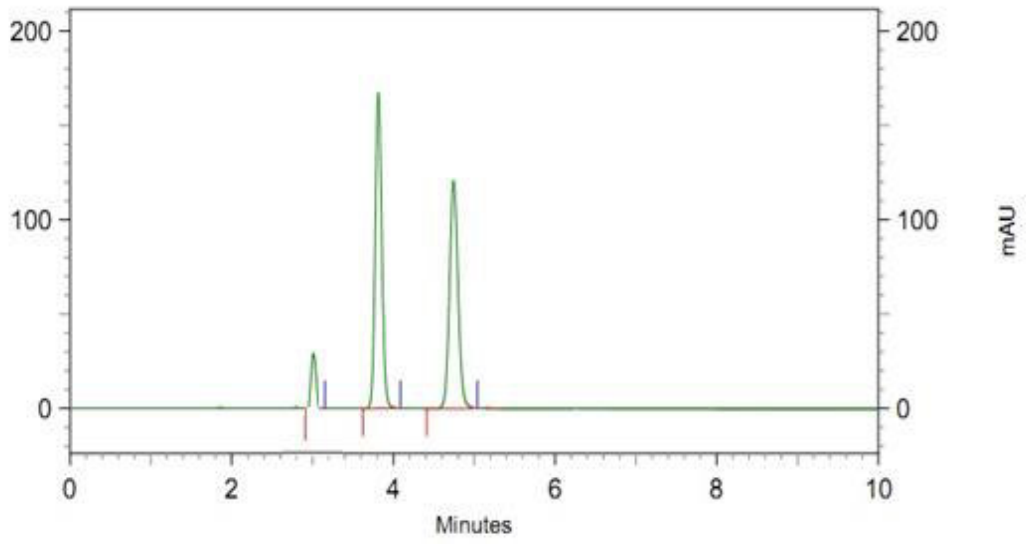


Fig. 5.15: Chromatogram of test sample

Table 5.9: Assay of test sample

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