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Stability indicating RP-HPLC method for simultaneous estimation of darunavir and ritonavir in pure drug and tablet dosage form

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ABSTRACT

This project presents a sensitive, rapid, precise and accurate stability indicating RP-HPLC technique for the estimation of two antiviral drug combination, ritonavir and darunavir in bulk and tablet form. Ritonavir and darunavir are extracted with mobile phase from the tablets. Analysis was done on a C8 YMC column by detection with photodiode array detector. Mobile phase composition with 0.1M potassium dihydrogen phosphate and methanol in proportion of 65:35 v/v is employed for analysis. The limits of quantification were 0.160 µg/ml and 2.359 µg/ml for ritonavir and darunavir, respectively.The limits of detection for ritonavir and darunavir are 0.048 µg/ml and 0.708 µg/ml, respectively. The precision (ritonavir – 0.142% RSD and darunavir – 0.116% RSD) and accuracy (ritonavir – 99.77% to 99.94% recovery and darunavir – 99.39% to 99.65% recovery) remained within standard limits while validation study. Ritonavir and darunavir were degraded applying acid, alkali, oxidative, neutral, thermal and photo degradation conditions. While degradation study, no substantial interfering peaks are seen in chromatograms of all tested degradation conditions at the retention time of ritonavir and darunavir. Finally it was concluded that the quantitative RP-HPLC method developed and validated can be utilized in routine analysis of ritonavir and darunavir in tablets and for stability related studies of ritonavir and darunavir in tablets.

Keywords: Ritonavir and Darunavir, Potassium Dihydrogen Phosphate.

INTRODUCTION

Drugs selected

Two human immunodeficiency virus protease inhibitors, darunavir and ritonavir are selected to develop a stability indicating RP-HPLC method for their assay in bulk and dosage tablet.

High performance liquid chromatography

High-performance liquid chromatography is an analytical laboratory technique employed in

separation and identification of compounds. It belongs to column chromatography category. Column chromatography relies on various polarities shown by the compounds in a mixture solution so as to separate them. In HPLC, pressure is used to force the sample solution via the column quickly. Therefore, it produces quickerand more accurate, results.

Reversed phase chromatography

In this technique, the usage of alkaline chains bonded with particles of stationary phase is applied for production of hydrophobic stationary phase with stronger affinity for hydrophobic or less polar compounds. As the mobile phase and stationary phase is inverted, the use of a hydrophobic stationary phase use is necessarily the reverse of normal phase chromatography– and so the term reversed-phase chromatography [19,20]. An aqueous polar mobile phase is used in this technique. Subsequently the hydrophobic molecules of polar mobile phase are tempted to adsorb with hydrophobic stationary phase, will pass through the column and get eluted first [19,20]. The elution of hydrophobic molecules from the column decreases the polarity of the mobile phase with the support of an organic (non-polar) solvent that reduces hydrophobic interactions. The increase in hydrophobicity of the molecule increases the binding strength with stationary phase, and the higher the concentration of organic solvent which is required for molecule elution.

DRUG PROFILE
DARUNAVIR AND RITONAVIR

Darunavir

Darunavir has beenrecommended for patients with acquired immunodeficiency syndrome condition and human immunodeficiency viral infections. Darunavir belongs to human immunodeficiency virus protease inhibitor drugs category.Used generally in combination with other antiretroviral agents [28-30].

Iupac name

[(1R,5S,6R)-2,8-dioxabicyclo[3.3.0]oct-6-yl] N-[(2S,3R)-4- [(4-aminophenyl)sulfonyl- (2-methylpropyl)amino]-3-hydroxy-1-phenyl- butan-2-yl] carbamate

Empirical formula

$C_{27}H_{37}N_3O_7S$

Molecular mass

547.667 g/mol

Drug structure

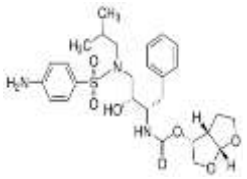

Figure 1: Darunavir chemical structure

Solubility

Slightly soluble in chloroform and methanol, and soluble in dimethylsulfoxide.

RITONAVIR

In low dose, ritonavir has been recommended as combination therapy for patients with acquired immunodeficiency syndrome condition and human immunodeficiency viral infections. Ritonavir belongs to human immunodeficiency virus protease inhibitor drugs category[31-33].

Iupac name

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] butanoyl] amino]-1,6-diphenylhexan-2-yl] carbamate

Empirical formula

$C_{37}H_{48}N_6O_5S_2$

Molecular mass

720.94 g/mol

Drug structure

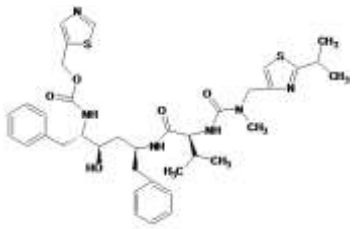

Figure 2: Ritonavir chemical structure

Solubility

Insoluble in water, soluble in methanol and ethanol.

EXPERIMENTAL

Materials

- Darunavir reference sample (gifted from Lara Drugs Private Limited, Telangana, India)
- Ritonavir reference sample (gifted from Lara Drugs Private Limited, Telangana, India)
- Durart R 450 tablets labeled to have 400 mg darunavir and 50 mg ritonavir (Mylan Pharmaceuticals Pvt Ltd, India)
- Milli Q water (Millipore, USA)
- Methanol (HPLC grade, Merck specialities Ltd, India)
- Potassium dihydrogen phosphate (Analytical grade, SD Fine-Chem Limited, India)
- Hydrochloric acid (Analytical grade, Fisher Scientific International, Inc, India)
- Sodium hydroxide (Analytical grade, SD Fine - Chem Limited, India)
- Orthophosphoric acid (Analytical grade, Fisher Scientific International, Inc, India)

Apparatus

- Model 2695 Waters HPLC Alliance system equipped by photodiode array detector
- Waters Version 2 empower software
- YMC analytical column (C8, 25 cm × 4.6 mm, particle size 5 µm)
- Ultrasonicator (Labline stock center, India)

Mobile phase

Methanol and 0.1M potassium dihydrogen phosphate are mixed as 35:65 (*v/v*) ratio. This is also employed for standard solutions preparation.

Conditions set for rp-hplc analysis

Isocratic elution is done at a flow rate of 1.0 ml/min. Sample injection vol is 10 µl. Column is set at 25^{o}C Temp. Detection and quantification is done at 275 nm.

Stock darunavir and ritonavir solution

Stock solution of darunavir and ritonavir is prepared in a 100 ml dry volumetric flask with mobile phase at a concentration of 4000 µg/ml (darunavir) and 500 µg/ml (ritonavir). For this, 400 mg of darunavir and 50 mg of ritonavir is dissolved in mobile phase of volume 100 ml.

Darunavir and ritonavir calibration solutions

The stock darunavir and ritonavir solution is successively diluted with solvent (mobile phase) to prepare calibration solutions. Darunavir and ritonavir calibration solutions were made by diluting 0.5, 0.75, 1.0, 1.25 and 1.5 ml of stock solution by 10 mlmobile phase in volumetric flask to get five point calibration concentrations:

- 200 µg/ml, 300 µg/ml, 400 µg/ml, 500 µg/ml, 600 µg/ml – darunavir
- 25 µg/ml, 37.5 µg/ml, 50 µg/ml, 62.5 µg/ml and 75 µg/ml – ritonavir

RESULTS AND DISCUSSION

Assay of darunavir and ritonavir in tabelt

Five Durart R 450 tablets are weighed followed by powdering in mortar pestle to made homogenous mixture. A quantity of mixture corresponding to weight of one tablet (having 400 mg of darunavir and 50 mg of ritonavir) is weighed and liquefied with mobile phase (30 ml). Following 10 min of sonication, the solution mixture was filtered through a membrane filter and transferred to 100 ml standard flask and filled to the mark with mobile phase. The prepared solution is stock tablet solution (4000 µg/ml - darunavir and 500 µg/ml - ritonavir). 1.0 ml of stock tablet solution is transferred to 10 ml standard flask and filled with mobile phase to get working test tablet solution with concentration 400 µg/ml of darunavir and 50 µg/ml of ritonavir. Ten µl of working test tablet solution is injected into the column. Isocratic elution with optimized mobile phase is done at a flow rate of 1.0 ml/min. Column is set at 25^{o}C Temp. Detection and quantification is done at 275 nm. The peak areas of darunavir and ritonavir are determined. From the response, the content of darunavir and ritonavir in tablets were evaluated using regression equation or calibration curve of the selected drugs.

Darunavir and ritonavir stress degradation evalation

Evaluation of darunavir and ritonavir stress degradation [39] was made by subjecting 1 ml of stock tablet solution ((4000 µg/ml - darunavir and 500 µg/ml - ritonavir) to diverse stress conditions as shown below:

- **Acidic stress:**1 ml of 0.1N hydrochloric acid followed by 30 min sonication at room temperature
- **Basic stress:**1 ml of sodium hydroxide followed by 30 min sonication at room temperature
- **Oxidative stress:**1 ml of 30% hydrogen peroxide followed by 30 min sonication at room temperature
- **Neutral stress:**1 ml of distilled water followed by 30 min sonication at room temperature
- **Photo stress:** Exposing1 ml of tablet stock solution to direct sunlight for 24 hr
- **Dry heat stress:** Exposing 1 ml of tablet stock solution to 105 ^{o}Cfor 30 min in hot air oven.

After degradation by applying stress, the samples were diluted to 10 ml with mobile phase (concentration: 400 µg/ml – darunavir; 50µg/ml – ritonavir) in 10 ml standard flask. After filtration, samples are assayed using proposed method. The percentage degradation of ritonavir and darunavir are assessed to check their stability.

Method development

During process of method development, different columns, solvents and mixture of solvent with buffers were checked to elute ritonavir and darunavirwith better resolution, low tailing factor and high plate count.

Table 1: Conditions used in different trails

Trail	Column	MP (*v/v*)	FR (ml/min)	Temp (^{o}C)	IV (µl)	RT (min)
1	Waters, C18, 25 cm × 4.6 mm, 5 µm	0.1% orthophosphoric acid: methanol (60:40)	1	25	10	10
2	Thermo, C18, 25cm × 4.6mm, 5µm	0.1M Na_2HPO_4: Methanol (60:40)	1	25	10	8
3	Kromosil, C18, 25 cm × 4.6mm, 5µm	0.1M Na_2HPO_4: Methanol (60:40)	1	25	10	10
4	Kromosil C8, 25cm × 4.6mm, 5µm	0.1M KH_2PO_4: Methanol (55:45)	1	25	10	10
5	YMC, C8, 25cm × 4.6mm, 5µm	0.1M KH_2PO_4: Methanol (65:35)	1	25	10	6

MP-mobile phase; FR – flow rate; Temp – temperature; IV – injection volume; RT – runtime

Table 2: Values obtained during different trails

Trail	Drug	RT	Area	Resolution	PT	PC
1	DAR	3.674	1975064	-	1.97	1320
	RIT	5.325	1521996	2.94	2.27	1131
2	DAR	4.310	1909029	-	0.70	6158
	RIT	4.982	1609951	2.34	0.85	3798
3	DAR	4.335	3550084	-	1.36	3787
	RIT	4.793	996211	1.31	2.08	2244
4	DAR	4.241	3178770	-	1.77	2539
	RIT	4.960	2605361	1.80	1.86	2135
5	DAR	3.711	3506792	-	1.56	9509
	RIT	4.752	2860531	5.26	1.62	7106

DAR – darunavir; RIT – ritonavir; RT – retention time; PT – peak tailing; PC – plate count

The chromatograms obtained during different trails are shown in the following figures.

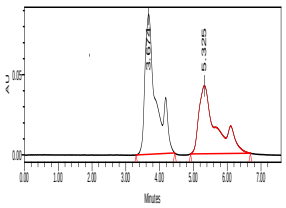

Figure 3: Chromatogram with trial 1 conditions

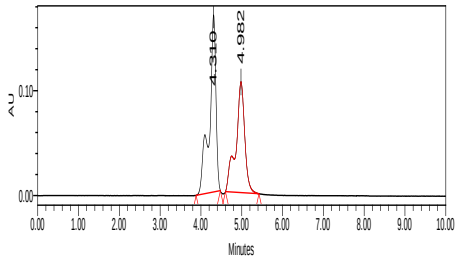

Figure 4: Chromatogram with trial 2 conditions

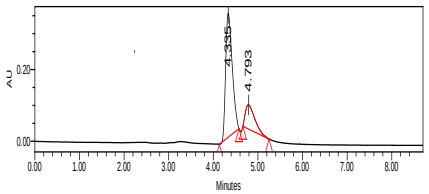

Figure 5: Chromatogram with trial 3

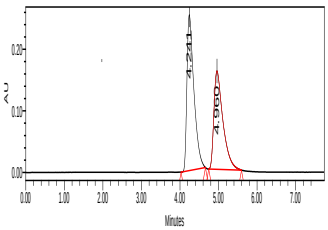

Figure 6: Chromatogram with trial 4 conditions

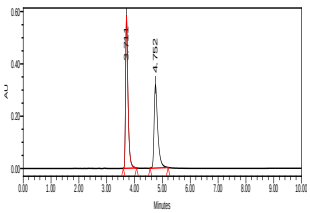

Figure 7: Chromatogram with trial 5 conditions

From the chromatograms and values obtained in all the trails tested, better results were obtained when trail 5 conditions are used. In trail 5 conditions, the ritonavir and darunavir peaks were

well shaped with less peak tailing. The resolution between ritonavir and darunavir is high in trail 5 conditions. The plate count and response for ritonavir and darunavir is more in trail 5 conditions. Hence the conditions used in trail 5 have been chosen as optimized conditions for the assay of ritonavir and darunavir simultaneously.

METHOD VALIDATION

Developed method is validated for the below explained parameters following set of laws of ICH [40].

Test for system suitability

Suitability of system to assay ritonavir and darunavirwas tested through six injections of working ritonavir (50 µg/ml) and darunavir(400 µg/ml) solution. The parameters measured are:

.

Table 3: System suitability results for darunavir and ritonavir

Sample No.	RET	PAR	TP	PA	RES
Darunavir(400µg/ml)					
1	3.712	3434442	9186	1.57	-
2	3.711	3396372	9619	1.55	-
3	3.71	3418479	9168	1.57	-
4	3.71	3438207	9131	1.56	-
5	3.71	3424197	9097	1.58	-
Mean,	**3.711,**	**3422339,**	**9240,**	**1.566,**	-
RSD (%)	**0.024**	**0.483**	**0.322**	**0.728**	
Ritonavir (50 µg/ml)					
1	4.757	2777053	6919	1.62	5.2
2	4.755	2738973	7266	1.6	5.33
3	4.756	2770852	6885	1.62	5.21
4	4.755	2777883	6894	1.6	5.2
5	4.755	2771190	6888	1.61	5.18
Mean,	**4.756,**	**2767190,**	**6970,**	**1.610,**	**5.224,**
RSD (%)	**0.019**	**0.582**	**0.378**	**0.621**	**1.153**
Recommended limit	**RSD ≤ 2**	**RSD ≤ 2**	**> 2000**	**≤ 2**	**> 1.5**

RET-retention time; TP-theoretical plates; PAR-peak area response; PA-peak asymmetry; RES-resolution

All values are inside the recommended limits. Hence the method passed system suitability test and is suitable for assay of ritonavir and darunavir simultaneously.

Test for selectivity

Selectivity was tested by injecting mobile phase and placebo and chromatographic procedure mentioned for proposed method is applied. Mobile phase chromatogram and placebo chromatogram did not show interfering peaks at ritonavir and darunavir retention time. Similarly the working standard and tablet sample solution with amount 400 µg/ml of darunavir and 50 µg/ml of ritonavir are also injected and chromatograms are compared. The retention times of ritonavir and darunavir both chromatograms are same.

No interfering peaks other than ritonavir and darunavir peaks are seen in chromatograms. This indicating that excipients of tablet do not interfered in assay of ritonavir and darunavir and demonstrated the selectivity of method.

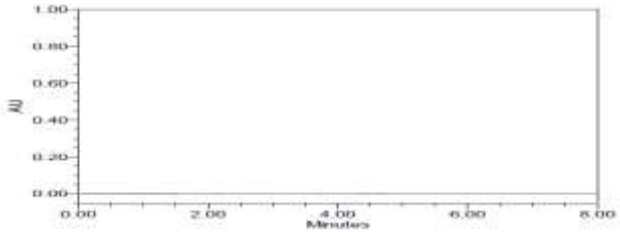

Figure 9: Mobile phase chromatogram

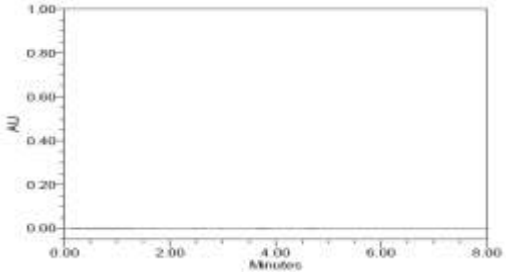

Figure 10: Placebo chromatogram

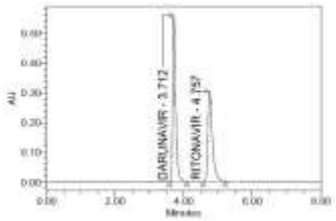

Figure 11: Ritonavir and darunavir working standard chromatogram

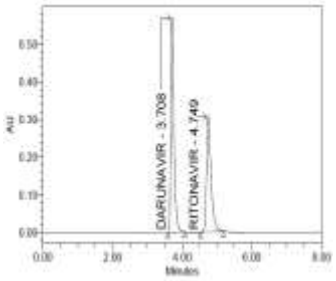

Figure 12: Ritonavir and darunavir tablet sample chromatogram

Linearity and range

The ritonavir and darunavir calibration curves are obtained via linear least square regression process at five diverse concentration levels from 200-600 µg/ml (darunavir) and 25-75 µg/ml (ritonavir) by plotting peak areas in opposition to concentrations. The representative linear equations are:

- Y = 8559 c + 863.6 (R^2 = 0.9999) --- Darunavir
- Y = 55414 c– 3193 (R^2 = 0.9998) ---- Ritonavir

Linear regression facts for calibration plots for daruanvir and ritonavir was demonstrative of good linearity between peak area and concentration in the series 200-600 µg/ml (darunavir) and 25-75 µg/ml (ritonavir).

Table 4: Darunavir and ritonavir linearity data

Conc %	Darunavir		Ritonavir	
	Area	µg/ml	Area	µg/ml
50	1713323	200	1381833	25

75	2566960	300	2070296	37.5
100	3429686	400	2767626	50
125	4277480	500	3455671	62.5
150	5135870	600	4158907	75.00

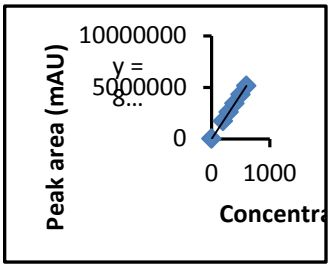

Figure 13: Darunavir linearity graph

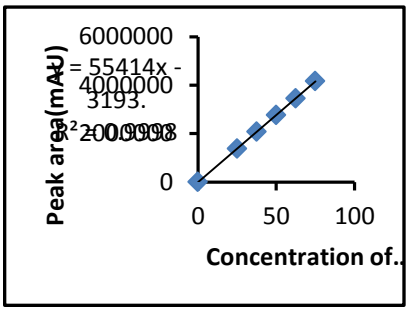

Figure 14: Ritonavir linearity graph

Limit of detection and limit of quantitation

These two parameters are calculated by taking signal to noise ratio of three for limit of detection and signal to noise ratio of ten for limit of quantitation. The values calculated were:

- Limit of detection – 0.708 µg/ml (darunavir) and 0.048 µg/ml (ritonavir)
- Limit of quantitation -2.359 µg/ml (darunavir) and 0.161 µg/ml (ritonavir)

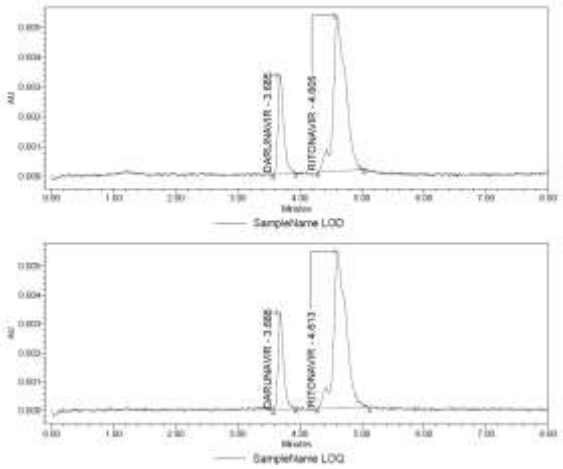

Figure 16: Limit of detection and limit of quantitation chromatograms

Precision

To evaluate precision, daruanvir(400 µg/ml) and ritonavir (50 µg/ml) standard solution was injected 6 times. Precision is estimated as percent relative standard deviation of six peak areas and percent assay of ritonavir and darunavir. Percent relative standard deviationis being lower than 2% for ritonavir and darunavirdemonstrates precision of method.

Table 5: Darunavir and ritonavir precision data

S.No	Peak area response		Percent assay	
	Darunavir	Ritonavir	Darunavir	Ritonavir
1	3422480	2766858	99.40	99.79
2	3420418	2768393	99.34	99.84
3	3429977	2769205	99.62	99.87
4	3427139	2761519	99.54	99.60
5	3429710	2768405	99.61	99.84
6	3428955	2761559	99.59	99.60
Average	**3427240**	**2765816**	**99.52**	**99.76**
RSD	**0.116**	**0.142**	**0.118**	**0.128**

Accuracy

Method accuracy is tested by determining recoveries of darunavir and ritonavir by standard addition technique procedure. Reference standards of ritonavir and darunavirknown amounts are added to prior-quantified tablet sample solution at 50%, 100% and 150% level of labeled claim. The quantity of ritonavir and darunavir was assayed by applying attained peak area values to theirindividual regression line equations and calibration curves.

Table 6: Darunavir accuracy data

Spiked Level	Peak area	µg/ml of DAR added	µg/ml of DAR found	% Recovery	% Mean
50%	1713534	200.000	199.07	99.54	99.65
	1716271	200.000	199.39	99.70	
	1716580	200.000	199.43	99.71	
100%	3429913	400.000	398.48	99.62	99.52
	3421934	400.000	397.55	99.39	
	3427989	400.000	398.26	99.56	
150%	5132711	600.000	596.31	99.38	99.39
	5134448	600.000	596.51	99.42	
	5131802	600.000	596.20	99.37	

Table 7: Ritonavir accuracy data

Spiked Level	Peak area	µg/ml of RIT added	µg/ml of RIT found	% Recovery	% Mean
50%	1389984	25.000	25.07	100.26	99.94
	1384556	25.000	24.97	99.87	
	1382069	25.000	24.92	99.69	
100%	2766079	50.000	49.88	99.76	99.77
	2765097	50.000	49.86	99.72	
	2767683	50.000	49.91	99.82	
150%	4150640	75.000	74.85	99.80	99.87
	4158387	75.000	74.99	99.98	
	4151782	75.000	74.87	99.82	

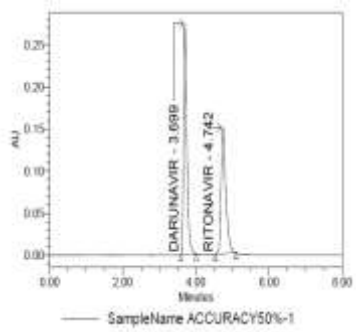

Figure 18: Accuracy chromatogram 1 (50%)

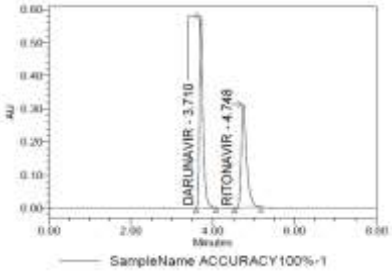

Figure 19: Accuracy chromatogram 2 (100%)

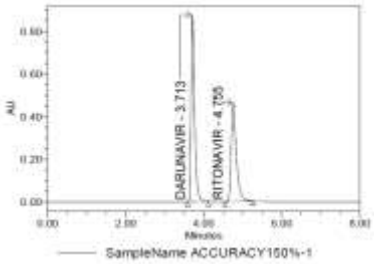

Figure 20: Accuracy chromatogram 3 (150%)

Degradation study

The tablet sample solution (daruanvir - 400 µg/ml and ritonavir - 50 µg/ml) was subjected to degradation by acid, base, oxidant, water, thermal and photo conditions. This experiment demonstrates the specificity, stability indicating nature and stability of ritonavir and darunavir under different applied conditions.

Table 8: Darunavir and ritonavir degradation data

Condition	Percent assay		Percent degradation	
	Darunavir	Ritonavir	Darunavir	Ritonavir
0.1 N HCl	86.17	86.55	13.83	13.45
0.1N NaOH	87.07	87.98	12.93	12.02
30% H_2O_2	90.51	89.77	9.49	10.23
105^oC	86.19	84.67	13.81	15.33
Sunlight	89.91	89.02	10.09	10.98
Water	93.24	91.89	6.76	8.11

Ritonavir and darunavir is more sensitive to acid condition and more resistance to water condition. The degradant peaks were well resolved from the ritonavir and darunavir peaks. No interference seen. Therefore, the method is specific and stability indicating.

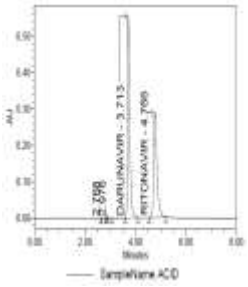

Figure 21: Acid degraded sample chromatogram

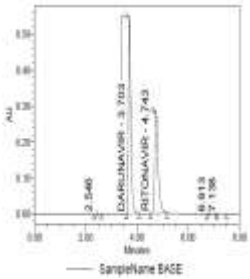

Figure 22: Base degraded sample chromatogram

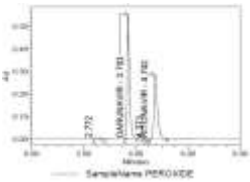

Figure 23: Oxidant degraded sample chromatogram

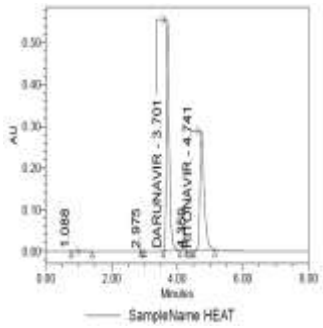

Figure 24: Thermal degraded sample chromatogram

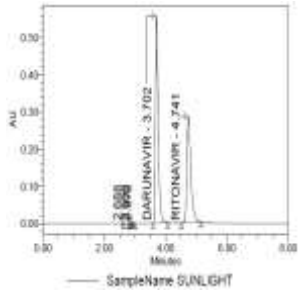

Figure 25: Photo degraded sample chromatogram

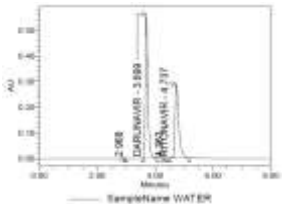

Figure 26: Water degraded sample chromatogram

Robustness test

Robustness test is done to check whether the system suitability parameter values of ritonavir and darunavir remains stable when minor changes made in the chromatographic conditions like:

- Flow rate –1.0 ±0.1 ml/min
- Mobile phase ratio - ±5% organic phase
- Temperature - 25˚C ±2 ^{o}C
- Detection wavelength – 275 nm ± 2 nm

The values in the table showed no significant changes in the system suitability parameter values of ritonavir and darunavir determined when minor changes made in above said conditions. Therefore the method is robust.

Table 9: System suitability values for darunavir and ritonavir while testing robustness

Parameter	Analyzed value	TP	PA	Res
Darunavir				
Flow rate (ml/min)	1.0 - 0.1	8302	1.54	-
	1.0 + 0.1	8930	1.56	-
Detection wavelength (nm)	275 - 2	9200	1.56	-
	275 + 2	9649	1.55	-
Mobile phase ratio (*v/v*)	70:30	8302	1.54	-
	60:40	9258	1.60	-
Temperature (^{o}C)	25˚C + 2 ^{o}C	9258	1.60	-
	25˚C - 2 ^{o}C	9541	1.63	-
Ritonavir				
Flow rate (ml/min)	1.0 - 0.1	6469	1.52	4.99
	1.0 + 0.1	6907	1.54	5.11
Detection wavelength (nm)	275 - 2	6937	1.62	5.21
	275 + 2	7251	1.59	5.33
Mobile phase ratio (*v/v*)	70:30	6469	1.52	4.99
	60:40	7062	1.60	5.16
Temperature (^{o}C)	25˚C + 2 ^{o}C	7062	1.60	5.16
	25˚C - 2 ^{o}C	7376	1.61	5.33

TP - theoreticalplates ; PA - peak asymmetry; Res - resolution

CONCLUSION

This investigational project describes a simple and rapid stability indicating method for the combined assay of ritonavir and darunavir in tablets and bulk form. While degradation Study, no substantial interfering peaks are seen in chromatograms of all tested degradation conditions at the retention time of ritonavir and darunavir. The proposed method finely fulfilled the validation parameter required criteria. Consequently, this quantitative RP-HPLC method can be utilized in routine analysis of ritonavir and darunavir in tablets and for stability studies of ritonavir and darunavir in tablets.

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