Research Article

International Journal of Indigenous Herbs and Drugs
Open Access ISSN: 2456-7345

Development and Validation of New Analytical Method for The Simultaneous Estimation of Darunavir And Ritonavir in Pharmaceutical Dosage Form

Pemra Raju*[1], K. Thejomoorthy[2], P.Sreenivasa Prasanna[3]

1 Department of Pharmaceutical analysis, M.L.College of Pharmacy, S. Konda-523101.

2 Head, Department of Pharmaceutical analysis, M.L.College of Pharmacy, S. Konda-523101.

3 Principal, M.L.College of Pharmacy, S.Konda-523101.

Article Info :

Article History
Received on: 03-03-2021
Revised on : 1-04-2021
Accepted on : 25-04-2021

Keywords:
Darunavir, Ritonavir, RP-HPLC.

Abstract

A simple, Accurate, precise method was developed for the simultaneous estimation of the Darunavir and Ritonavir in Tablet dosage form. The chromatogram was run through Agilent C18 150 x 4.6 mm, 5m. Mobile phase containing Buffer 0.1% Formic acid: Acetonitrile, taken in the ratio 70:30 was pumped through the column at a flow rate of 0.95 ml/min. The temperature was maintained at 30°C. The optimized wavelength selected was 293 nm. The retention times of Darunavir and Ritonavir were found to be 2.369min and 2.911. %RSD of the Darunavir and Ritonavir were and found to be 0.7 and 0.5 respectively. %Recovery was obtained as 99.67% and 99.78% for Darunavir and Ritonavir respectively. LOD, LOQ values obtained from regression equations of Darunavir and Ritonavir were 1.49, 5.191and 0.37, 1.11 respectively. Regression equation of Darunavir is y = 5421x + 640.7, and y = 3870.x + 5191 of Ritonavir. Retention times were decreased and run time was decreased, so the method developed was simple and economical that can be adopted in regular Quality control tests in Industries.

***Corresponding Author**
Pemra Raju
Email: mlcollegeofpharmacy@gmail.com

Doi: https://doi.org/10.46956/ijihd.vi.157
Production and Hosted By
Saap.org.in

Introduction

Pharmaceutical products formulated with more than one drug, typically referred to as combination products. These combination products can present daunting challenges to the analytical chemist responsible for the development and validation of analytical methods. The development and validation of analytical methods Spectrophotometric, High performance liquid chromatography (HPLC) and High-performance thin layer chromatography (HPTLC) for drug products containing more than one active ingredient. The official test methods that result from these processes are used by quality control laboratories to ensure the identity, purity, potency, and performance of drug products. The number of drugs introduced into the market is increasing every year. These drugs may be either new entities or partial structural modification of the existing ones. Very often there is a time lag from the date of introduction of a drug into the market to the date of its inclusion in pharmacopoeias. This happens because of the possible uncertainties in the continuous and wider usage of these drugs, reports of new toxicities (resulting in their withdrawal from the market), development of patient resistance and introduction of better drugs by competitors. Under these conditions, standards and analytical procedures for these drugs may not be available in the pharmacopoeias. It becomes necessary, therefore to develop newer analytical methods for such drugs[3] Darunavir [1] (DRV) is chemically (3R,3aS,6aR)-hexahydrofuro[2,3-b]furan-3-yl N-[(2S,3R)- 3-hydroxy-4-[N-(2-methylpropyl)4- aminobenzenesulfonamido]-1-phenylbutan2yl] carbamate Figure 1. It is a protease inhibitor used to treat HIV. It acts on the HIV aspartyl protease which the virus needs to cleave the HIV polyprotein into its functional fragments

SOUTH ASIAN ACADEMIC PUBLICATIONS

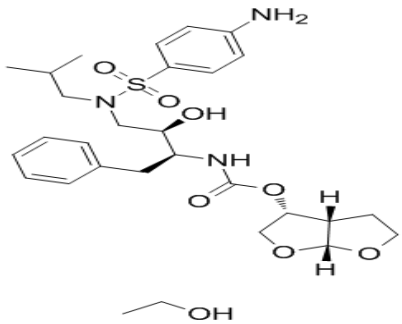


Figure 1: chemical structure of Darunavir

Ritonavir[2] (RTV) is chemically 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 Figure 2. It is an HIV protease inhibitor that interferes with the reproductive cycle of HIV.

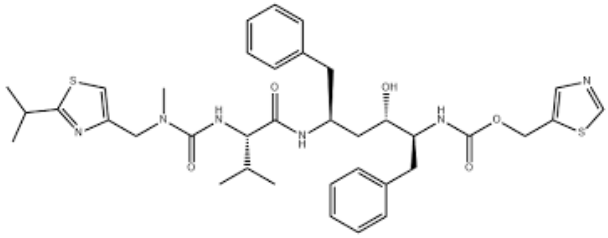


Figure 2: chemical structure of Ritonavir

Although it was initially developed as an independent antiviral agent, it has been shown to possess advantageous properties in combination regimens with low-dose ritonavir and other protease inhibitors.[3-7] There are few methods reported in the literature of Darunavir and Ritonavir alone or in combination with other drugs in the pure and pharmaceutical formulation by UV, HPLC and UPLC-MS [8-20]. In view of the need of suitable, cost effective RP HPLC method for routine analysis of simultaneous estimation of RTV and DRV in bulk and synthetic mixture (tablet dosage form), attempts we made do develop a simple, accurate, precise and cost effective analytical method for the estimation of RTV and DRV. The purpose of stability testing is to check the drug quality under the action of many environmental factors like temperature, acid, base and oxidative condition. This is necessary for establishment of re-test period for the drug products and for recommendation conditions for their storage. ICH guidelines therefore emphasize stability-indicating analytical methods[4] . Efforts were therefore made to develop a novel, fast and validated stability indicating HPLC procedure for determining simultaneously both the drugs in tablet dosage forms The proposed method will be validated as per ICH guidelines.

Experimental work

Materials and Methods

Materials

Ritonavir and Darunavir pure drugs (API), Combination Ritonavir and Darunavir, Distilled water, Acetonitrile, Phosphate buffer, , Methanol, Potassium dihydrogen ortho phosphate buffer, Ortho-phosphoric acid. Allthe above chemicals and solvents are from Rankem

Instruments

Electronics Balance-Denver , p^H meter -BVK enterprises, India ,Ultrasonicator-BVK enterprises, WATERS HPLC 2695 SYSTEM equipped with quaternary pumps,Photo Diode Array detector and Auto sampler integrated with Empower 2 Software.UV-VIS spectrophotometer PG Instruments T60 with special bandwidth of 2mm and 10mm and matched quartz cells integrated with UV win 6 Software was used for measuring absorbances of Ritonavir and Darunavir solutions.

Methods

Diluent

Based up on the solubility of the drugs, diluent was selected, Methanol and Water taken in the ratio of 50:50

Preparation of Standard stock solutions

Accurately weighed 12.5 mg of Ritonavir, 100mg of Darunavir and transferred to 25ml volumetric flask and 3/4 th of diluents was added to these flask and sonicated for 10 minutes. Flask were made up with diluents and labeled as Standard stock solution. (500µg/ml of Ritonavir and 4000µg/ml of Darunavir)

Preparation of Standard working solutions (100% solution)

1ml from each stock solution was pipetted out and taken into a 10ml volumetric flask and made up with diluent. (50µg/ml Ritonavir of and 400µg/ml of Darunavir)

Preparation of Sample stock solutions

5 tablets were weighed and the average weight of each tablet was calculated, then the weight equivalent to tablet was transferred into a 100 ml volumetric flask, 5ml of diluents was added and sonicated for 25 min, further the volume was made up with diluent and filtered by HPLC filters (500µg/ml of Ritonavir and 4000µg/ml of Darunavir)

Preparation of Sample working solutions (100% solution)

1ml of filtered sample stock solution was transferred to 10ml volumetric flask and made up with diluent.(50µg/ml of Ritonavir and 400µg/ml of Darunavir)

Preparation of buffer

0.1%OPA Buffer

1ml of ortho phosphoric acid was diluted to 1000ml with HPLC grade water.

Method Validation [21-22]

System suitability parameter

The system suitability parameters were determined by preparing standard solutions of Ritonavir (50ppm) and Darunavir (400ppm) and the solutions were injected six times and the parameters like peak tailing, resolution and

USP plate count were determined. The % RSD for the area of six standard injections results should not be more than 2%.

Specificity

Checking of the interference in the optimized method.We should not find interfering peaks in blank and placebo at retention times of these drugs in this method. So this method was said to be specific.

Precision

Preparation of Standard stock solutions Accurately weighed 12.5 mg of Ritonavir, 100mg of Darunavir and transferred to 25ml volumetric flask and 3/4 th of diluents was added to these flask and sonicated for 10 minutes. Flask were made up with diluents and labeled as Standard stock solution. (500µg/ml of Ritonavir and 4000µg/ml of Daruna)

Preparation of Standard working solutions (100% solution) 1ml from each stock solution was pipetted out and taken into a 10ml volumetric flask and made up with diluent. (50µg/ml of Ritonavir and 400µg/ml of Darunavir)

Preparation of Sample stock solutions 5 tablets were weighed and the average weight of each tablet was calculated, then the weight equivalent to tablet was transferred into a 10 ml volumetric flask, 5ml of diluents was added and sonicated for 25 min, further the volume was made up with diluent and filtered by HPLC filters (500µg/ml of Ritonavir and 4000µg/ml of Darunavir)

Preparation of Sample working solutions (100% solution) 1ml of filtered sample stock solution was transferred to 10ml volumetric flask and made up with diluent.(50µg/ml of Ritonavir and 400µg/ml of Darunavir)

Linearity

Preparation of Standard stock solutions Accurately weighed 12.5 mg of Ritonavir, 100mg of Darunavir and transferred to 25ml volumetric flask. and 3/4 th of diluents was added to these flask and sonicated for 10 minutes. Flask were made up with diluents and labeled as Standard stock solution. (500µg/ml of Ritonavir and 4000µg/ml of Daruna)

25% Standard solution 0.25ml each from two standard stock solutions was pipetted out and made up to 10ml. (12.5µg/ml of Ritonavir and 100µg/ml of Darunavir)

50% Standard solution 0.5ml each from two standard stock solutions was pipetted out and made up to 10ml. (25µg/ml of Ritonavir and 200µg/ml of Darunavir)

75% Standard solution 0.75ml each from two standard stock solutions was pipetted out and made up to 10ml. (37.5µg/ml of Ritonavir and 300µg/ml of Darunavir)

100% Standard solution 1.0ml each from two standard stock solutions was pipetted out and made up to 10ml. (50µg/ml of Ritonavir and 400µg/ml of Darunavir)

125% Standard solution 1.25ml each from two standard stock solutions was pipetted out and made up to 10ml. (62.5µg/ml of Ritonavir and 500µg/ml of Darunavir)

150% Standard solution 1.5ml each from two standard stock solutions was pipettede out and made up to 10ml (75µg/ml of Ritonavir and 600µg/ml of Darunavir)

Accuracy

Preparation of Standard stock solutions Accurately weighed 12.5 mg of Ritonavir, 100mg of Darunavir and transferred to 25ml volumetric flask. and 3/4 th of diluents was added to these flask and sonicated for 10 minutes. Flask were made up with diluents and labeled as Standard stock solution. (500µg/ml of Ritonavir and 4000µg/ml of Darunavir)

Preparation of 50% Spiked Solution 0.5ml of sample stock solution was taken into a 10ml volumetric flask, to that 1.0ml from each standard stock solution was pipetted out, and made up to the mark with diluent.

Preparation of 100% Spiked Solution 1.0ml of sample stock solution was taken into a 10ml volumetric flask, to that 1.0ml from each standard stock solution was pipetted out, and made up to the mark with diluent.

Preparation of 150% Spiked Solution

1.5ml of sample stock solution was taken into a 10ml volumetric flask, to that 1.0ml from each standard stock solution was pipetted out, and made up to the mark with diluent.

Acceptance Criteria

The % Recovery for each level should be between 98.0 to 102

Robustness Small deliberatechanges in method like Flow rate, mobile phase ratio, and temperature are made but there were no recognized change in the result and are within range as per ICH Guide lines.

Robustness conditions like Flow minus (1ml/min), Flow plus (1.2ml/min), mobile phase minus, mobile phase plus, temperature minus (25°C) and temperature plus(35°C) was maintained and samples were injected in duplicate manner. System suitability parameters were not much effected and all the parameters were passed. %RSD was within the limit.

LOD sample Preparation 0.25ml each from two standard stock solutions was pipetted out and transferred to two separate 10ml volumetric flasks and made up with diluents. From the above solutions 0.1ml each of Ritonavir, Darunavir, solutions respectively were transferred to 10ml volumetric flasks and made up with the same diluents

LOQ sample Preparation 0.25ml each from two standard stock solutions was pipetted out and transferred to two separate 10ml volumetric flask and made up with diluent. From the above solutions 0.3ml each of Ritonavir, Darunavir, solutions respectively were transferred to 10ml volumetric flasks and made up with the same diluent.

Degradation studies [23]

Oxidation

To 1 ml of stock solution of Ritonavir and Darunavir, 1 ml of 20% hydrogen peroxide (H_2O_2) was added separately. The solutions were kept for 30 min at 60^0c.

For HPLC study, the resultant solution was diluted to obtain 50 µg/ml & 400 µg/ml solution and 10 µl were injected into the system and the chromatograms were recorded to assess the stability of sample.

Acid Degradation Studies

To 1 ml of stock ssolution Ritonavir and Darunavir, 1 ml of 2N Hydrochloricacidwasadded and refluxed for 30mins at 60^{0}c.The resultant solution was diluted to obtain 50 µg/ml & 400 µg/ml solution and10µl solutions were injected into the system and the chromatograms were recorded to assess the stability of sample.

AlkaliDegradat ionStudies:

To 1 ml of stock solutionRitonavir and Darunavir, 1 ml of 2N sodium hydroxidewasadded and refluxed for 30mins at 60^{0}c. Theresultantsolutionwas diluted to obtain 50µg/ml&400µg/ml solution and 10µl were injected into the system and the chromatograms were recordedtoassessthestabilityofsample.

Dry Heat Degradation Studies

Thestandarddrug solution was placedinovenat105°C for1h tostudydryheat degradation.ForHPLCstudy,the resultant solution was diluted to 50µg/ml&400µg/ml solution and10µl were injected into the system and the chromatogramswererecordedtoassessthestability ofthesample.

Photo Stability studies

The photochemical stability of the drug was also studied by exposing the 500µg/ml &4000µg/ml solution to UV Light by keeping the beaker in UV Chamber for 1days or 200 Watt hours/m^2 in photo stability chamber. For HPLC study, the resultant solution was diluted to obtain 200µg/ml&300µg/ml solutions and 10µl were injected into the system and the chromatograms were recordedtoassessthestabilityofsample.

.

NeutralDegradationStudies

Stress testing under neutral conditions was studied by refluxingthedruginwaterfor1hrs atatemperature of 60º. For HPLC study, the resultant solution was diluted to 50µg/ml&400µg/ml solution and 10µl were injected intothesystemandthechromatogramswererecorded toassessthestabilityofthesample.

Results and Discussion

Optimized method

Chromatographic conditions

Mobile phase : 70% Formic acid (0.1%): 30% Acetonitrile

Flow rate : 1ml/min

Column : Azilent C18 (4.6 x 150mm, 5µm)

Detector wave length : 260nm

Column temperature : 30°C

Injection volume : 10µL

Run time : 6 min

Diluent : Water and Acetonitrile in the ratio 50:50

Results : Both peaks have good resolution, tailing Factor, theoretical plate count and resolution.

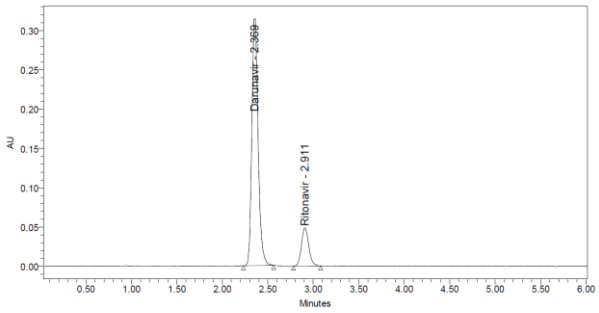


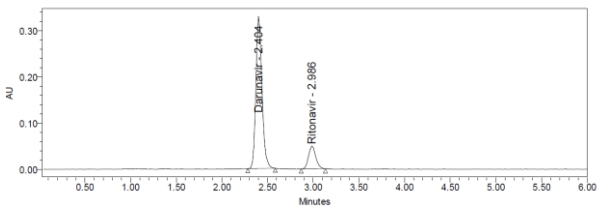
Fig 3 Optimized Chromatogram

System suitability: All the system suitability parameters were within the range and satisfactory as per ICH guidelines

Table:1 Systemsuitability parameters forDarunavir and Ritonavir

Sno	Darunavir			Ritonavir			
Inj	RT(min)	USP Plate Count	Tailing	RT(min)	USP Plate Count	Tailing	Resolution
1	2.404	6178	1.19	2.986	6601	1.13	4.4
2	2.405	6198	1.16	2.986	7121	1.12	4.4
3	2.405	6089	1.20	2.988	6573	1.15	4.3
4	2.413	5924	1.17	2.998	6244	1.09	4.2
5	2.421	5892	1.18	3.013	6859	1.13	4.4
6	2.433	6253	1.18	3.036	6763	1.10	4.5

Fig 4 Systemsuitability Chromatogram



Discussion

According to ICH guidelines plate count should be more than 2000, tailing factor should be less than 2 and resolution must be more than 2. All the system suitable parameters were passed and were within the limits.

Validation

Specificity

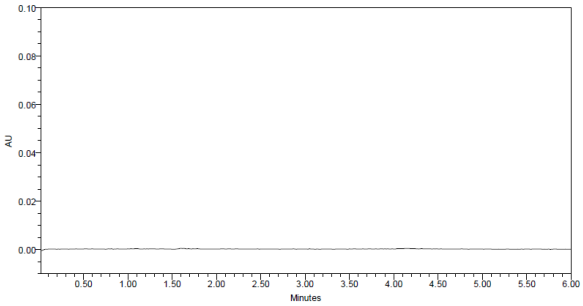


Figure No. 5. Chromatogramof blank

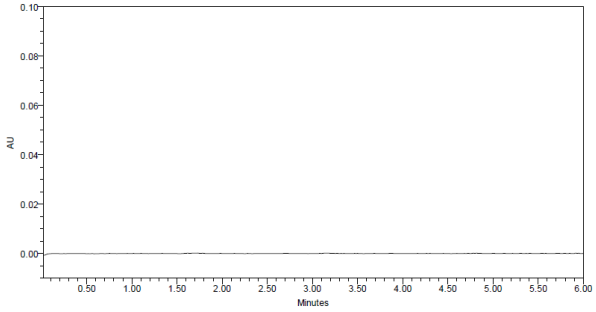


Figure No. 6 Chromatogramof placebo

Linearity:

Table 2 Linearity table for Darunavir and Ritonavir.

Darunavir		Ritonavir	
Conc (µg/mL)	Peak area	Conc (µg/mL)	Peak area
0	0	0	0
100	390764	12.5	67733
200	786093	25	134305
300	1174300	37.5	205232
400	1563383	50	277599
500	1907925	62.5	338712
600	2341934	75	404125

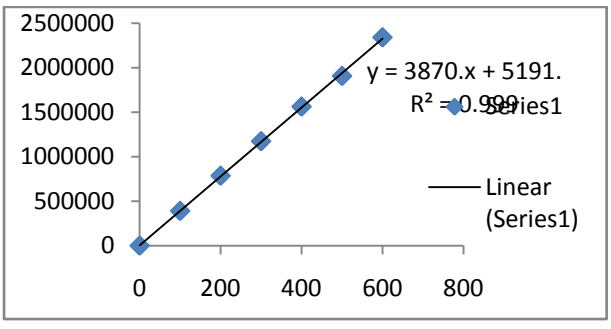


Fig No. 7 CalibrationcurveofDarunavir

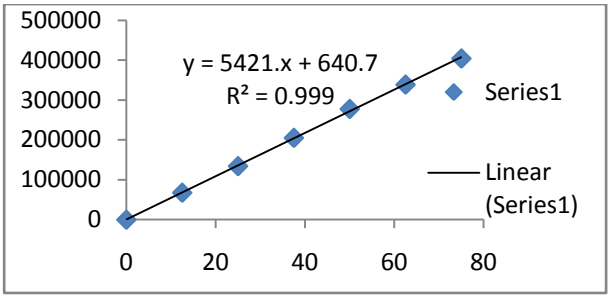


Fig No. 8 CalibrationcurveofRitonavir

Discussion

Six linear concentrations of Darunavir (100-600µg/ml) and Ritonavir (12.5-75µg/ml) were injected in a duplicate manner. Average areas were mentioned above and linearity equations obtained for Darunavir was y = 3870.x + 5191 and of Ritonavir was y = 5421.x + 640.7 Correlation coefficient obtained was 0.999 for the two drugs.

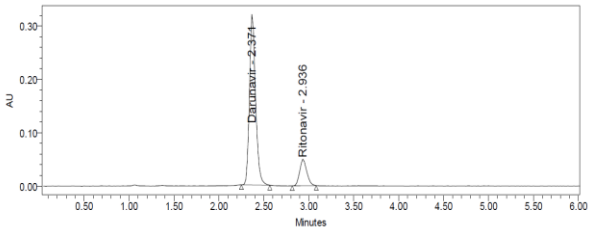


Fig 9 Typical Chromatogram

Discussion

Retention times of Darunavir and Ritonavir were 2.371 min and 2.938 min. respectively. We did not found and interfering peaks in blank and placebo at retention times of these drugs in this method. So this method was said to be specific.

Precision

System Precisio

Table 3 System precision table of Darunavir and Ritonavir

S. No	Area of Darunavir	Area of Ritonavir
1.	1562412	272468
2.	1565061	277211
3.	1568363	271649
4.	1566157	270677
5.	1566158	273575
6.	1561519	272713
Mean	1564945	273049
S.D	2560.9	2264.3
%RSD	0.2	0.8

Repeatability

Table 4 Repeatability table of Darunavir and Ritonavir

S. No	Area of Darunavir	Area of Ritonavir
1.	1563796	273031
2.	1563323	274473
3.	1570928	275737
4.	1585721	275393
5.	1590713	273519
6.	1582460	272597
Mean	1576157	274125
S.D	11729.6	1282.7
%RSD	0.7	0.5

Discussion: Multiple sampling from a sample stock solution was done and six working sample solutions of same concentrations were prepared, each injection from each working sample solution was given and obtained areas were mentioned in the above table. Average area, standard deviation and % RSD were calculated for two drugs and obtained as 0.7% and 0.5% respectively for Darunavir and Ritonavir. As the limit of Precision was less than "2" the system precision was passed in this method.

Intermediate precision (Day_ Day Precision)

Table 5 Intermediate precision table of Darunavir and Ritonavir

S. No	Area of Darunavir	Area of Ritonavir
1.	1508957	270706
2.	1506297	270017
3.	1488309	274639
4.	1509602	268996
5.	1502940	267494
6.	1499562	270510
Mean	1502611	270394
S.D	7958.4	2393.2
%RSD	0.5	0.9

Discussion

Multiple sampling from a sample stock solution was done and six working sample solutions of same concentrations were prepared, each injection from each working sample solution was given on the next day of the sample preparation and obtained areas were mentioned in the above table. Average area, standard deviation and % RSD were calculated for two drugs and obtained as 0.5% and 0.9% respectively for Darunavir and Ritonavir. As the limit of Precision was less than "2" the system precision was passed in this method.

Accuracy

Table 6 Accuracy table of Darunavir

% Level	Amount Spiked (µg/mL)	Amount recovered (µg/mL)	% Recovery	Mean %Recovery
50%	200	200.00	100.00	99.67%
	200	202.03	101.02	
	200	196.43	98.22	
100 %	400	396.33	99.08	
	400	398.75	99.69	
	400	400.62	100.15	
150 %	600	596.41	99.40	
	600	590.94	98.49	
	600	605.68	100.95	

Table 7 Accuracy table of Ritonavir

% Level	Amount Spiked (µg/mL)	Amount recovered (µg/mL)	% Recovery	Mean %Recovery
50%	25	24.94	99.75	99.57%
	25	24.80	99.19	
	25	25.03	100.13	
100 %	50	49.10	98.21	
	50	49.30	98.60	
	50	49.57	99.13	
150 %	75	74.33	99.10	
	75	74.83	99.78	

	75	74.68	99.57	

Discussion

Three levels of Accuracy samples were prepared by standard addition method. Triplicate injections were given for each level of accuracy and mean %Recovery was obtained as 99.67% and 99.57% for Darunavir and Ritonavir respectively.

Sensitivity

Table 8 Sensitivity table of Darunavir and Ritonavir

Molecule	LOD	LOQ
Darunavir	1.49	4.51
Ritonavir	0.37	1.11

Robustness

Table 9 Robustness data for Darunavir and Ritonavir.

S.no	Condition	%RSD of Darunavir	%RSD of Ritonavir
1	Flow rate (-) 0.9ml/min	1.1	1.1
2	Flow rate (+) 1.1ml/min	0.3	1.0
3	Mobile phase (-) 65:35A	0.8	1.0
4	Mobile phase (+) 75B:25A	0.5	0.7
5	Temperature (-) 25°C	0.5	0.4
6	Temperature (+) 35°C	0.8	0.7

Discussion

Robustness conditions like Flow minus (0.85ml/min), Flow plus (1.15ml/min), mobile phase minus (65B:35A), mobile phase plus (75B:25A), temperature minus (25°C) and temperature plus(35°C)was maintained and samples were injected in duplicate manner. System suitability parameters were not much affected and all the parameters were passed. %RSD was within the limit.

Assay

Mylan pharmaceuticals(Durart R 450 Tablet), bearing the label claim Darunavir 400mg, Ritonavir 50mg. Assay was performed with the above formulation. Average % Assay for Darunavir and Ritonavir obtained was 100.62% and 100.29% respectively

Table 10Assay Data of Darunavir

S.no	Standard Area	Sample area	% Assay
1	1562412	1563796	99.83
2	1565061	1563323	99.80
3	1568363	1570928	100.28
4	1566157	1585721	101.23
5	1566158	1590713	101.54
6	1561519	1582460	101.02
Avg	1564945	1576157	100.62
Stdev	2560.9	11729.6	0.75
%RSD	0.2	0.7	0.7

Table 11 Assay Data of Ritonavir

S.no	Standard Area	Sample area	% Assay
1	272468	273031	99.89
2	277211	274473	100.42
3	271649	275737	100.88
4	270677	275393	100.76
5	273575	273519	100.07
6	272713	272597	99.73
Avg	273049	274125	100.29
Stdev	2264.3	1282.7	0.5
%RSD	0.8	0.5	0.5

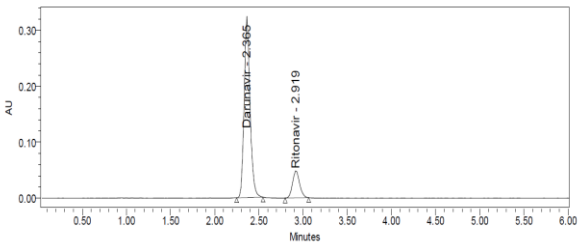


Fig 08 Chromatogram of working standard solution

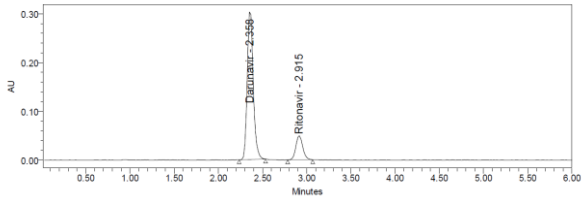


Fig No. 09 Chromatogram of working sample solution

Degradation data

Table 12 Degradation data for Darunavir and Ritonavir

Type of degradation	Darunavir			Ritonavir		
	AREA	%RECOVERED	% DEGRADED	AREA	%RECOVERED	% DEGRADED
Acid	1420809	90.70	9.30	256076	93.69	6.31
Base	1471589	93.94	6.06	260788	95.41	4.59
Peroxide	1453963	92.82	7.18	257555	94.23	5.77
Thermal	1522949	97.22	2.78	265997	97.32	2.68
Uv	1552374	99.10	0.90	269020	98.43	1.57
Water	1556561	99.36	0.64	270988	99.15	0.85

Conclusion

A simple, Accurate, precise method was developed for the simultaneous estimation of the Darunavir and Ritonavir in Tablet dosage form. Retention time of Darunavir and Ritonavir were found to be 2.369min and 2.911. %RSD of the Darunavir and Ritonavir were and found to be 0.7 and 0.5 respectively. %Recovery was obtained as 99.67% and 99.78% for Darunavir and Ritonavir respectively. LOD, LOQ values obtained from regression equations of Darunavir and Ritonavir were 1.49, 5.191and 0.37, 1.11 respectively. Regression equation of Darunavir isy = 5421x + 640.7, and y = 3870.x + 5191 of Ritonavir. Retention times were decreased and run time was decreased, so the method developed was simple and economical that can be adopted in regular Quality control test in Industries.

Author Contribution

All authors are Contributed Equally.

Funding

No Funding.

Conflict of Intrest

Authors are Declared no Conflict of Intrest.

References

1. https://www.chemsrc.com/en/cas/635728-49-3_1170683.html
2. https://www.chemicalbook.com/ChemicalProduct Property_EN_CB0119429.htm
3. Peter John, Waqar Azeem, Muhammad Ashfaq, Islam Ullah Khan, Syed Naeem Razzaq and Salah Ud-Din Khan. StabilityIndicating RP-HPLC method for Simultaneous Determination of Methoxsalen and p-aminobenzoic acid in binary combination. 2015; 29 (1), 27-39.
4. ICH, Validation of Analytical Procedures; Methodology, Q2 (R1), International Conference on Harmonization, IFPMA, Geneva, 1996.
5. ICH Q1A (R2) Guidelines, Stability testing of new drug substances and products, 2003.
6. Ravindra B Nehete and Pushpendra Sharma. Analytical Method Validation: Planning and its significance. Journal of Harmonized Research in Applied Science. 2017; 5(4), 172-177.
7. Kala Shweta and Singh Anita. A Review on Analytical Method Validation. International Journal of Pharma Research and Review. 2016; 5(9), 30-36.
8. Tulsi Modi, Bhumi Patel and Jaimin Patel. Development and Validation of Stability Indicating RP-HPLC Method for Simultaneous Estimation of Lignocaine HCl and Nlfedipine in cream. Journal of Pharmaceutical Analysis, 2016; 5(1), 1-37.
9. Manisha b Mane, Pranali j Gaikawad, Anuja v Patil . RP-HPLC method for determination of darunavir in bulk and pharmaceutical preparations. International Journal of

Pharmaceutical Science Review and Research. 2013; 21(2), 20-23.

10. G.Raveendra babu, Lakshmana rao and J. Venkateswara rao. Development and validation of novel HPLC method for estimation of darunavir in pharmaceutical formulations, International Journal of Research in Pharmacy and Chemistry. 2013; 3(2), 2231-2781.
11. Nagendrakumar AVD, Sreenivasa Rao B and Basaveswara Rao MV. Validated RP - HPLC Method for the Determination of Darunavir in Bulk and Pharmaceutical Formulation. Research Journal of Pharmaceutical, Biological and Chemical Sciences, 2014; 5(3), 63-74.
12. Santosh V. Gandhi and Rasika R. Korhale. A RP-HPLC Method Development and Validation for the Estimation of Ritonavir in Bulk and Pharmaceutical Dosage Form, Journal of Chemical and Pharmaceutical Research. 2016; 8(7), 901-904.
13. Zakir Ahmad Sandeep s. sonawane Atharva s. bhalerao. Development of a validated RP-HPLC method for estimation of Darunarvir in spiked Human Plasma with UV Detection. International Research Journal of Pharmacy. 2016; 7(1), 24-28.
14. J. Sathish kumar reddy, k. R. S. Prasad and k. Suresh babu. AStability Indicating RP-HPLC method for Simultaneous Estimation of Darunavir and Cobicistat in bulk and Tablet Dosage Form. Der Pharmacia Lettre. 2016; 8 (12), 89- 96.
15. Sigamala S. Kumar, Donthireddy Sai Priyanka and Paul Richards M. RPHPLC Method Development and Validation for Simultaneous Estimation of Cobicistat and Darunavir in Tablet Dosage Form. World Journal of Pharmacy and Pharmaceutical Sciences. 2016; 5 (6), 490-499.
16. Venkateswara Rao B, Vidyadhara S and Ram Babu. R, Analytical Method Development and Validation for Simultaneous Estimation of Lopinavir and Ritonavir by RPHPLC. International Journal of Research & Development in Pharmacy & Life Science.2014; 3 (4), 1074-1079.
17. C.Varaprasad and K.Ramakrishna. Simultaneous Quantification of Atazanavir and Ritonavir in Pharmaceutical Dosage Form by Validated RP-HPLC Method. International Journal of Pharmacy & Pharmaceutical Research. 2015; 3 (4), 26-37.
18. Alagar Raja. M, Bhavana, Rao. K N V. Simultaneous Estimation of Method Development and Validation of Atazanavir and Ritonavir by RPHPLC Method. Asian Journal of Pharmaceutical Analysis and Medicinal Chemistry. 2015; 3(3), 89 - 99.
19. T Sankarshana and Md. Musthafa. RP-HPLC Method Development & Validation for Simultaneous Estimation of Cobicistat and Dorunavir in Bulk and Pharmaceutical Dosage Form. International Journal of Medical Sciences & Pharma Research. 2017; 3(2), 13-22.
20. Tulsidas mishra and Pranav S. Shrivastav. Validation of Simultaneous Quantitative Method of HIV protease inhibitors Atazanavir, Darunavir and Ritonavir in human plasma by UPLC-MS/MS. The Scientific World Journal. 2014; 3(2), 1-13.
21. Rao CP, Rahaman SA, Prasad YR, Reddy PG. RP-HPLC method of simultaneous estimation of amlodipine besylate and metoprolol in combined dosage form. International Journal of Pharmaceutical Research and Development. 2010 Nov;2(9):69-76.
22. Rao CM, Konda R, Ramanjeneeyulu S. Estimation of Nevirapine Anhydrous Bulk Formulation by Using IR, RP-HPLC, GC Methods. Research Journal of Pharmacy and Technology. 2010 Dec 28;3(4):1088-92.
23. Ch M M Prasada et al, Development and Validation of a Novel Stability Indicating RP-HPLC Method for The Estimation of Entecavir In Tablet Formulation, EJBPS, 2017, 4(7):176-180
24. Kumaraswamy.Gandla, R.Lalitha, Dara Varun Kumar, and P.VR Teja Shruthi. "Analytical Method Development & Validation for the Simultaneous Estimation of Ledipasvir and Sofosbuvir in Bulk and it's Dosage Form by Rp-Hplc". *International Journal of Pharmaceutics and Drug Analysis*, Apr. 2020, pp. 6-15,
25. Z. M. Sayyed, Aijaz A. Sheikh, Zakirhussain A. Shaikh, S. A. Shinde, . J. Chaware5, and K. R. Biyani. "DEVELOPMENT AND VALIDATION OF ANALYTICAL METHOD FOR SIMULTANEOUS ESTIMATION OF TENO-FOVIR AND EMTRICITABINE IN PHARMACEUTICAL DOSAGE FORMS BY HPLC". *International Journal of Pharmaceutics and Drug Analysis*, Jan. 2016, pp. 24-29,
26. Gokul S. Sanap, Nilesh S. Zarekar, and Sarita S. Pawar. "REVIEW ON METHOD DEVELOPMENT AND VALIDATION". *International Journal of Pharmaceutics and Drug Analysis*, May 2017, pp. 177-84,
27. K, V. G., T. K, and S. P. P. "A New Stability Indicating Analytical Method Development And Validation for The Quantitative Determination of Emitricitabine And Lamivudine By RP-HPLC". *World Journal of Current Medical and Pharmaceutical Research*, Vol. 2, no. 2, May 2020, pp. 184-90, doi:10.37022/WJCMPR.2020.2219.