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New Analytical HPLC Method Development and Validation for the Simultaneous Quantification of Paritaprevir Ombitasvir and Ritonavirin Spiked Human Plasma.

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ABSTRACT

A rapid HPLC bio-analytical method has been developed and validated for the simultaneous quantification of Ombitasvir (OMBTSR), Ritonavir (RTNVR), Paritaprevir (PTVR) in human plasma. OMBTSR and PTVR are used to control Hepatitis-C infection. RTNVR is used in the treatment of HIV/AIDS. The method was developed with Column (Intersil ODSC 18, 250 mm × 4.6 mm × 5µ) at 230 nm wave length and at flow rate of 1.0 mL/min. The mobile phase consisted of 20% Acetonitrile, 20% Methanol, 60% 1mM $NH_4H_2PO_4$Buffer(P^H 6.5 v/v). The retention times of RTNVR, OMBTSR, PTVR are 5.7 min. 7.8 min. and 12.8 min. respectively. The method was developed and validated in terms of linearity, interday precision, intraday precision, accuracy, LOQ, LOD and stability study. The proposed method is useful in pharmacokinetic studies using HPLC or LC-MS.

Key words: Ombitasvir, Ritonavir, Paritaprevir, HPLC Method, Bio analytical method.

INTRODUCTION

Ritonavir (RTNVR) is an antiretroviral drug used along with other medications to treat HIV/AIDS and hepatitis C.[1-3]. Ombitasvir (OMBTSR) is an antiviral drug for the treatment of hepatitis C virus. Mostly it is used in the combinations with in combination with Paritaprevir, Ritonavir and Dasabuvir[4]. Paritaprevir (PTVR) is best drug in the treatment of hepatitis C. The usual side effects of this combination are Diarrhea, Vomiting, loss of appetite and numbness of the hands and feet. The serious side effects are liver problems, pancreatitis, allergic reactions, and Arrhythmias[5]. From last few decades, Pharmacokinetics playing major important role in drug development. At the time of identification of drug's biological properties, metabolic feature of the drug and understanding of pharmacokinetic the bioanalytical methods playing key role. The bioanalytical method used to determine the drug and its metabolites in serum, plasma, urine[6-12].

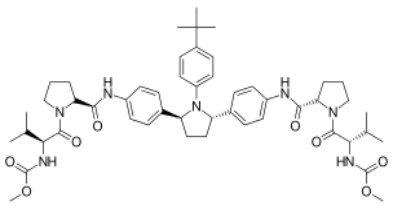


Fig. 1. Structure of OMBTSR

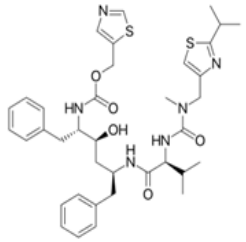


Fig. 2. Structure of RTNVR

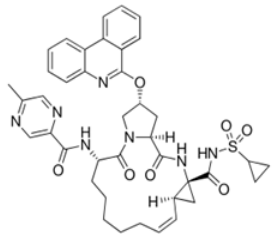


Fig. 3. Structure of PTVR

Literature survey revealed that Nourah Zoman *et al* [12] submitted a method for the estimation of RTNVR, OMBTSR, PTVR in pharmaceutical formulations. Other methods are reported for analysis of individual and combination of two drugs. From the literature survey there is no bio analytical method reported. Andrew J Ocque *et al* [13] developed a method to determination of Paritaprevir and Ritonavir in rat liver tissue samples. In this method accuracy range is 6.68 to 10.1%. Jyoti. M. Salunke *et al* [14] developed a method to estimate and RTNVR and Lopinavir in combined dosage form with C18 column (250 x 4.6mm id,5µm) at 1.5 ml/min. flow rate. Methanol, Acetonitrile and Potassium Dihydrogen Phosphate as a mobile phase. Rajasekhar *et al*[15] developed a method for LCMS method to determination of Lopinavir and RTNVR in Human Plasma by Protein Precipitation method. The method was developed with mobile phase consisting of CH_3CN and 5mM CH_3COONH_4buffer. The linearity range of the method is 50.67–10,008.82 ng/mL for Lopinavir and 5.066–1,000.693 ng/ml for Ritonavir. K Vinod Kumar *et al* [16] reported a HPLC method developed for the simultaneous quantification of RTNVR and Lopinavir in tablet dosage form and in plasma. The concentration range of Ritonavir and Lopinavir are 5-50 µg/ml, 20-200 µg/ ml respectively. The method was carried out on RP C18 column and methanol: water (85:15v/v) as a mobile phase. M Jagadees waran *et al*[17] submitted a method developed a method for quantitative estimation of Lopinavir and RTNVR. The method was reported with reversed-phase C18 Column, mixture of Buffer: Acetonitrile (45:55 v/v) as mobile phase at p^H 4.5.The recovery of the method was between 102.1% and 100.1% for Lopinavir and Ritonavir respectively. Suneetha. A *et al*[18] developed a method for estimation of Lopinavir and RTNVR in combined dosage. The method was developed with Potassium di hydrogen phosphate buffer, CH_3CN and CH_3OH in the ratio of 50:35:15v/v at pH 6.0 linear in the ranges are 400-600µg/ml for Lopinavir and 100-150 µg/ml for RTNVR.

Objective

The present study is concerned with the development and validation of RTNVR, OMBTSR and PTVR in spiked human plasma by HPLC.

MATERIALS AND METHODS

Apparatus

The new method was developed and validated with Peak LC P7000 HPLC (Isocratic) system rheodyne injector with 20 µL and UV/Vis detector UV7000 and PEAK Chromatographic version 1.06. The Ombitasvir, Ritonavir Paritaprevir were scanned with UV-Visible spectrophotometer (Tech comp-UV 2301, make Japan) with Hitachi software. OMBTSR, RTNVR and PTVR were obtained from Manus Aktteva Biopharma LLP (India). HPLC grade solvents Water, Acetonitrile, Methanol and Tri Ethyl Amine (TEA) were procured from Merck, Mumbai. Method was developed at 230 nm with Intersil ODS C18 column (250 mm × 4.6 mm×5µ).

Chromatographic conditions

The HPLC method conditions were optimized by using different columns, different mobile phases and different buffers. The finalized mobile phase is 20% Acetonitrile, 20% Methanol,

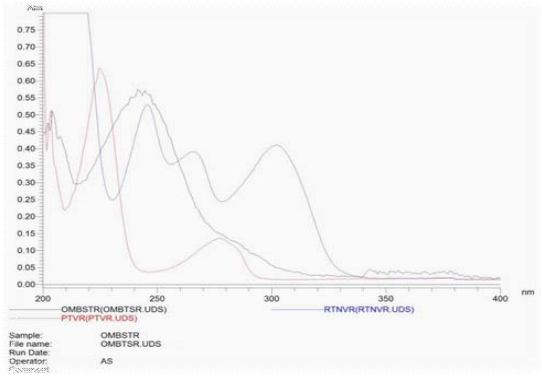


Fig. 4. Overlay of RTNVR, OMBTSR and PTVR

60% 0.001 M $NH_4H_2PO_4$ Buffer v/v. The mobile phase P^H was adjusted to 6.5 with TEA for perfect separation. Analysis was performed with flow rate 1.0 ml/min. at ambient temperature.

Standard stock solution preparation [19]

The plasma samples was extracted by liquid-liquid extraction process. The fixed dosages (10mg) are spiked in to 10ml plasma and stored in freezer for 24 hours. For processing, the stored spiked samples were taken out from freezer and allowed to thaw at room temperature. An aliquot of 500µL was transferred to prelabeled 10.0ml polypropylene centrifuge tubes. Extraction solvent, 5.0ml of ethyl acetate, was then added to extract the drug. The samples were then kept on a vibramax unit and vortexed for 15 minute. Samples were centrifuged at 5000 rpm for 5 min. in a refrigerated centrifuge (4°C). Supernatant solution, 1 ml was then transferred into pre-labeled polypropylene tubes and was allowed to evaporate to dryness under N_2 at 40°C. The dried residue dissolved in 200µL of mobile phase and transferred into shell vials containing vial inserts for analysis. Samples, 20µL by volume, were then injected into the column and analyzed by HPLC on the same day to avoid any degradation. The column temperature was maintained at ambient temperature.

Validation

Linearity Test

The Linearity test was conducted in the range of 25% to 200%. For RTNVR and PTVR the concentrations ranges are 0.15 to 1.2µg/ml. For OMBTR the linearity range is 0.25 µg/ml to 2.0 µg/ml . The results are showed in below Table.1. The target concentration solutions are prepared by

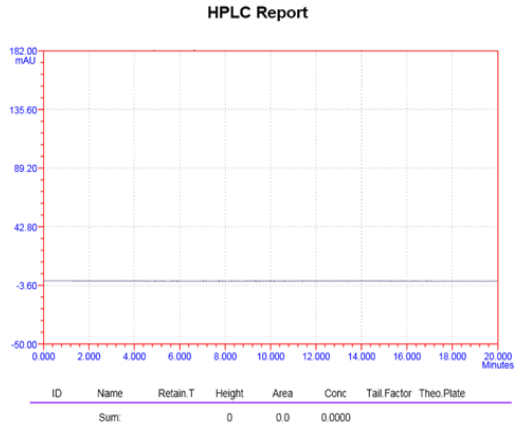


Fig..5 Blank chromatogram

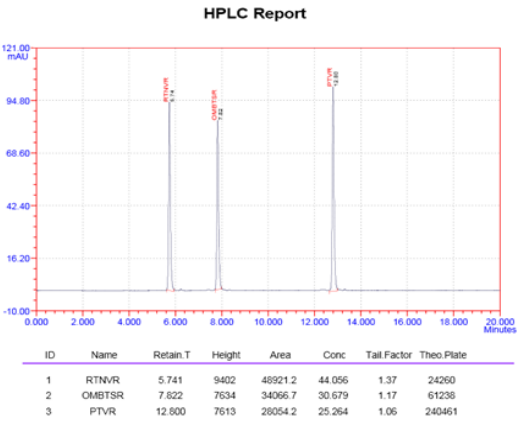


Fig.6. Standard chromatogram of RTNVR.OMBTSR and PTVR

serial dilution from standard stock solution. The linearity graph was plotted with peak area against concentration. The intercept, slope and the correlation coefficient were determined.

Precision

Intraday precision and Interday precision tests were conducted at standard concentrations of RTNVR, OMBTSR and PTVR i.e 0.6 µg/ml, 1.0µg/ml and 0.6µg/ml respectively. The Precision was expressed as the percentage of RSD(Relative Standard Deviation). The calculated percentage RSD of intraday and inter day precision test of each drug is below 2.0.

Recovery

The recovery test was conducted at three different concertinos levels 50%, 100% and 150%. Recovery of RTNVR, OMBTSR and PTVR was evaluated by comparing of observed peak area with

Table.1: Linearity results of developed method

S.No	Percentage of Concentration	Concentration of RTNVR (µg/ml)	Peak Area	Concentration of OMBTSR (µg/ml)	Peak area	Concentration of PTVR (µg/ml)	peak area
1	25%	0.15	26025	0.25	14083	0.15	10440
2	50%	0.3	33830	0.5	20759	0.3	16734
3	100 %	0.6	48921	1.0	34066	0.6	28054
4	150%	0.9	67191	1.50	50463	0.9	42285
5	200%	1.2	79973	2.0	62630	1.2	55252
6	r^2	0.9996	0.9990	0.9993			
7	Slope	51833.7	28204.73	42731.1			
8	Intercept	18273.97	6785.232	3632.409			

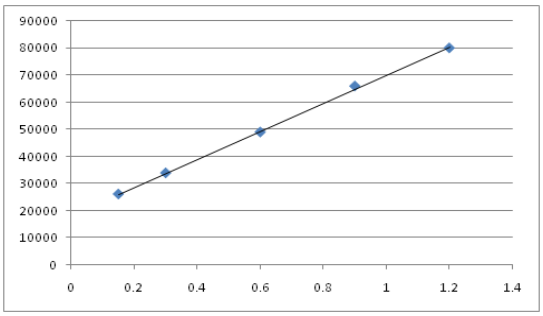


Fig. 7. Linearity graph of RTNVR

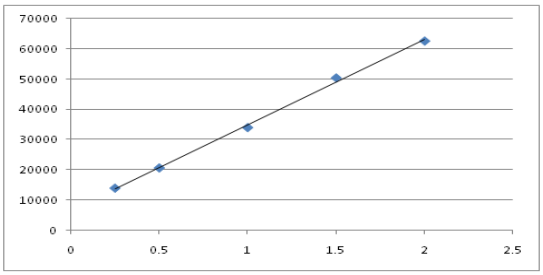


Fig. 8. Linearity graph of OMBTSR

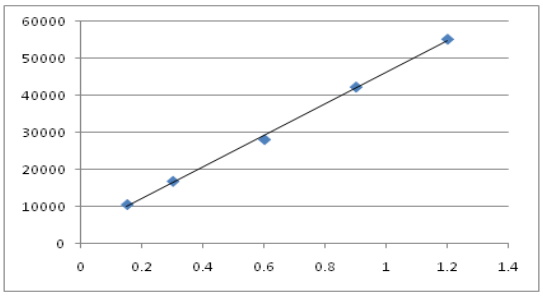


Fig. 9. Linearity graph of PTVR

standard peak area. The average recovery of RTNVR is 99.81, OMBTSR is 101.42 and PTVR is 100.38. The results were showed in Table.2.

Sensitivity

The sensitivity of method was calculated by signal to noise ratio. The plasma sample solution was diluted serially and injected at developed conditions. Similarly, blank plasma samples were also processed and injected. The results were showed in Table.3.

Stability

The stability experiments were aimed at testing all possible conditions that the samples might experience after collecting and prior the analysis. Short term BT (Bench-Top) stability test was evaluated after 24 h. at room temperature. Autosampler stability test was evaluated on QCs extracts maintained in the auto sampler at 10°C for 24 h, by comparing their concentrations with freshextracts. Freeze and Thaw stability test evaluated after three cycles at -20°C to room temperature, by comparison with freshly prepared.Stability of RTNVR, OMBTSR and PTVR solutions was observed at room and temperature and in refrigerated conditions for period of 48 hours. Acceptable stability has been considered as percent difference in concentration lower than 5%. The stability study results were showed in Table.4.

Table. 2: Recovery results of developed method

Concentration level	RTNVR	True area	% of recovery	OMBTSR	True area	% of recovery	PTVR	True area	% of recovery
50%	33982	33830	100.44	21011	20759	101.21	16554	10734	98.32
100%	48689	48921	99.52	34784	34066	102.1	28884	28054	102.95
150%	66845	67191	99.48	50676	50463	100.42	42237	42285	99.88
Average recovery			99.81			101.42			100.38

Table. 3: LOQ and LOD results of developed method

S. No	Test	RTNVR	OMBTSR	PTVR
1	LOQ	0.0375 µg/ml	0.0625 µg/ml	0.375 µg/ml
2	LOD	0.009 µg/ml	0.0156 µg/ml	0.009 µg/ml

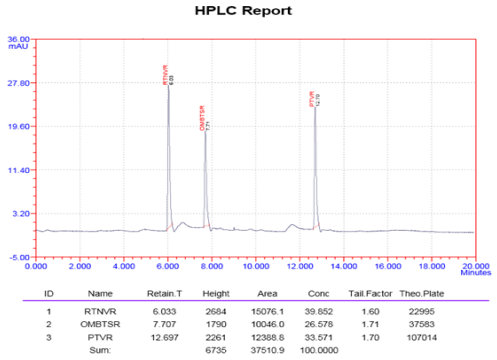


Fig.10. LOQ test chromatogram of RTNVR, OMBTSR and PTVR

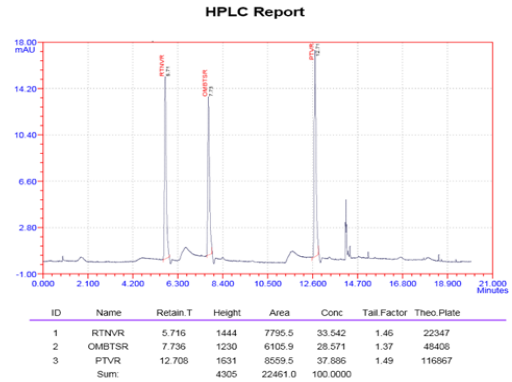


Fig. 11. LOD test chromatogram of RTNVR, OMBTSR and PTVR

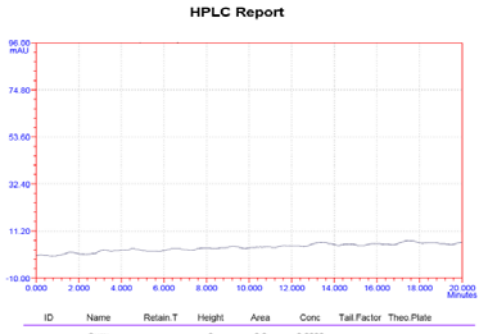


Fig.12. Blank chromatogram of stability test

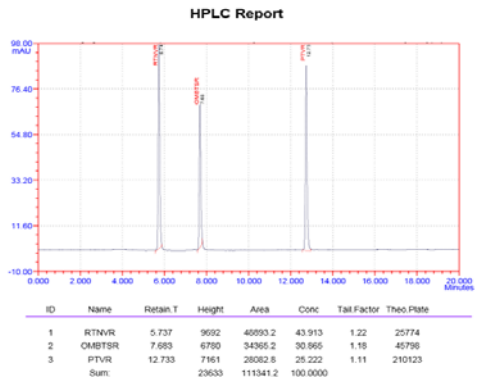


Fig. 13. 1. Stability test chromatogram of RTNVR, OMBTSR and PTVR (Freshly prepared plasma sample)

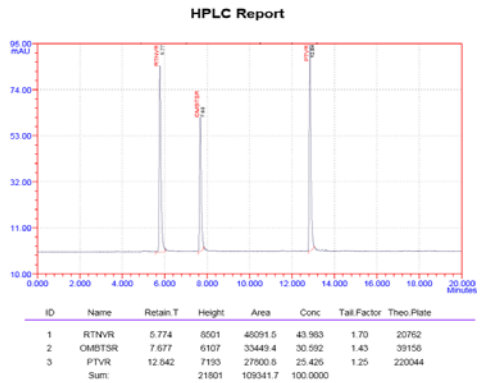
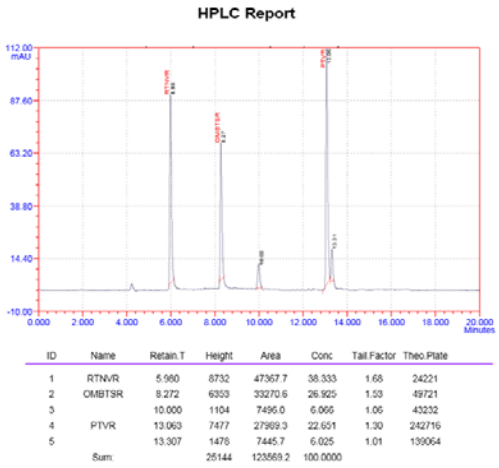


Fig.13. 2. Bench-topstability test chromatogram of RTNVR, OMBTSR and PTVR



13. 3. Autosamplerstability test chromatogram of RTNVR, OMBTSR and PTVR

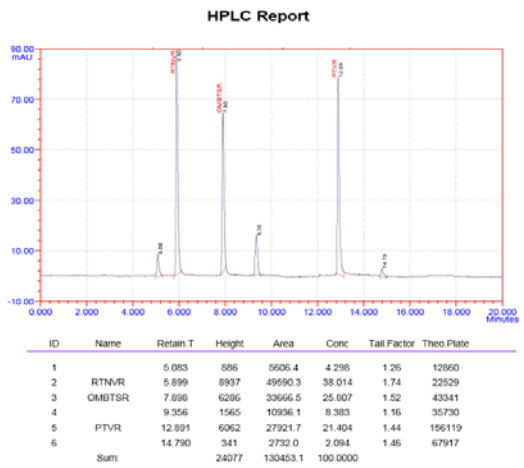


Fig.13. 4. Freeze& Thawstability test chromatogram of RTNVR, OMBTSR and PTVR

Table. 4: Stability results of developed method

S.No	Stability test	RTNVR	percentage of change	OMBTSR	Percentage of change	PTVR	percentage of change
1	Blank	00	00	00	00	00	00
2	Freshly prepared	48893	0.0	34365	0.0	28082	0.0
3	Bench top	48091	1.65	33449	2.67	27800	1.01
4	Auto sampler	47367	3.13	33270	3.19	27989	0.34
5	Freeze &Thaw	49590	1.42	33666	2.04	27921	0.58

RESULTS AND DISCUSSION

The developed method was simple, accurate and precise than reported methods. In this method the primarily chromatographic experiments were set to elute selected analytes in biological fluid. The method was validated over the concentration range of 0.15-1.2 µg/ml for RTNVR, PTVR and 0.25-2.0 µg/ml for OMBTSR. The mean percent recovery of RTNVR is 99.81%, OMBTSR is 101.42% and PTVR is 100.38. The intraday and inter day precision were conducted at standard concentration limits, the Percentage of RSD is below 2%. The LOQ concentrations are 0.0375 µg/ml for RTNVR, PTVR and 0.0625 µg/ml for OMBTSR. Stability (Bench top, Auto sampler, Freeze thaw) of compounds was established in a series of stability studies.

CONCLUSION

This method is suitable use in clinical studies to determination optimal therapeutic ranges for RTNVR, OMBTSR and PTVR and then for regular analysis and the rapeutically drug monitoring of RTNVR, OMBTSR and PTVR.

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