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A NEW UPLC METHOD FOR THE SEPARATION AND QUANTITATION OF PROCESS AND DEGRADATION RELATED IMPURITIES IN THE COMBINED DOSAGE TABLETS OF DARUNAVIR AND RITONAVIR

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ABSTRACT

Aim: The objective of the present study is to develop a new UPLC method for the simultaneous determination of process and degradation related impurities in the combined dosage tablets of darunavir and ritonavir.

Method: Separation of the analytes was achieved on an Aeris Wide Pore C4 column (100 × 2.1 mm; 3.6µm) by gradient programming of mobile phase-A (mixture of 2.72 g of potassium dihydrogen phosphate, 3.5 g of disodium hydrogen phosphate and 2.0 g of tetra N-butyl ammonium hydrogen sulphate in 1000 mL of water; pH adjusted to 6.5 with orthophosphoric acid) and mobile phase-B (mixture of acetonitrile and tetrahydrofuran in the ratio of 90:10 v/v) at a flow rate of 0.5 mL/min. The analytes in the eluate were monitored at 240 nm.

Results: By applying the proposed method, the relative retention times of darunavir related compound-01, darunavir, darunavir related compound-02, ritonavir impurity-E, ritonavir impurity-F, ritonavir impurity-L, ritonavir, ritonavir impurity-O, and ritonavir impurity-T were found to be 0.22, 1.00, 5.37, 0.55, 0.58, 0.95, 1.00, 1.04 and 1.54 respectively. The relative response factor values for darunavir related compound-01, darunavir related compound-02, ritonavir impurity-E, ritonavir impurity-F, ritonavir impurity-L, ritonavir impurity-O, and ritonavir impurity-T were found to be 1.19, 0.67, 1.05, 0.71, 1.62, 3.21, and 1.65 respectively. The proposed method was validated for other parameters like accuracy, precision, LOD, LOQ, forced degradation studies, robustness and solution stability.

Conclusion: The proposed stability-indicating UPLC method was proved to be fast, sensitive, robust, precise and accurate. Hence, the proposed method can be used for the identification and quantitation of the process-related and degradation impurities of darunavir and ritonavir in tablet dosage forms.

KEY WORDS
Darunavir, Ritonavir, Impurities, UPLC, Gradient elution.

INTRODUCTION

Ritonavir (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) belongs to the class of protease inhibitors [1, 2]. HIV-1 protease is an enzyme required for the proteolytic cleavage of the viral polyprotein precursors into the individual functional proteins found in infectious HIV-1. Ritonavir binds to the protease active site and inhibits the activity of the enzyme. This inhibition prevents cleavage of the viral polyproteins resulting in the formation of immature non-infectious viral particles. Darunavir ([(3aS,4R,6aR) -2,3,3a,4,5,6a-hexahydrofuro [2,3-b]furan-4-yl] N-[(2S,3R) -4-[(4-amino phenyl) sulfonyl- (2-methylpropyl) amino] -3-hydroxy-1-phenylbutan-2-yl] carbamate) is a second

generation protease inhibitor drug used to treat HIV infection [3]. It was developed to maintain activity against multi-drug resistant strains of HIV-1 [4]. To achieve optimal therapeutic effect, darunavir must be co-administered with ritonavir. Failure to do so may lead to suboptimal therapeutic levels reducing the antiviral activity of darunavir. Darunavir acts on the HIV aspartyl protease which the virus needs to cleave the HIV polyprotein into its functional fragments. This mechanism prevents the maturation of viral particles into infectious virions [5].

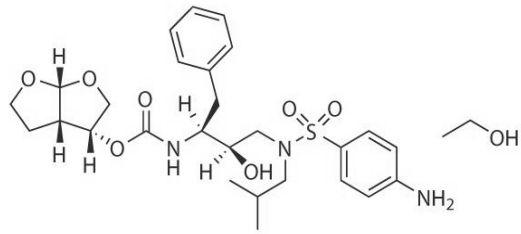


Fig. 1: Chemical structure of darunavir ethanolate

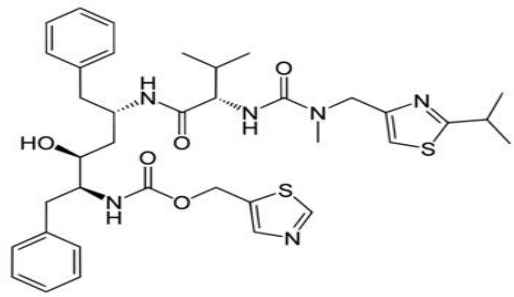


Fig. 2: Chemical structure of ritonavir

A literature survey revealed one RP-HPLC method for the separation and characterization of unknown impurities of darunavir by ESI-MS and 2D NMR spectroscopy [6]. Method for the separation and estimation of impurities from ritonavir tablets was recommended by USP 37 [7]. No liquid chromatographic method was reported for the simultaneous determination of process and degradation related impurities of darunavir and ritonavir in pharmaceutical dosage forms.

The present investigation by the author describes an accurate and precise RP-HPLC method for the simultaneous determination of the following process and degradation related impurities in the combined dosage tablets of darunavir and ritonavir.

1. 4-Amino-N- (2R, 3S) (3-amino-2-hydroxy-4-phenyl-butyl)-N-isobutylbenzene sulfonamide. (Darunavir related compound-01)
2. [(1S, 2S)-3 [[(4-Amino-phenyl) sulfonyl] (2-methylpropyl)amino]-2-hydroxy-1-(phenylmethyl) propyl] carbamic acid (3R, 3aS, 6aR) - hexahydrofuro [2,3-b]-furan-3-yl ester. (Darunavir related compound-02)
3. Thiazol-5-ylmethyl (2S,3S,5S)-3-hydroxy-5-[2-(3-{[2-(2-hydroxypropan-2- yl)thiazol-4-yl]methyl}-3-methylureido)acetamido]-1,6-diphenylhexan-2-ylcarbamate. (Ritonavir impurity-E)
4. Thiazol-5-ylmethyl(2S,3S,5S)-3-hydroxy-5-[(S)-4-isopropyl-2,5-dioxoimidazolidin-1-yl]-1,6-diphenylhexan-2-ylcarbamate.(Ritonavir impurity-F)
5. (4*S*,5*S*)-4-benzyl-5-[(2*S*)-2-[[(2*S*)-3-methyl-2-[[methyl[[2-(1-methylethyl)thiazol-4yl]methyl] carbamoyl]amino]butanoyl]amino]-3 phenylpropyl]oxazolidin-2-one.(Ritonavir impurity- L)
6. Thiazol-5-ylmethyl[(1*S*,2*R*,4*S*)-1-benzyl-2-hydroxy-4-[[(2*S*)-3-methyl-2-[[methyl[[2-(1methylethyl)thiazol-4-yl]methyl] carbamoyl]amino]butanoyl]amino]-5-phenylpentyl] carbamate. (Ritonavir impurity- O)
7. (2*S*)-*N*-[(1*S*,2*S*,4*S*)-1-benzyl-2-hydroxy-4-[[(2*S*)-3-methyl-2-[[methyl[[2-(1-methylethyl)thiazol-4yl] methyl]carbamoyl]amino]butanoyl]amino]-5-phenylpentyl]-3-methyl-2-[[methyl[[2-(1-methylethyl)thiazol-4-yl] methyl] carbamoyl] amino]butanamide. (Ritonavir impurity- T)

MATERIALS AND METHODS

Drugs and chemicals

Reference standard samples of darunavir ethanolate (purity 99.9 %), ritonavir (purity 99.4 %), darunavir related compound-01 (purity 96.1 %), darunavir related compound-02 (purity 94.7 %), ritonavir impurity-E (purity 93.9 %), ritonavir impurity-F (purity 89.6 %), ritonavir impurity-L (purity 95.9 %), ritonavir impurity-O (purity 95.4 %), ritonavir impurity-T (purity 96.8 %), ritonavir related compounds mixture and in-house tablets of darunavir and ritonavir combination (Each tablet containing 400 mg of darunavir and 50 mg of ritonavir) were obtained from Hetero Labs Ltd. (Hyderabad, India). AR grade ammonium acetate, potassium dihydrogen phosphate, disodium hydrogen phosphate, tetra N-butyl ammonium hydrogen sulphate and glacial acetic acid were purchased from Sigma-Aldrich Limited. HPLC grade acetonitrile, methanol and tetrahydrofuran were purchased from Merck Limited. HPLC grade water was prepared by using Millipore Milli-Q system.

Instrumentation

The chromatographic system consisted of a Waters Acquity UPLC H-Class system fitted with a photodiode array detector and an autosampler using Empower2 data handling system. An Aeris Wide Pore C4 column (100 × 2.1 mm; 3.6 µm) was used for the separation of the analytes. Solubility of all the compounds was enhanced by sonication on an ultrasonicator. All the weighings in the experiments were done with Sartorius balances (model CPA225D and ME36S).

Preparation of mobile phase-A

2.72 g of potassium dihydrogen phosphate, 3.5 g of disodium hydrogen phosphate and 2.0 g of tetra N-butyl ammonium hydrogen sulphate were accurately weighed and dissolved in a beaker containing 1000 mL of water. The pH of the solution was adjusted to 6.5 with orthophosphoric acid and was filtered through a 0.45 µm membrane filter followed by sonication.

Preparation of mobile phase-B

A mixture of acetonitrile and tetrahydrofuran in the ratio of 90:10 v/v was used as mobile phase-B.

Preparation of the buffer (pH 6.5)

0.77 g of ammonium acetate was weighed and dissolved in a beaker containing 1000 mL of water. The pH of the solution was adjusted to 6.5 with glacial acetic acid and was filtered through a 0.45 µm membrane filter followed by sonication.

Preparation of the diluent

The above prepared buffer, acetonitrile and methanol were mixed in the ratio of 30:25:45 v/v and used as a diluent for preparation of various drug solutions.

Preparation of mixed working standard solution of the drugs

About 43 mg of darunavir ethanolate and 25 mg of ritonavir were weighed individually and transferred into two separate 100 mL volumetric flasks. 60 mL of the diluent was added into both the volumetric flasks and sonicated to dissolve. The contents were made up to volume with the diluent and mixed. These solutions were filtered through 0.45 µm membrane filters (The first few mL the filtrates were discarded). These solutions were used as standard stock solutions of darunavir and ritonavir. 4.0 mL of standard stock solution of darunavir and 2.0 mL of standard stock solution of ritonavir was transferred in to a 100 mL volumetric flask and diluted to volume with the diluent to make a mixed working standard solution containing 17.2 µg/mL of darunavir ethanolate (15.87 µg/mL of darunavir) and 5 µg/mL of ritonavir.

Preparation of placebo solution

Ten typical placebo tablets were crushed and finely powdered. From this, a quantity equivalent to the weight of a tablet was a transferred into a 50 mL volumetric flask containing 30 mL of diluent. The contents were mixed well and sonicated for 30 minutes with occasional shaking (The temperature of water-bath of the sonicator was maintained at 20-25°C). The volume of the mixture was made up to the volume with the diluent and mixed. A portion of this mixture was filtered through a 0.45 µm membrane filter (The first few mL of the filtrate was discarded). This placebo solution was later used for the testing the interference of the excipients used in tablets.

Preparation of formulation sample solution

Ten tablets (Each tablet contains 400 mg of darunavir and 50mg of ritonavir) were crushed and ground to a fine powder. Tablet powder equivalent to 50 mg of ritonavir was accurately weighed and transferred into a 50 mL volumetric flask. About 30 mL of diluent was added into it and sonicated for 30 minutes (20-25°C) with occasional shaking. The contents were made up to volume with diluent, mixed well and filtered through a 0.45 µm membrane filter (The first few mL of the filtrate was discarded). This solution was used formulation sample solution (8000 µg/mL of darunavir and 1000 µg/mL of ritonavir).

Preparation of individual standard solutions of related compounds and impurities

About 4 mg each of darunavir related compound 01 and darunavir related compound 02, 3 mg of ritonavir impurity-E, 7 mg of ritonavir impurity-F, and 2 mg each ritonavir impurity-L, ritonavir impurity-O and ritonavir impurity-T were weighed individually and transferred into separate 10 mL volumetric flasks. 5.0 mL of diluent was added into each of the above volumetric flasks and sonicated for 10 minutes. The volumes were made up with the diluent and mixed well. These solutions were used as stock solutions of impurities. Using the above stock solutions, dilutions containing 16 µg/mL each of darunavir related compound 01 and darunavir related compound 02, 2 µg/mL each of ritonavir impurity-L, ritonavir impurity-O and ritonavir impurity-T, 3 µg/mL solution of ritonavir impurity-E and 25 µg/mL of ritonavir impurity-F were prepared. These solutions were used as individual standard solutions of related compounds and impurities (100% concentration level).

Preparation of resolution solution

About 2 mg of ritonavir related compounds mixture was accurately weighed and transferred into a 25 mL volumetric flask. 10 mL of the diluent was added into it and sonicated to dissolve. The contents were made up to volume with the diluent and mixed.

Optimization of the chromatographic conditions

Initial method development trials were performed on C18 and C8 based columns using different buffers like potassium dihydrogen phosphate and ammonium acetate with different pH together with organic modifiers like acetonitrile and methanol. Co-elution of ritonavir impurity-E and ritonavir impurity-F as well as poor peak shape of ritonavir impurity-E was observed. Satisfactory chromatographic separation was achieved on Aeris WIDEPORE C4 column (100 × 2.1 mm; 3.6 µm). A mixture of potassium dihydrogen phosphate, disodium hydrogen phosphate and tetra butyl ammonium hydrogen sulphate in water (pH adjusted to 6.5 with orthophosphoric acid) was used as mobile phase-A and a mixture of acetonitrile and tetrahydrofuran in the ratio of 90:10 v/v was used as mobile phase-B.

Mobile phase-A and mobile phase-B were pumped through the column in gradient proportions at a flow rate of 0.5 mL/min. The gradient time program was set as T/%B: 0/20, 30/40, 35/80, 40/80, 40.5/20, and 45/20. The injection volume was 3 µL and the column was kept at 30°C. Sample temperature was set to 10°C. The detector wavelength was set at 240 nm. Prior to injection of the drug solution, the column was equilibrated with the initial composition of the mobile phase for 30 minutes.

Typical chromatograms of the analysis of the blank solution, mixed working standard solution, placebo sample solution, formulation sample solution, formulation sample solution spiked with impurities and resolution solution are shown in the Fig. 3, 4, 5, 6, 7 and 8 respectively.

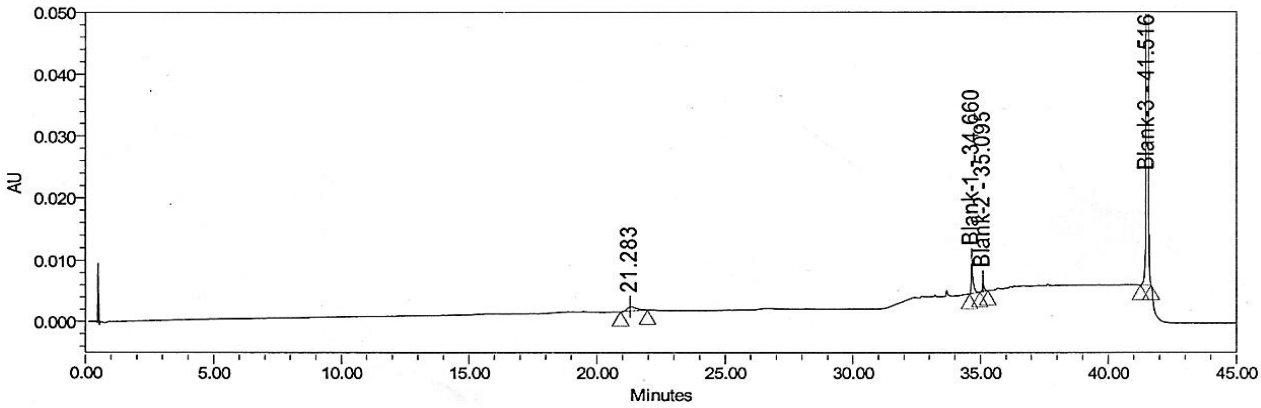


Fig. 3: Representative chromatogram of the blank solution.

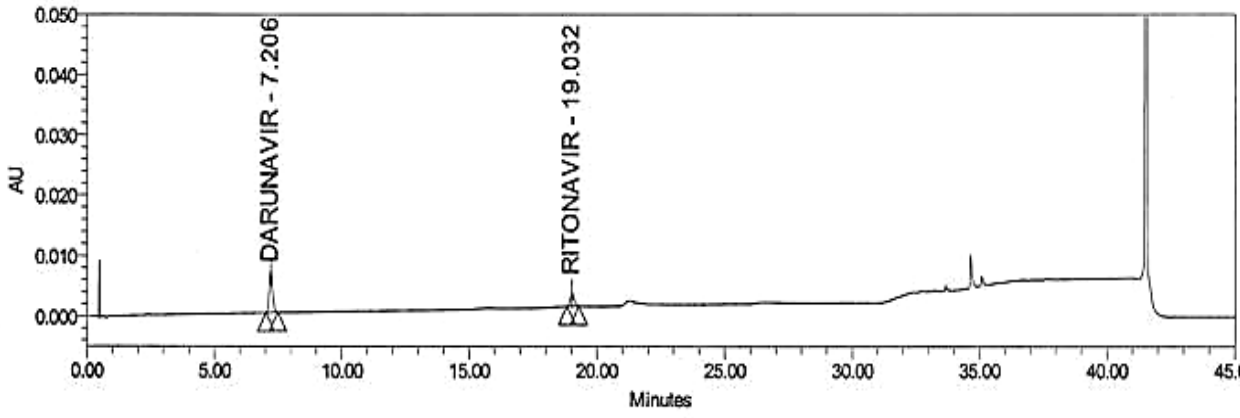


Fig. 4: Representative chromatogram obtained from the analysis of mixed working standard solution.

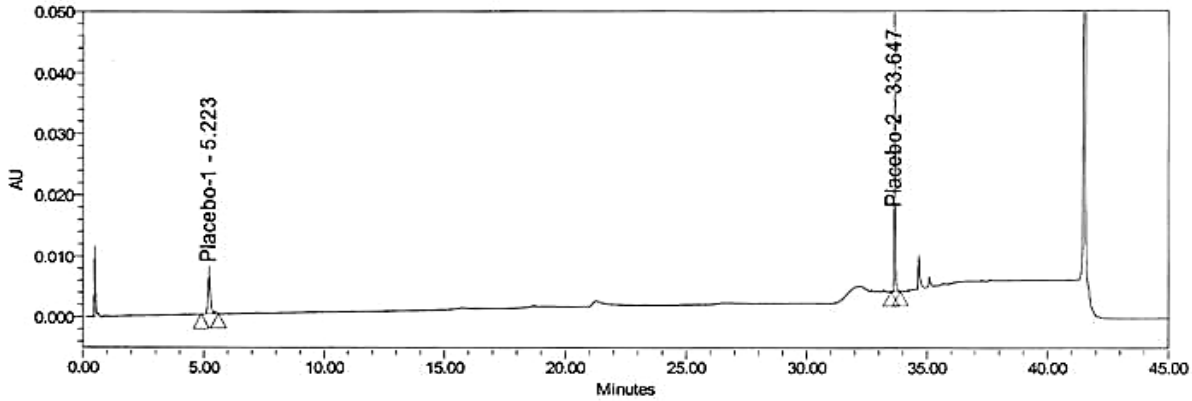


Fig. 5: Representative chromatogram of the placebo sample solution.

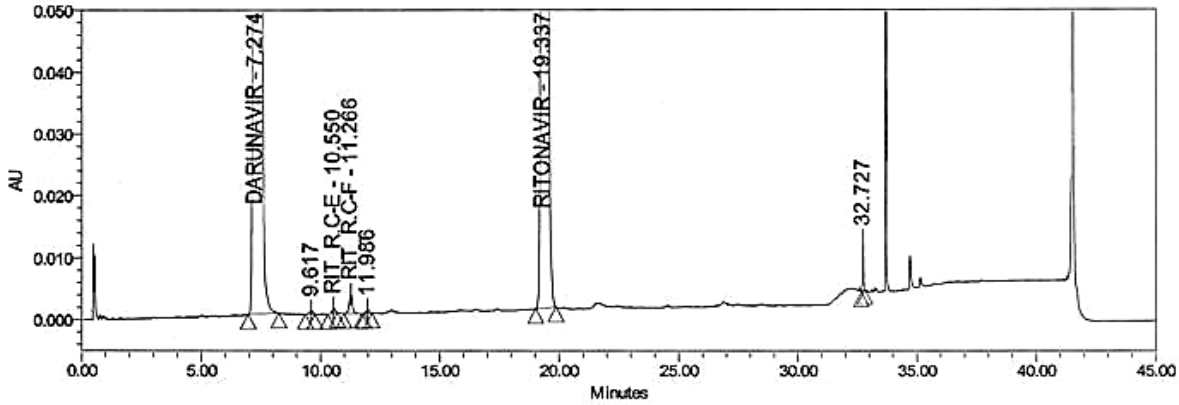


Fig. 6: Representative chromatogram obtained from the analysis of the formulation sample solution.

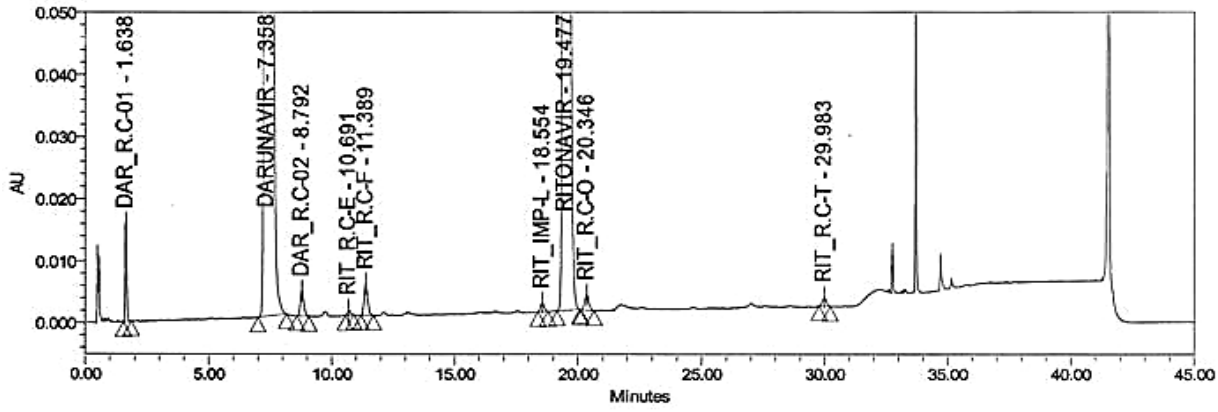


Fig. 7: Representative chromatogram obtained from the analysis of formulation sample solution spiked with the impurities.

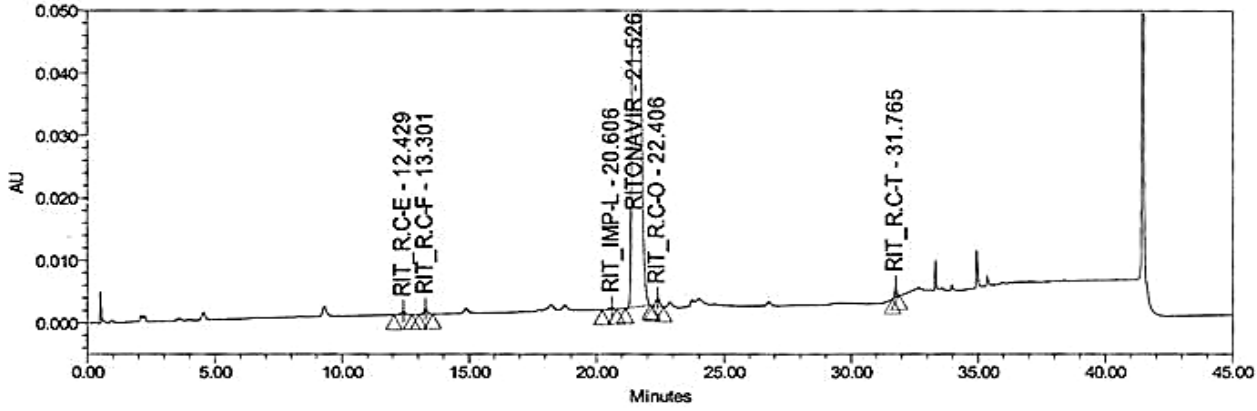


Fig. 8: Representative chromatogram obtained from the analysis of the resolution solution.

RESULTS AND DISCUSSION

The described method has been extensively validated was according to ICH guideline Q2 (R1) for specificity, linearity, accuracy, precision, LOD, LOQ, and robustness [8]. Solution stability studies and forced degradation studies were also performed.

Specificity

Individual solutions of darunavir, ritonavir, impurities at working concentration level, mixed standard solution, formulation sample solution and formulation sample solution spiked with known impurities at standard working concentration levels were analyzed in six replicates by HPLC. The retention times obtained for the drugs and impurities for the mixed working standard solution, formulation sample solution and formulation sample solution spiked with known impurities were compared with those of the respective reference compounds.

The blank (diluent) and placebo solutions were injected into the chromatographic system. No interfering peaks were observed at the retention times of the analytes and the known impurities due to the presence of excipients.

System suitability

For system suitability, six replicates of the mixed standard solution were injected and the parameters like peak area, number of theoretical plates and tailing factor of the peaks were calculated. These results are shown in the Table 2. High number of theoretical plates describes that the method is very efficient. Resolution between ritonavir impurity-E and F is also an integral part of system suitability studies. This was determined by analyzing the resolution solution (Fig. 8). Resolution between ritonavir impurity-E and F was found to be 3.6.

Linearity and range

Six linearity study solutions (calibration) were prepared by using working standards of darunavir ethanolate, ritonavir, darunavir related compound 01, darunavir related compound 02, ritonavir impurity-E, ritonavir impurity-F, ritonavir impurity-L, ritonavir impurity-O and ritonavir impurity-T at different concentration levels ranging from LOQ to 150% of working concentration level for impurities and working standard concentration for darunavir and ritonavir. LOQ level and highest level were analyzed in six replicates and other levels in duplicate. From these chromatograms, the mean peak areas were calculated and linearity plots of mean peak areas over concentrations were constructed for individual compounds. The results are shown in Tables 3 and 4.

Limit of detection (LOD) and Limit of quantitation (LOQ)

The LOD and LOQ values of darunavir, ritonavir and their impurities were estimated by preparing the solutions at lower concentrations and injecting the solutions into the chromatographic system and calculating the S/N ratio (signal/noise). LOD and LOQ are the concentrations where S/N ratio is 3.3 and 10 respectively. The LOD and LOQ values are shown the Table 5.

Accuracy

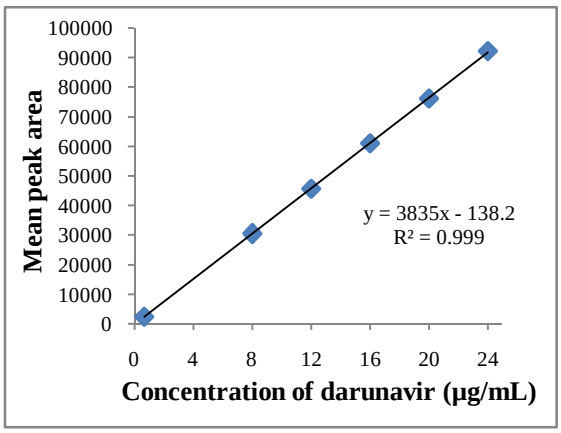
Accuracy was performed by spiking darunavir related compound 01, darunavir related compound 02, ritonavir impurity-E, ritonavir impurity-F, ritonavir impurity-L, ritonavir impurity-O and ritonavir impurity-T to the placebo solution at 50%, 100% and 150% of specification level in triplicate at each level. These solutions were injected into the chromatographic

Table No 1: Identification of the peaks by confirmation of retention times

Component	Retention time (min)	
	Mixed standard solution	**Formulation sample solution**
Darunavir	7.206	7.274
Ritonavir	19.032	19.337
Individual reference solutions		
Darunavir related compound-01	1.618	
Darunavir	7.299	
Darunavir related compound-02	8.788	
Ritonavir impurity-E	10.617	
Ritonavir impurity-F	11.312	
Ritonavir impurity-L	18.497	
Ritonavir	19.419	
Ritonavir impurity-O	20.322	
Ritonavir impurity-T	29.863	
Spiked test solution		
Darunavir related compound 01	1.638	
Darunavir	7.358	
Darunavir related compound 02	8.792	
Ritonavir impurity-E	10.691	
Ritonavir impurity-F	11.389	
Ritonavir impurity-L	18.554	
Ritonavir	19.477	
Ritonavir impurity-O	20.346	
Ritonavir impurity-T	29.983	

Table No 2: System suitability parameters of the proposed method

S.No.	Peak area		Theoretical plates		Tailing factor	
	Darunavir	**Ritonavir**	**Darunavir**	**Ritonavir**	**Darunavir**	**Ritonavir**
1	61118	21803	15862	73265	1.17	1.11
2	60728	21974	16054	73954	1.17	1.10
3	59964	21289	15788	73184	1.17	1.11
4	60819	21639	15890	73937	1.18	1.11
5	61001	21321	15927	73619	1.17	1.10
6	61313	21893	16001	73829	1.17	1.10
Mean	60823.8	21653.2	-	-	-	-
SD	470.26	291.91	-	-	-	-
%RSD	0.773	1.348	-	-	-	-



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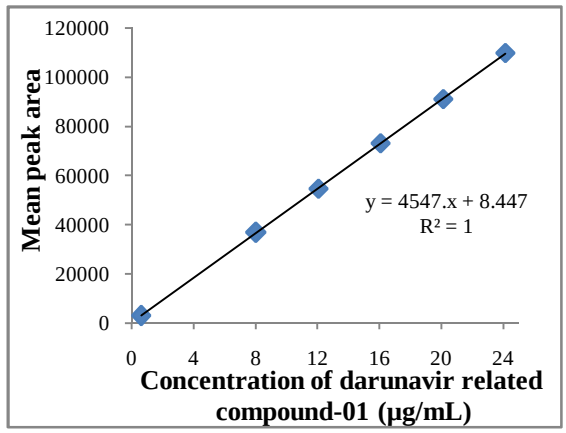

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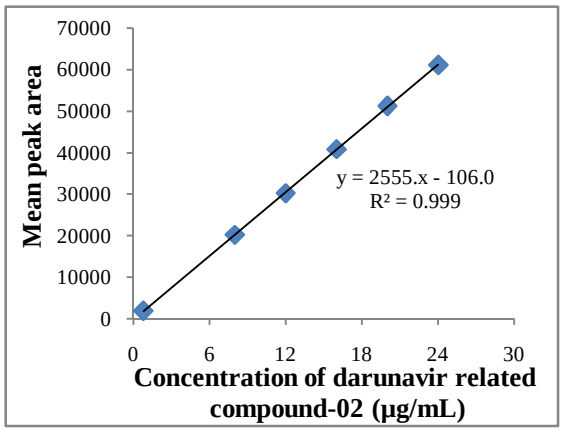
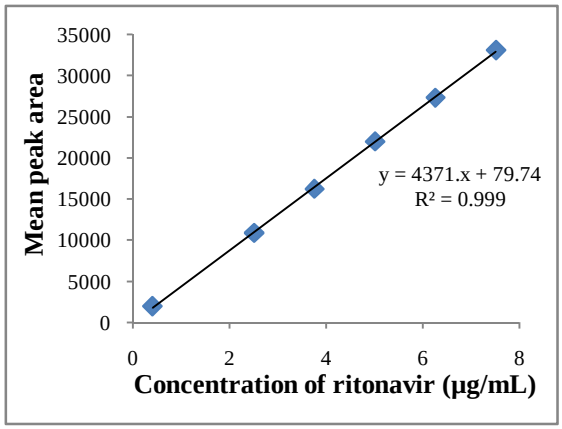


(c)

Fig. 9: Linearity plots of darunavir and its impurities

Table No 3: Linearity data of the proposed method

Component	Linearity range (µg/mL)	Regression equation and coefficient
Darunavir	0.640-23.990	y = 3835x - 137.3 (R^2 = 0.999)
Darunavir related compound-01	0.643-24.113	y = 4547.x + 9.378 (R^2 = 1)
Darunavir related compound-02	0.801-24.035	y = 2555.x - 106.3 (R^2 = 0.999)
Ritonavir	0.400-7.509	y = 4371.x + 78.08 (R^2 = 0.999)
Ritonavir impurity-E	0.593-4.444	y = 4604.x + 48.61 (R^2 = 0.999)
Ritonavir impurity-F	1.004-37.654	y = 3089.x - 93.59 (R^2 = 1)
Ritonavir impurity-L	0.298-2.985	y = 7092.x + 98.14 (R^2 = 0.999)
Ritonavir impurity-O	0.203-3.035	y = 14033x + 58.79 (R^2 = 0.999)
Ritonavir impurity-T	0.421-3.161	y = 7206.x + 9.650 (R^2 = 0.999)



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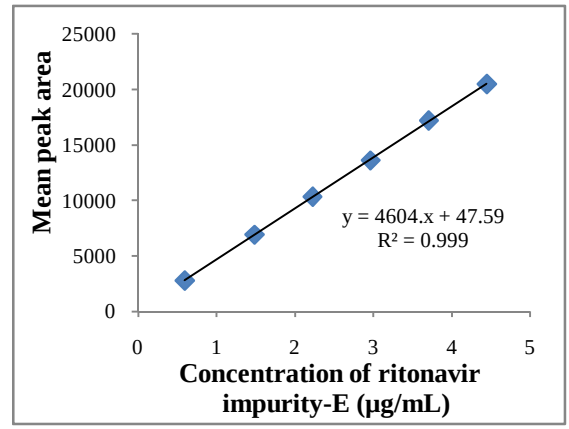

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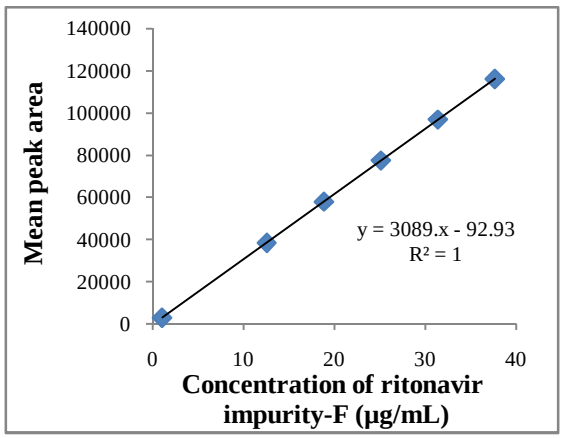

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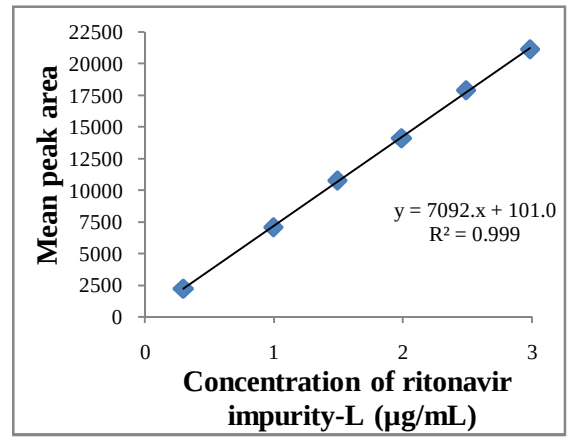

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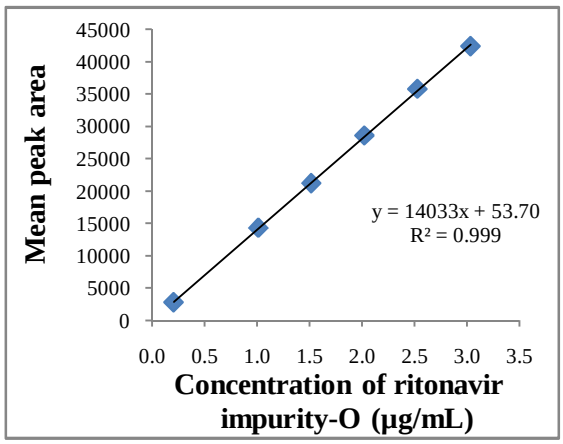

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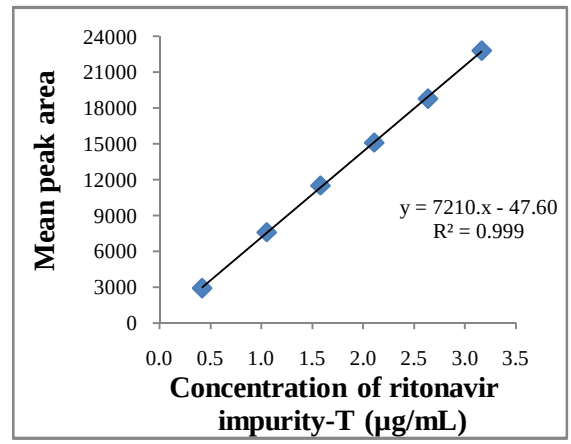

(f)

Fig. 10: Linearity plots of ritonavir and its impurities

Table No 4: Relative response factors of the impurities

Component	Slope	Relative response factor
Darunavir	3834.97	-
Ritonavir	4371.42	-
Darunavir related compound-01	4547.84	1.19
Darunavir related compound-02	2555.54	0.67
Ritonavir impurity-E	4604.71	1.05
Ritonavir impurity-F	3089.41	0.71
Ritonavir impurity-L	7092.92	1.62
Ritonavir impurity-O	14033.05	3.21
Ritonavir impurity-T	7206.33	1.65

Table No 5: Limits of detection and quantitation

S.No.	Compound Name	LOD (µg/mL)	LOQ (µg/mL)
1	Darunavir related compound-01	0.212	0.643
2	Darunavir	0.211	0.640
3	Darunavir related compound-02	0.264	0.801
4	Ritonavir impurity-E	0.196	0.593
5	Ritonavir impurity-F	0.331	1.004
6	Ritonavir impurity-L	0.099	0.298
7	Ritonavir	0.132	0.400
8	Ritonavir impurity-O	0.067	0.203
9	Ritonavir impurity-T	0.139	0.421

Table No 6: Results obtained from the recovery studies

Compound name	Mean percent recovery at different levels			
	LOQ level	**50% level**	**100% level**	**150% level**
Darunavir related compound-01	100.32	99.76	99.85	100.13
Darunavir related compound-02	99.89	99.69	99.96	99.73
Ritonavir impurity-E	100.94	100.20	99.91	100.36
Ritonavir impurity-F	99.32	99.46	99.99	100.02
Ritonavir impurity-L	100.01	100.27	100.04	99.89
Ritonavir impurity-O	99.97	99.47	99.99	99.09
Ritonavir impurity-T	100.55	100.44	100.05	100.50

system and the percent recovery was calculated. Accuracy at LOQ was performed by darunavir related compound 01, darunavir related compound 02, ritonavir impurity-E, ritonavir impurity-F, ritonavir impurity-L, ritonavir impurity-O and ritonavir impurity-T to the placebo in triplicate and these solutions were injected into the chromatographic system and the percent recovery was calculated. The percent recoveries of impurities at all the levels were between 85.0 and 115.0. Hence the method is very accurate.

Precision

System precision was studied by preparing working standard solution and analyzing them in six replicates. Peak areas of darunavir and ritonavir were measured and their percent relative standard deviations were found to be 0.90 and 1.18 respectively. Repeatability and intermediate precision was studied by preparing formulation sample solution and formulation sample solution spiked with known impurities at working concentration level and analyzed in six replicates.

%RSD values of recoveries are found to be very small which describes that the method is very precise. The results of repeatability and intermediate precision studies are shown in the Table 7.

Forced degradation studies

Ten tablets were crushed and grinded to a fine powder. This powdered tablet was then subjected to various stress conditions like acid (1M HCl, 80°C, 2 hr), base (0.25M NaOH, 80°C, 1 hr), peroxide (3% H_2O_2, 2 hr), photo degradation (254 nm, 168 hr), thermal degradation (90°C, 2 hr) and humidity induced degradation (90% relative humidity). These stressed samples were analyzed and was found that darunavir was highly susceptible to base-degradation. Darunavir and ritonavir were found to be relatively stable to photolysis and humidity degradation.

Robustness

The formulation sample solution spiked with impurities and mixed standard solution were prepared and analyzed in three and six replicates respectively, after deliberately changing the chromatographic parameters (one at a time) like flow rate of the mobile phase, temperature of the column and pH of the buffer.

The system suitability parameters obtained after analyzing the mixed standard solution were summarized in the Table 8. The overall percent relative standard deviations of recoveries of known impurities and total impurities from this study were summarized in the Table 9.

Solution stability

Formulation sample solution spiked with known impurities at working concentration levels was prepared and injected into the chromatographic system

Table No 7: Repeatability and intermediate precision data

Compound name	%RSD of recoveries	
	Repeatability studies	Intermediate precision studies
Formulation sample solution		
Darunavir related compound-01	NA	NA
Darunavir related compound-02	NA	NA
Ritonavir impurity-E	1.84	1.64
Ritonavir impurity-F	1.07	0.90
Ritonavir impurity-L	NA	NA
Ritonavir impurity-O	NA	NA
Ritonavir impurity-T	NA	NA
MSUI	1.97	1.96
Total impurities	1.12	1.02
Formulation sample solution spiked with impurities		
Darunavir related compound-01	0.26	0.27
Darunavir related compound-02	0.27	0.26
Ritonavir impurity-E	0.56	0.37
Ritonavir impurity-F	0.31	0.32
Ritonavir impurity-L	0.26	0.26
Ritonavir impurity-O	0.26	0.27
Ritonavir impurity-T	0.36	0.25
MSUI	2.53	1.83
Total impurities	0.23	0.23

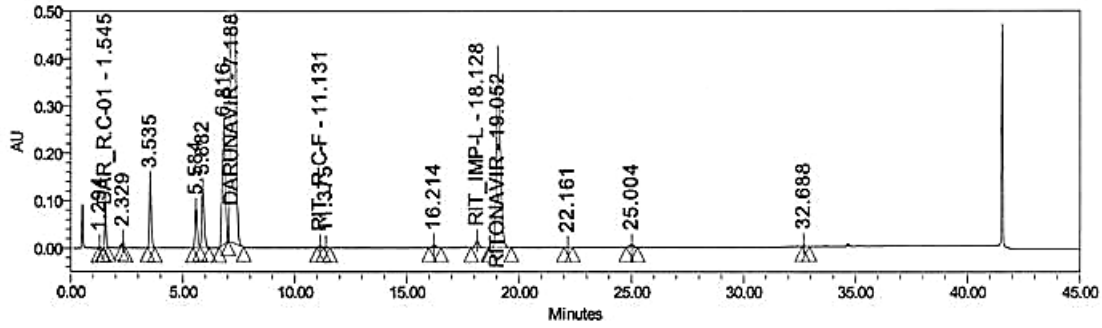

Fig. 11: Representative chromatogram of the formulation sample subjected to acid-degradation.

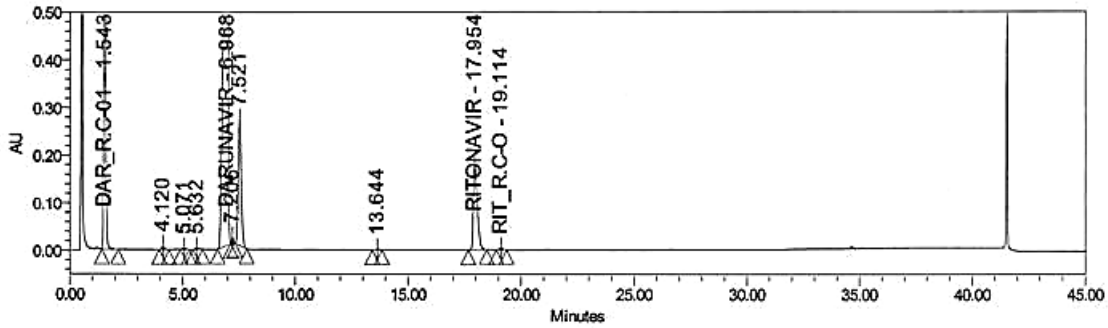

Fig. 12: Representative chromatogram of the formulation sample subjected to base-degradation.

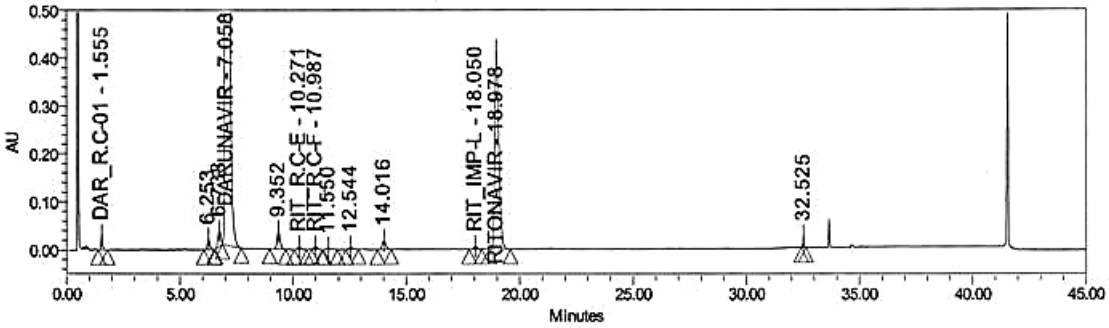

Fig. 13: Representative chromatogram of the formulation sample subjected to peroxide-degradation.

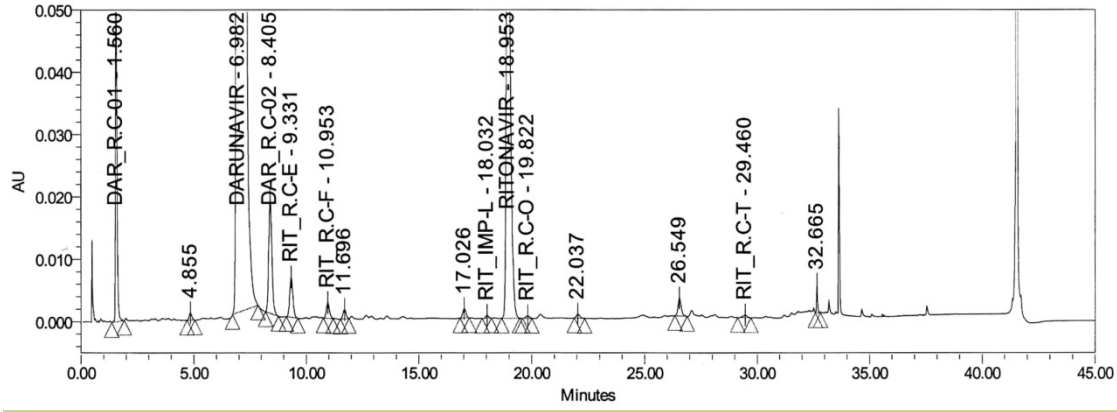

Fig. 14: Representative chromatogram of the formulation sample subjected to thermal-degradation.

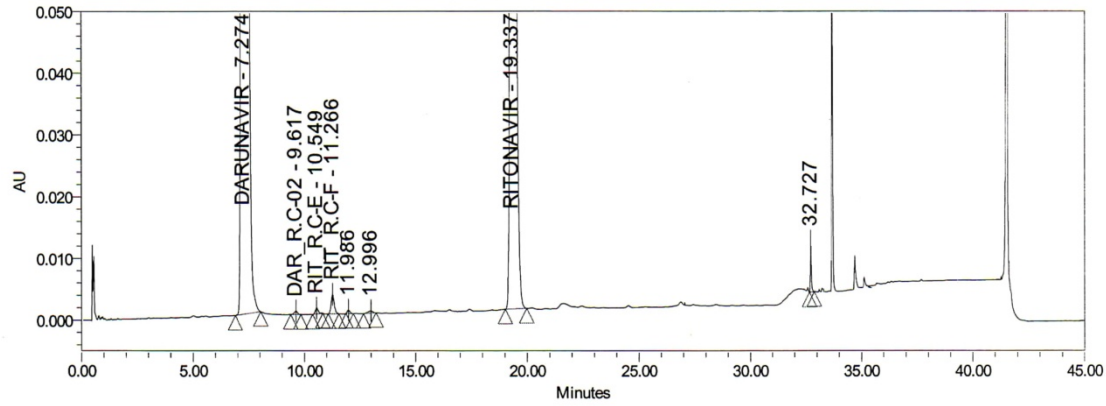

Fig. 15: Representative chromatogram of the formulation sample subjected to photo degradation

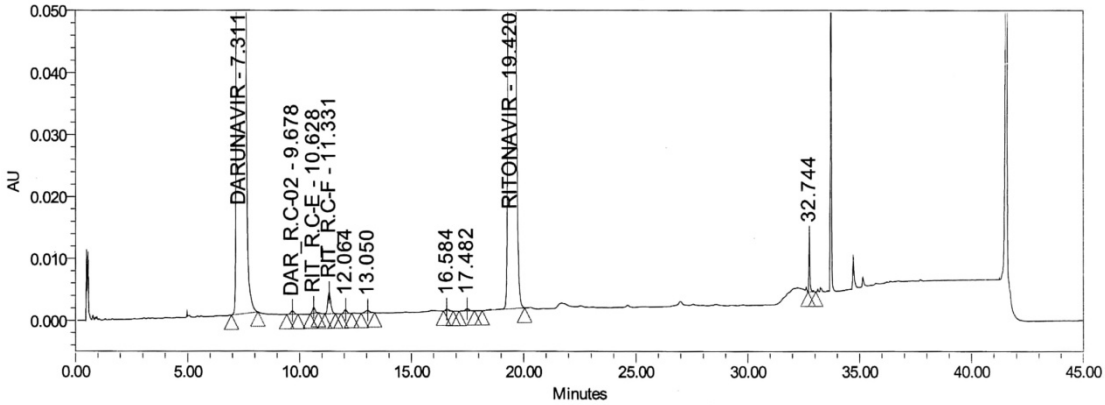

Fig. 16: Representative chromatogram of the formulation sample subjected to humidity-degradation

Table No 8: Summary of results obtained after analyzing mixed standard solution (n=6)

Variation in chromatographic condition		Peak area %RSD		Minimum number of theoretical plates		Maximum tailing factor	
		Darunavir	Ritonavir	Darunavir	Ritonavir	Darunavir	Ritonavir
Unchanged condition		0.773	1.348	15788	73184	1.18	1.11
Flow rate (0.5 mL/min)	0.45 mL/min	0.563	1.082	15936	75823	1.13	1.10
	0.55 mL/min	0.897	1.561	15302	69783	1.09	1.08
Column oven temperature (30°C)	28°C	0.978	1.615	15082	68186	1.17	1.09
	32°C	0.498	1.087	16163	75304	1.08	1.02
Change in pH of buffer (6.5)	6.4	1.028	1.102	14893	72049	1.21	1.13
	6.6	0.844	1.003	15447	71833	1.17	1.11

Table No 9: Summary of results obtained after analyzing formulation sample solution spiked with known impurities (n=3)

Chromatographic condition		%RSD values of recoveries							
		Darunavir related compound 01	Darunavir related compound 02	Ritonavir impurity-E	Ritonavir impurity-F	Ritonavir impurity-L	Ritonavir impurity-O	Ritonavir impurity-T	Total impurities
Flow rate (0.5 mL/min)	0.45 mL/min	1.467	1.637	2.304	2.468	1.982	2.083	2.243	1.984
	0.55 mL/min	2.528	1.534	1.394	3.294	2.102	1.655	1.521	2.934
Column temperature (30°C)	28°C	1.284	1.732	2.743	2.039	1.772	3.398	2.357	3.029
	32°C	3.298	2.338	1.839	3.134	2.091	2.217	3.063	3.882
Change in pH of buffer (6.5)	6.4	2.447	1.692	1.920	2.099	2.113	1.384	1.962	2.327
	6.6	2.390	2.039	2.225	2.194	2.538	1.539	2.724	2.928

at periodic intervals of 0 hours (initial time), 24 hours and 50 hours by storing the sample solution at room temperature. Recoveries of impurities (%w/w) and their difference in recoveries with time were calculated. The formulation sample solution spiked with impurities was stable for 50 hours at room temperature. The difference in recoveries (%w/w) of known impurities and maximum single unspecified impurity was less than 0.05. The difference in recovery of total impurities was less than 0.2.

CONCLUSION

The proposed stability-indicating UPLC method has been extensively validated according to ICH guidelines and was proved to be fast, sensitive, robust, precise and accurate. Hence, the proposed method can be used for the identification and quantitation of the process-related and degradation impurities of darunavir and ritonavir in tablet dosage forms.

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